Supporting Online Material

Materials and Methods

Animals

We used C57BL/6 (wild-type; WT) mice, and congenic GSNOR$^{-/-}$ mice (males, 6-9 weeks old) that had been backcrossed 10 consecutive times with C57BL/6 mice (*S1*). (In a separate analysis of 6 GSNOR$^{-/-}$ female mice, no differences were seen vs. males at baseline and in the response to OVA, and similar protection was conferred vs. female WT; n=6). All mice were fed with standard mouse chow and housed in a pathogen-free facility. The experimental protocol and the facility housing the animals were approved by the Institutional Animal Care and Use Committee of Duke University, Durham, North Carolina.

Asthma induced by ovalbumin sensitization and challenge

Both wild-type and GSNOR$^{-/-}$ mice were divided into two groups: ovalbumin (OVA) and alum-only (control) group. Mice in the OVA group (n = 7-9) were sensitized on days 1 and 8 with an intraperitoneal (IP) injection of 10.0 μg ovalbumin (chicken egg, grade V, Sigma, St. Louis, MO) emulsified in 2.0 mg aluminum hydroxide (Alum) (AlumInject, Pierce Chemical Company, Rockford, IL) in a total volume of 100 μl. Control mice (n = 7-9) were sensitized with an IP injection of 2.0 mg alum alone in a total volume of 100 μl on the same days.

On days 15, 16 and 17, mice in OVA and control groups were exposed respectively to aerosolized OVA (3% OVA) and phosphate buffered saline (PBS; pH 7.4)

for 60 minutes. A separate group of mice received 1400 W, a selective iNOS inhibitor, 1 μg/g twice a day on days 15, 16, 17. For aerosol exposures, mice were placed in a whole-body plastic exposure chamber (16 L), and the ovalbumin or PBS solution was delivered via an ultrasonic nebulizer (model 9306, Fairchild Industrial Products Company, Winston-Salem, NC). 4.5 g of ovalbumin emulsified in 150 ml PBS was added to the reservoir of the nebulizer. Approximately 16 L/min of diluted air was forced through 3 of the 6 jets. This generated the aerosol and produced air flow through the nebulizer and exposure system. The aerosol was delivered to the chamber in a continuous flow (dynamic) mode. On day 18 (i.e., a 72-hour post-OVA exposure time when C57BL/6 animals show marked airway inflammation (*S2*), airway responsiveness was measured, and bronchoalveolar lavage fluid and tissues were then collected.

Determination of airway reactivity (Flexivent)

Airway responsiveness to MCh was determined using a constant flow method (*S3*). Briefly, mice were anesthetized with pentobarbital (150 mg/kg, IP) and tracheotomized with a cannula, which was attached to a ventilator that delivered a constant flow of air. Animal weight (W; kg) was used to determine breathing frequency (50 x $[W]^{-0.25}$/minute) and tidal volume (7.5 ml/W). Mice were given pancuronium bromide (0.8 mg/kg, IP) to block spontaneous breathing. After baseline airway resistance stabilized, increasing doses of MCh (0.1 mg/ml; 10-1000 μg/kg) were infused intravenously over 2 seconds with a syringe pump, allowing an interval of at least 2 minutes for recovery. Total respiratory system resistance (R_t) was determined immediately before MCh infusion, and then every 6 seconds following infusion for a period of 1 minute. MCh-induced increases in R_T were

calculated as [peak R_T - baseline R_T] using the baseline value immediately prior to each dose of MCh.

Bronchoalveolar lavage (BAL)

PBS supplemented with 20 μM diethylenetriamine pentaaetic acid (DTPA) was infused into the lungs to total lung capacity and then collected. A total of 4 ml of saline was infused into the lungs and the volume of retrieved fluid (ALF) was noted. Cells in ALF were isolated by centrifugation at 1000 g for 10 min. The supernatant was stored at -80°C for later assessment of nitrite, nitrate, cytokines, and GSNOR activity. The cells were re-suspended in 200 μl of PBS and counted using a hemacytometer. Cells in 200 μl of the original ALF were also centrifuged onto cytoslides (Cytospin 3, Shandon Inc., Pittsburg, PA) and stained with hematoxylin and eosin (Hema 3, Wright-Giemsa stain, Biochemical Sciences Inc., Swedesboro, NJ). Differential counts of pulmonary inflammatory cells (alveolar macrophages, neutrophils, lymphocytes, and eosinophils) were determined via light microscopy, and the number of eosinophils, neutrophils and other cells per milliliter of lavage fluid were calculated.

Cytokine analysis

Concentrations of IL-13 in ALF were determined using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

ALF nitrite and nitrate

Nitrite and total NO_x present in ALF were detected using an NOA 280 (Sievers) (*S4*). Nitrite was determined using a 1% w/vol solution of KI in glacial acetic acid, and nitrate plus nitrite were assayed in a saturated solution of VCl_3 in 0.8 M HCl (*S4*). Nitrite and nitrate standards displayed linearity between 0.03 and 25 μM ($r^2 \geq 0.98$ for all experiments) and were detected with equal efficiency (< 15% difference in detection at any concentration).

Assessment of GSNOR activity

GSNO reductase (GSNOR) activity in ALF was measured in Tris-buffered saline (20 mM pH 8.0), containing 100 μM DTPA. Homogenates of mouse lung were prepared, following removal of blood by perfusion, in lysis buffer (20 mM Tris-HCl, pH 8.0; 0.5 mM EDTA; 100 μM DTPA; 0.1% NP-40; 1 mM pheynylmethylsulfonyl fluoride). The protein concentration of each sample was measured. Using equal concentrations of protein, absorbance at 340λ was measured over time in the presence of NADH (final concentration 100 μM) and after the addition of GSNO (final concentration 100 μM). NADH-dependent GSNO reductase activity is the difference obtained in the presence and absence of GSNO.

Tracheal bioassay

Mice were sacrificed with an intraperitoneal injection of 15 mg pentobarbitone sodium (Nembutal, Abbott Laboratories, North Chicago, IL). Tracheas were resected *in toto* and transferred into modified oxygenated Krebs' bicarbonate solution (118.1 mmol/L NaCl; 4.8 mmol/L KCl; 2.5 mmol/L $CaCl_2 \cdot 6H_2O$; 1.2 mmol/L $MgSO_4 \cdot 6H_2O$; 25 mmol/L

$NaHCO_3$; 1.2 mmol/L $KH_2PO_4 \cdot H_2O$; 11.0 mol/L glucose). After removal of excess tissue, the trachea (nine tracheal rings caudal to the larynx) were dissected into ring segments of approximately 4 mm in length. Rings were mounted on tension transducers in organ baths filled with Krebs' solution, continuously gassed with O_2 (95%)/CO_2 (5%). The temperature was maintained at 37˚C. Changes in isometric tension were recorded with a polygraph. The optimal basal tension was set at 1 g (*S5, S6*). After an equilibration period of 2 hr, rings were contracted with increasing cumulative concentrations of carbachol to determine the EC_{50}. Following a 30-minute washout period, rings were precontracted with carbachol at the predetermined EC_{50} (5.62×10^{-7} M) and their relaxation in response to increasing doses of isoproterenol or GSNO was then measured to obtain cumulative concentration-relaxation curves.

Immunohistochemical detection of GSNOR and iNOS

Following sacrifice by exposure to concentrated carbon dioxide, mouse lungs were perfused *in situ* with physiological saline delivered through a cannula placed in the pulmonary artery, then inflation-fixed for 30 min with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) delivered through a tracheal cannula. Fixed tissues were embedded in paraffin, and sections were cut at 5 μM thickness, mounted on slides, and deparaffinized in xylene. Sections were treated with 0.3 % hydrogen peroxide for 1 hr to block endogenous peroxidase activity. Non-specific antibody binding was blocked by incubation for 1 hr with 5% goat serum/ 5% powdered milk/ 1% bovine serum albumin in PBS. Sections were labeled with either a rabbit polyclonal antiserum raised to a peptide comprising the residues (50-63) of human GSNOR, or an affinity-purified rabbit anti-

human iNOS antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Sections were exposed to primary antibody overnight at 4°C followed by 1 hr incubations with biotinylated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) and streptavidin-horseradish peroxidase (HRP). Control sections were incubated overnight with non-specific rabbit IgG. HRP reaction product was generated with diaminobenzidine as substrate, and all sections were counterstained with hematoxylin-eosin before coverslipping.

MUC5AC Real-time PCR

Total RNA was isolated and purified from lung tissues using the RNeasy purification kit (QIAGEN, Valencia, CA). Reverse transcriptase/polymerase chain reactions (RT-PCR) was performed on 2.5 μg of total RNA by using oligo(dT) primer, and complementary DNA (cDNA) corresponding to 250 ng of total RNA was amplified by real-time PCR with specific primers for *MUC5AC*. Primers used for *MUC5AC* detection were 5'-atcccacctcacatggagtc-3' and 5'-ggtgtcttttgtgggagagg-3'. Primers used for *β-actin* internal control were *β-actin* 5'-agtatcccgggtaacccttct-3', and *β-actin* 5'-agcagaaactgcaaagatcca-3'. Real-time PCR of *MUC5AC* and *β-actin* were performed in duplicate on an iCycler iQ Real-Time PCR detection system (Bio-Rad, Hercules, CA) using 0.5 μM (final concentration) of each primer, 2 μl of cDNA, and 10 μl of iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). Amplification conditions for all reactions were done at 95°C for 3 minutes, 95°C for 15 seconds, and 61°C for 45 seconds, for 40 cycles, followed by 80 steps of 10 seconds with a temperature gradient increase of 0.5°C per step from 55-95°C. This last step allowed the melting curve of the PCR products, and, consequently,

their specificity to be determined. For each run, a standard dilution of cDNA was used to check the relative efficiency and quality of primers. A negative control was included in all real-time PCR assays.

Statistical Analysis

Data were analyzed by ANOVA and Student's t-test.

References and Notes*:*

S1. L. Liu *et al.*, *Cell* **116**, 617 (2004).

S2. G. S. Whitehead, J. K. Walker, K. G. Berman, W. M. Foster, D. A. Schwartz, *Am J Physiol Lung Cell Mol Physiol* **285**, L32 (2003).

S3. S. H. Gavett *et al.*, *J Clin Invest* **104**, 721 (1999).

S4. B. Gaston *et al.*, *Proc Natl Acad Sci U S A* **90**, 10957 (1993).

S5. C. S. Hooker, P. J. Calkins, J. H. Fleisch, *Blood Vessels* **14**, 1 (1977).

S6. T. E. Bartell, W. W. Busse, *Allergy* **35**, 291 (1980).

S7. L. Kobzik *et al., Am J Respir Cell Mol Biol* **9**, 371 (1993).

S8. K. Asano *et al.*, *Proc Natl Acad Sci U S A* **91**, 10089 (1994).

SUPPLEMENTAL FIGURES

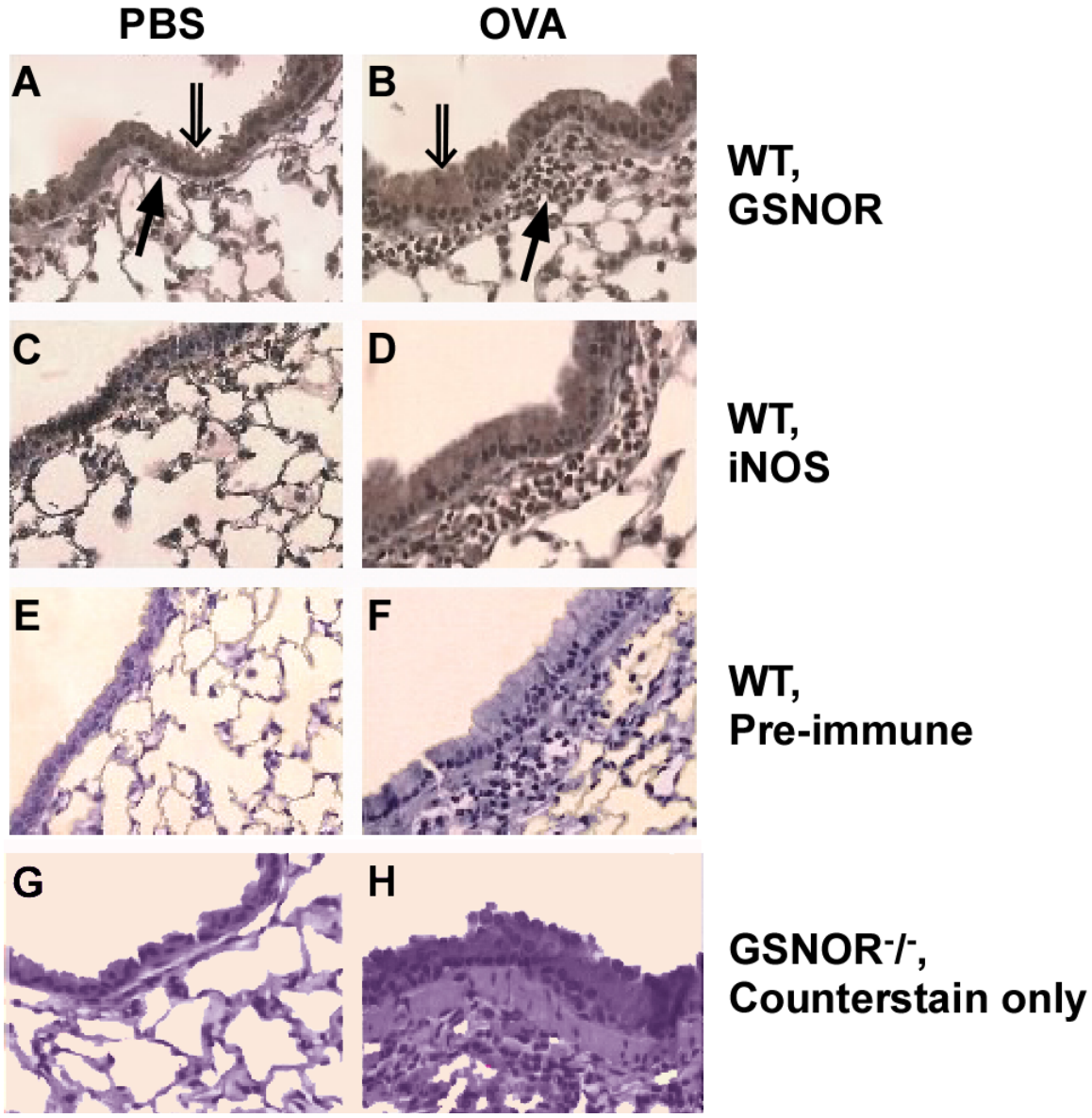

Fig. S1. Allergen-induced inflammation, and immunohistochemical distribution of GSNOR and iNOS, in the lungs of PBS- and ovalbumin (OVA)-treated WT and GSNOR$^{-/-}$ mice. All sections depicted were counterstained with hematoxylin-eosin, and are representative of results from six mice in each group. **A-D** GSNOR and iNOS co-localize

in lungs of both control (PBS-treated) and allergen-challenged (OVA-treated) WT mice, and both enzymes are particularly prominent in bronchial epithelial cells (open arrows). Constitutive expression in the lung of iNOS and of GSNOR is consistent with previous observations (*S1, S7, S8*). The abundance of both GSNOR and iNOS increases in WT lungs following OVA challenge, reflecting the presence of both enzymes in lung inflammatory cells (solid arrows), as well as the hyperplasia of enzyme-positive epithelial cells (open arrows). **E,F** No staining was detected in sections exposed to non-specific rabbit IgG. **G,H** Infiltration by inflammatory cells and epithelial cell hyperplasia, evident in hematoxylin-eosin stained sections, were indistinguishable in OVA-challenged WT and $GSNOR^{-/-}$ mice.

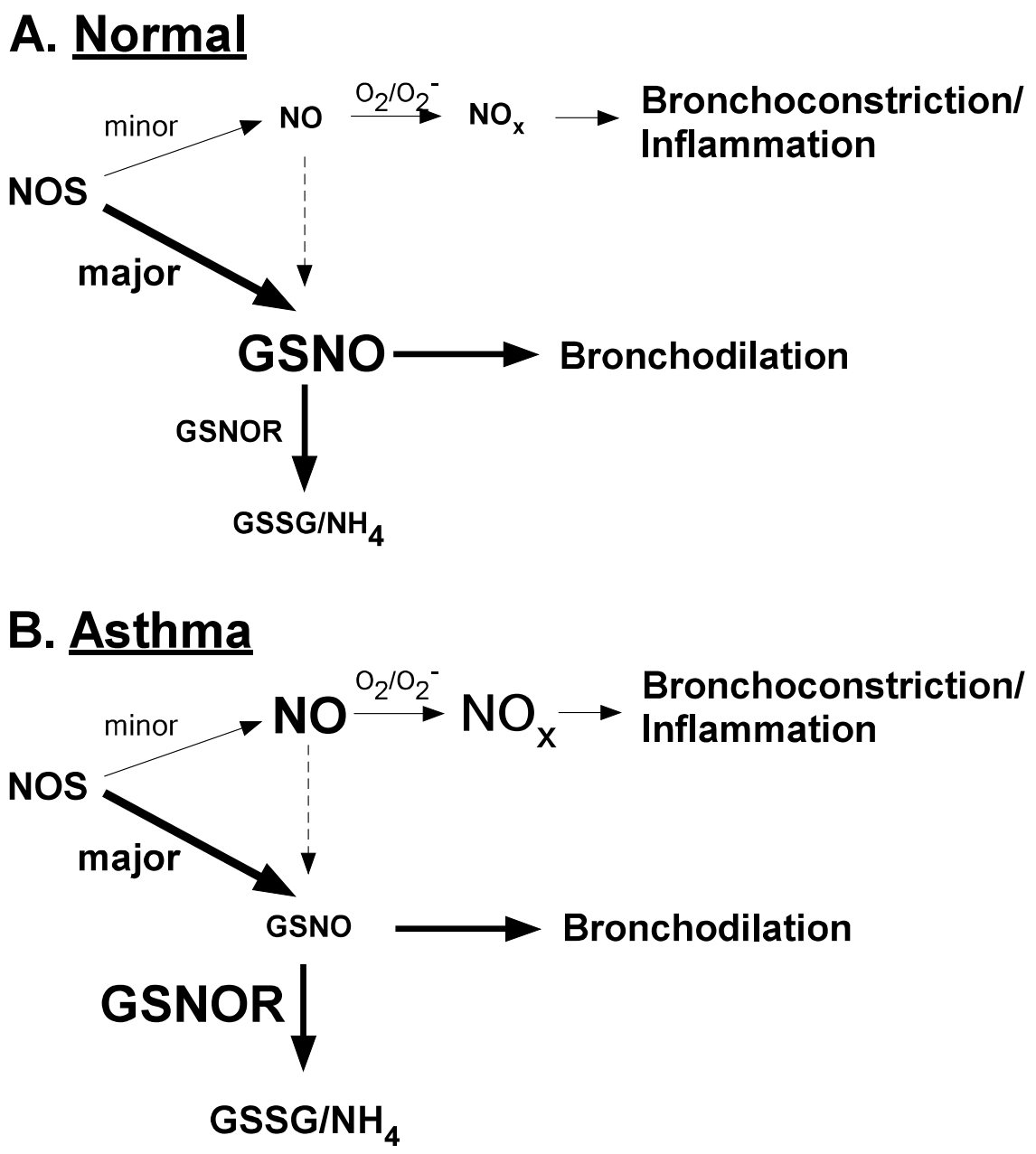

Fig. S2. Schematic illustration of NO/SNO metabolism in normal and asthmatic airways.

A. Normal, **B.** Asthma.