Expression of nitric oxide synthase-2 in the lungs decreases airway resistance and responsiveness

Josephine Hjoberg,1,2 Stephanie Shore,1 Lester Kobzik,1 Shoji Okinaga,3 Arlene Hallock,2 Joseph Vallone,1 Venkat Subramaniam,1 George T. De Sanctis,2 Jack A. Elias,4 Jeffrey M. Drazen,1,2 and Eric S. Silverman1,2

1Physiology Program, Department of Environmental Health, Harvard School of Public Health, 2Pulmonary and Critical Care Medicine, Brigham and Women’s Hospital, Harvard Medical School, and 3Perlmutter Laboratory, Children’s Hospital, Harvard Medical School, Boston, Massachusetts 02115; and 4Section of Pulmonary and Critical Care Medicine, Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut 06520

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THE AVERAGE CONCENTRATION of nitric oxide (NO) in exhaled air of untreated asthmatic patients is increased compared with individuals without asthma (1, 28, 34, 40). When measurements of NO are made with standardized methods based on chemiluminescence (2), individuals without asthma have a fraction of expired NO (FENO) that is usually between 5 and 15 parts per billion (ppb) (25). In contrast, untreated asthmatic patients have an FENO that is often >15 ppb, with values as high as 100 ppb in some individuals (19).

The anatomic, cellular, and molecular bases of increased NO in the exhaled air of asthmatic subjects are controversial; several tissue compartments and mechanisms are probably involved. Although NO is produced along the entire respiratory tract, it is likely that most of the increase originates from the lower airways (27, 35). NO may be produced in the airways by all three nitric oxide synthases (NOS-1, or neuronal; NOS-2, or inducible; NOS-3, or endothelial) as well as by enzymatic and nonenzymatic sources (15, 21, 29, 53). However, it is NOS-2, induced in airway epithelial cells by inflammatory cytokines, that is thought to be the major source of increased NO in asthmatic subjects (9, 16, 17). This belief is supported by studies with NOS-2 knockout mice. NOS-2 is a major source of NO in exhaled air in mice (49) and is the isoenzyme responsible for most of the increased NOS activity in allergic models of airway inflammation (7).

The roles of NO in airway homeostasis and the pathogenesis of asthma are even more controversial, but it is clear that NO is not just a marker of airway inflammation or hyperresponsiveness. NO is a weak bronchodilator and may have beneficial effects on pulmonary function in asthmatic patients by attenuating bronchoconstriction and airway reactivity. In contrast, NO may be toxic to airway tissues and may increase inflammation and airway hyperresponsiveness in asthmatic subjects (56). In short, it has been difficult to separate the effects of NO from all the inflammatory mediators expressed in the airways of asthmatic subjects. Studies with NOS inhibitors, NO donors, and NOS knockout mice have been helpful in defining some of the roles of NO; however, results have been conflicting (7, 55), and it is still unclear whether NO per se is primarily beneficial or harmful (6).

To explore the roles of NO in airway biology, we have developed an externally regulatable transgenic mouse (CC10-rtTA-NOS-2) capable of overexpressing NOS-2 in the airways and of increasing FENO to levels comparable to those of asthmatic subjects. The CC10-rtTA-NOS-2 mouse contains two transgenes: a reverse tetracycline transactivator under the control of the Clara cell protein promoter and the mouse nitric oxide synthase-2 (NOS-2) coding region under control of a tetracycline operator. This mouse presents an important advantage in that the CC10-rtTA-NOS-2 mouse contains two transgenes, a reverse tetracycline transactivator under the control of the Clara cell protein promoter and the mouse nitric oxide synthase-2 (NOS-2) coding region under control of a tetracycline operator. Addition of doxycycline to the drinking water of CC10-rtTA-NOS-2 mice causes an increase in nitric oxide synthase-2 that is largely confined to the airspace epithelium. The fraction of expired nitric oxide increases over the first 24 h from ∼10 parts per billion to a plateau of ∼20 parts per billion. There were no obvious differences between CC10-rtTA-NOS-2 mice, with or without doxycycline, and wild-type mice in lung histology, bronchoalveolar protein, total cell count, or count differentials. However, airway resistance was lower in CC10-rtTA-NOS-2 mice with doxycycline than in CC10-rtTA-NOS-2 mice without doxycycline or wild-type mice with doxycycline. Moreover, doxycycline-treated CC10-rtTA-NOS-2 mice were hyporesponsive to methacholine compared with other groups. These data suggest that increased nitric oxide in the airways has no proinflammatory effects per se and may have beneficial effects on pulmonary function.

asthma; inducible transgene; mouse; epithelial cell; methacholine
line lung structure and function. We hypothesized that the induction of NO in airway epithelium in these mice would have proinflammatory effects on the lung and cause an increase in airway responsiveness. Contrary to our expectations, we find that NO per se has no proinflammatory effects on the lung and decreases airway responsiveness.

MATERIALS AND METHODS

Generation and maintenance of the transgenic mouse. The mouse NOS-2 open reading frame was amplified from pMac-NOS by PCR with primers NOSacF 5'-CCGCGGAACGGAGAACGTTGGAT-TTG and NOXbaR 5'-TCTAGGAGGGAGGAGGAGGAGAG and pfu DNA polymerase (Stratagene, La Jolla, CA). The PCR product was agarose gel purified with QIAEX II gel extraction system (Qiagen, Valencia, CA), ligated into the pCR-Blunt II-TOPO (Invitrogen, Carlsbad, CA) and removed from the plasmid backbone by digestion with SacII and XhoI. The modified cDNA was gel agarose purified and ligated into the multiple cloning site of pTRE (BD Bioscience, Franklin Lakes, NJ) to form construct pTRE-NOS-2-SV40. pMac-NOS was obtained from James M. Cunningham at Brigham and Women's Hospital (32). Construct pCC10-tTA-hGH has been described in detail (42). All constructs were checked for orientation by restriction fragment digest and aDNA and sequenced. Constructs pTRE-NOS-2-SV40 and pCC10-tTA-hGH were linearized by digestion with XhoI and NotI and XhoI and HindIII, respectively, and purified by agarose gel electrophoresis and DEAE Elutip chromatography (Schleicher & Schuell, Keene, NH). Transgenic mice were prepared by simultaneously microinjecting both DNA fragments into the pronuclei of FVB mice (Charles River Laboratories, Wilmington, MA) by standard techniques. The presence or absence of the transgenes in offspring was determined by examining DNA prepared by X-ray film at ~80°C for 2–5 days. Autoradiographs were scanned and quantitated using Gel-Pro Analyzer densitometry software.

Present experiments were normalized for total protein content (10 μg) as measured by Bradford protein assay (Bio-Rad, Hercules, CA), separated by 8% SDS-polyacrylamide gel electrophoresis, and transferred to a polyvinylidene difluoride membrane. The membranes were probed with polyclonal antibodies to NOS-2 (Santa Cruz Biotechnology, Santa Cruz, CA) at dilutions of 1:1,000, followed by enhanced chemiluminescent detection (Amersham) with [α-32P]dCTP (DuPont, NEN, Boston, MA), using ExpressHyb hybridization solution (Clontech Laboratories, Palo Alto, CA) according to the manufacturer's instructions. The blots were washed with 0.1× SSC several times at 65°C and exposed to X-ray film at ~80°C for 2–5 days. The bands were scanned and quantitated using Gel-Pro Analyzer densitometry software. The evenness of protein loading was assessed by Coomasie blue staining.

Bronchoalveolar lavage, histological evaluation, and immunohistochemistry. After exsanguination, bronchoalveolar lavage (BAL) was performed with 35 ml/kg of PBS containing 0.6 mM EDTA, as previously described (47). Cells were pelleted by centrifugation at 600 g for 10 min at 4°C, and the supernatant was removed for analysis of protein by Bradford assay. The cell pellets were resuspended in 100 μl of red blood cell lysis buffer containing 0.15 M NH4Cl, 1.0 mM KHCO3, and 0.1 mM EDTA for 2 min at room temperature; diluted with 1 ml of PBS; and centrifuged at 600 g for 10 min at 4°C. The pellet was resuspended with 1 ml of cold PBS, and cells were counted with a hemocytometer. Differential counts were tabulated from cytocentrifuged prepared slides stained with H&E or Wright-Giemsa (Biochemical Sciences, Swedesboro, NJ).

After exsanguination, the lungs were inflated with 10% formalin at a pressure of 23 cmH2O, excised, fixed for 24 h in formalin, and stored in 70% ethanol. The fixed lungs were embedded in paraffin, cut in 5-μm sections, and stained with hematoxylin and eosin, periodic acid-Schiff (PAS)-Alcian blue (pH 2.5), and Masson trichrome. Sections were examined under light microscopy in a masked fashion and scored with a semiquantitative grading scale as previously described in detail (47).

Immunostaining for NOS-2 was performed on frozen lung sections. After exsanguination, lungs were inflated (35 ml/kg) with a 1:1 optimum cutting temperature medium and PBS mixture through a tracheal tube, embedded in optimum cutting temperature compound, immediately frozen in a 2-methylbutane-dry ice bath and stored at ~80°C until sectioning. Sections were cut to a thickness of 8 μm, fixed in 2% paraformaldehyde-methanol at 4°C for 10 min, and rehydrated in graded alcohols. The slides were blocked with 10% normal swine serum diluted in PBS with 2% bovine serum albumin. The primary antibody was rabbit polyclonal antiserum to NOS-2 (Upstate, Lake Placid, NY) at a dilution of 2 μg/ml. Nonspecific rabbit IgG was substituted for the primary antiserum as a negative control. After overnight incubation at 4°C, the slides were washed and inco-
bated in methanol containing 1% hydrogen peroxide for 10 min at room temperature to remove endogenous peroxidase activity. Immunopositivity was visualized with PBS containing 0.025% dianisobenzene and 0.1% hydrogen peroxide, after a standard peroxidase-anti-peroxidase protocol with swine anti-rabbit antibody (Dako, Santa Barbara, CA) as the secondary layer and rabbit peroxidase-anti-peroxidase complex (Dako) as the tertiary layer. Slides were counterstained with hematoxylin, dehydrated through graded alcohols and xylene, mounted, and coverslipped.

Measurement of NO in mice. The F\texttextsubscript{E}NO was measured by a modification of the technique described in detail by Weicker and colleagues (54). Briefly, unanesthetized and unrestrained mice were placed in an 85-ml sealed cylindrical Plexiglas chamber through which NO free air (BOC Gases, Port Allen, LA) was continuously flushed by a flow regulator (Dynamic Gas Calibration, model 146, Thermo Environmental Instruments, Woburn, MA) at a rate of 48 ml/min. This flow rate was chosen to approximate eucapnic ventilation of resting mice over the weight range studied (33, 50). The mice remained comfortable with this flow rate (54), and it allows for rough comparisons with F\texttextsubscript{E}NO reported in the literature. After a flushing and equilibrium period of 12 min, air passing out of the chamber was collected in a Mylar balloon for 6 min. The concentration of NO in the balloon was measured with a calibrated Sievers NO analyzer (model 280 ES, Boulder, CO). Two balloons were used for each mouse, and an average of the two measurements was used to express the NO concentration as ppb. The absence of leakage was confirmed by continuously monitoring the flow rate at the proximal and distal ends of the circuit and by frequent checks to make sure that an empty chamber yielded a concentration of NO < 1 ppb. The effect of NO produced from NOS-2 on airway physiology was evaluated by treating mice with the selective NOS-2 inhibitor 1400W (Calbiochem) at 10 mg/kg ip in 0.25 ml of pyrogen-free PBS for 4 days, with that treatment starting 1 day before dox treatment.

**Lung mechanics.** Mice were anesthetized with pentobarbital sodium (100 mg/kg ip), tracheostomized with a 20-gauge cannula, and mechanically ventilated with a small animal ventilator (flexiVent, SCIREQ, Montreal, PQ, Canada) at a tidal volume of 0.3 ml, a frequency of 2.5 Hz, and fraction of inspired O\textsubscript{2} of 0.21 (room air). SCIREQ, Montreal, PQ, Canada) at a tidal volume of 0.3 ml, a frequency of 2.5 Hz, and fraction of inspired O\textsubscript{2} of 0.21 (room air). Continuous monitoring of the flow rate at the proximal and distal ends of the circuit yielded a concentration of NO < 1 ppb. The effect of NO produced from NOS-2 on airway physiology was evaluated by treating mice with the selective NOS-2 inhibitor 1400W (Calbiochem) at 10 mg/kg ip in 0.25 ml of pyrogen-free PBS for 4 days, with that treatment starting 1 day before dox treatment.

**Generation of the CC10-rtTA-NOS-2 mice.** Two constructs were used to generate transgenic mice that would allow external regulatable expression of NOS-2 in the airway epithelium: 1) pCC10-rtTA-hGH, which consists of a reverse tetracycline transactivator under the transcriptional control of the rat Clara cell 10-kDa protein promoter (42); and 2) pTRE-NOS-2-SV40, which consists of the mouse NOS-2 cDNA under the transcriptional control of a tetracycline operator and minimal cytomegalovirus promoter (TetO/Pcmv min) (Fig. 1A). The constructs were injected simultaneously into the pronuclei of FVB mice by standard transgenic techniques and resulted in 21 offspring. One mouse with both constructs was bred onto an FVB background to generate heterozygous (H) transgenic offspring and transgene negative-wild-type (W) offspring as genotyped by PCR (Fig. 1B). The constructs appear to have been inserted in tandem by Southern blot analysis, and recombination events separating the two transgenes have not been detected in more than 100 offspring from five generations of H × W crosses (data not shown). The randomly inserted transgenes do not appear to confer a selective disadvantage, and the distribution of offspring was ~50% W and 50% H.

**Induction of NOS-2 in airway epithelium.** The proper functioning of CC10-rtTA-NOS-2 mice was determined by exposing adult (8 wk) H mice to dox (0.5 mg/ml) water for 3 wk and comparing them with similarly housed adults H mice given plain water. The effects of dox treatment were controlled for by also making comparisons with adult W mice exposed to dox water for 3 wk. We first measured NOS-2 mRNA by Northern blot analysis from whole lungs obtained at the end of the 3-wk treatment period. NOS-2 mRNA was not detected in the W mice with dox or the H mice without dox (Fig. 2A). However, H mice with dox had an NOS-2 mRNA signal at ~4,500 bp, consistent with NOS-2 transgene expression. Dox had no effect on W mice for any outcome (data not shown). These data indicate that dox induced NOS-2 mRNA in the CC10-rtTA-NOS-2, whereas NOS-2 mRNA was not detected in W mice receiving dox or CC10-rtTA-NOS-2 mice not receiving dox.

To detect the presence of a transgenic leak of NOS-2 mRNA (i.e., NOS-2 expression in the absence of dox), we performed the more sensitive RT-PCR technique on the whole-lung RNA samples. After 32 cycles of PCR, a faint NOS-2-specific amplicon was detected from the lungs of H mice without dox, whereas no amplicon was detected from the lungs of W mice with dox (Fig. 2B). The same RT-PCR protocol produced an intense amplicon from the lungs of H mice with dox. Thus there appears to be a small amount of transgenic NOS-2 mRNA leak in CC10-rtTA-NOS-2 mice without dox.

To detect induction of NOS-2 protein, we performed Western blot analysis on protein extracts made from whole lungs of H mice with dox and compared them with the two control groups. We found an intense band at ~130 kDa from lungs of
H mice with dox that is consistent with NOS-2 (Fig. 2C). In contrast, no band was detected from lungs of H mice without dox or W mice with dox. These data indicate that NOS-2 protein is increased in the lungs of CC10-rtTA-NOS-2 mice with dox but that NOS-2 protein remains undetectable in W mice with dox or in CC10-rtTA-NOS-2 mice without dox, even with the small amount of transgenic leak detected by RT-PCR.

Immunohistochemistry with an antibody to NOS-2 protein was performed on lung sections of the CC10-rtTA-NOS-2 mice to better characterize the induction and tissue-specific expression of the transgene. After 3 wk of dox treatment, H mice showed intense NOS-2 staining of the epithelium of large and small airways (Fig. 3, A–C). The staining of epithelial cells was heterogeneous, with about 50% of the epithelial cells having the characteristic brown appearance of positive cells. This percentage is roughly equivalent to the percentage of Clara cells in the mouse airway epithelium (10). Occasional staining was detected in alveolar epithelial cells and alveolar macrophages (Fig. 3C). In contrast, in the absence of dox, H mice had minimal staining of the airway epithelium or distal lung (Fig. 3D). W mice with (Fig. 3E) or without dox (data not shown) had no detectable NOS-2 staining in their airways or distal lung, except for a few alveolar macrophages. Controls demonstrated the absence of staining with serum IgG as the primary antibody (Fig. 3F). These data indicate that NOS-2-immunoreactive protein is dramatically increased in the lungs of CC10-rtTA-NOS-2 mice treated with dox compared with W mice with dox and CC10-rtTA-NOS-2 mice without dox. Moreover, NOS-2 immunostaining is confined primarily to airway epithelial cells, a pattern of tissue-specific expression consistent with transgenics using the Clara cell 10-kD protein promoter (42).

Regulatable levels of exhaled NO. The concentration of NO in expired air was measured to determine the enzymatic activity of NOS-2 overexpressed in the airways of CC10-rtTA-NOS-2 mice treated with dox. The baseline FE NO of H mice without dox (10.3 ± 1.3 ppb, n = 7) was slightly higher than that of W mice with dox (7.2 ± 0.5 ppb, n = 18, P = 0.003) (Fig. 4). Addition of dox to the drinking water of cages resulted in a significant increase in FE NO by 24 h (18.4 ± 1.6 ppb) that was sustained to at least 120 h (21.5 ± 1.1 ppb, n = 4 in each group, P < 0.001). Removal of dox from the drinking water of H mice resulted in a significant decline in FE NO by 12 h (14.2 ± 0.6 ppb, n = 4, P < 0.001) and a return to baseline values by 48 h.

The NOS-2-specific inhibitor 1400W was used to verify the source of the increased FE NO (38). When 1400W was administered to W mice with dox for 4 days, beginning 1 day before dox treatment, baseline FE NO decreased slightly but not significantly, compared with that of W mice with dox without 1400W (5.0 ± 1.4 ppb, n = 6 vs. 7.2 ± 0.5 ppb, n = 18, respectively, P = 0.08) (Fig. 4B). In contrast, when 1400W was administered to H mice with dox by the same protocol, FE NO was significantly decreased compared with levels in H mice with dox.
Effects of NO induction on lung histology. We speculated that the induction of NO in airway epithelium has proinflammatory effects on the lung. To test this hypothesis, we obtained lung tissue and BAL from H mice given dox for up to 3 wk and compared these specimens with identically processed specimens from W mice with dox and H mice without dox. We found no differences in inflammation as assessed by hematoxylin and eosin stain, and the lungs from H mice with dox appeared normal (data not shown). There were no differences in mucus production as assessed by PAS stain or in airway fibrosis as assessed by trichrome stain (data not shown). We could find no increases in the level of 3-nitrotyrosine by immunohistochemistry after NO induction per se (data not shown). Furthermore, there were no differences in total cell counts, cell differential, or total protein in BAL (Table 1). These data suggest that the induction of NO per se in the airway epithelium for up to 3 wk does not cause lung inflammation, fibrosis, mucus hypersecretion, or increased vascular permeability.

Effect of NO induction on lung mechanics. Because induction of NO does not cause inflammation and FeNO levels are relatively low, we hypothesized that NO induction in the normal mouse lung (i.e., in the absence of airway inflammation) would have no effect on the resistive, dissipative, or elastic properties of the lung. To test this hypothesis, we measured and compared total Rt as a function of ventilation frequency in H mice with dox for 3 days, H mice without dox, and W mice with dox for 3 days. Responses at low frequencies provide specific information about the tissue component of lung resistance, and responses at high frequencies allow for the estimation of Raw. There were no significant differences in Rt at lower frequencies between the three groups (Fig. 5A). There were also no differences in the imaginary part of impedance (Fig. 5A, which reflects lung compliance at frequencies below 20 Hz in the mouse). Gt, Ht, and hysteresivity, as calculated in accordance with the constant-phase model, were unaffected by NO induction (Table 2). However, at higher frequencies, Rt (and thus Raw) for H mice with dox was significantly lower than that for the other groups (Fig. 5). Raw for H mice with dox (0.26 ± 0.02 cmH2O·ml⁻¹·s⁻¹, n = 14) was lower than that for H mice without dox (0.42 ± 0.03 cmH2O·ml⁻¹·s⁻¹, n = 12) (P = 0.004) or W mice with dox (0.38 ± 0.05 cmH2O·ml⁻¹·s⁻¹, n = 11) (P = 0.018). There was no difference between H mice without dox and W mice with dox (P = 0.45). Moreover, the reduction in Raw in H mice with dox was abolished after their treatment with the NOS-2-specific inhibitor 1400W (n = 9, P = 0.94) (Table 2, Fig. 5B). These data indicate that NO induction in the airways per se decreases baseline airway resistance but has no significant effects on the dissipative and elastic properties of the normal mouse lung.

Effect of NO induction on airway responsiveness to methacholine. Because NO decreases Raw, we hypothesized that NO induction would decrease airway responsiveness to bronchoconstricting agonists. To test this hypothesis, we measured peak Rt at a ventilation frequency of 2.5 Hz after intravenous injection of methacholine and plotted the peak Raw of increasing methacholine dose for each mouse group. The dose-response curve showed a decrease in sensitivity (shift to right) and reactivity (decreased maximal response) to methacholine with a significantly lower Rt at doses 0.3, 1, and 3 mg/kg for H mice with dox (n = 14) compared with H mice without dox (n = 12, P = 0.006, 0.009, and 0.036, respectively) and W mice with dox (n = 11, P = 0.006, 0.001, and <0.001, respectively) (Fig. 6). Moreover, treatment of H mice with dox with 1400W (n = 9) shifted the dose-response curve toward values in W mice (P = 0.81) and H mice without dox (P = 0.18) but away from H mice with dox alone (P < 0.001), indicating that the induction of NO in CC10-rtTA-NOS-2 mice
decreases airway sensitivity and reactivity to methacholine. Peak R plotted as percent change from baseline as a function of increasing methacholine dose for each group shows a similar decrease in the H mice with dox group (data not shown).

DISCUSSION

To study the effects of NO on airway structure and function, we produced a transgenic mouse based on the CC10-rtTA system (42) that allows for the airway-specific and externally regulatable overexpression of NOS-2. A major advantage provided by this mouse is that NO induction per se can be studied in the absence of the many inflammatory mediators generally found at increased levels in the airways of asthmatic subjects. Moreover, studies with these mice are not confounded by the untoward effects of NO donors or NOS inhibitors or the developmental abnormalities found in mice with targeted deletions of NOS genes (20, 31). We hypothesized that the induction of NO in airway epithelium in these mice would have proinflammatory effects on the lung and cause an increase in airway responsiveness to methacholine. Contrary to our expectations, we find that NO per se has no proinflammatory effects on the lung and decreases airway responsiveness.

We chose to overexpress NOS-2 because it is increased in the airways of asthmatic subjects and is thought to be a major source of increased FeNO in asthma (17, 24). It is also the isoenzyme responsible for the greatest increase in NOS activity in mice and other animals with allergen-induced airway inflammation (7, 11). The CC10 promoter was chosen because it confers tissue-specific expression that is largely confined to airway epithelium (10, 41), the principal site of NOS-2 induc-

Fig. 3. Immunohistochemical localization of inducible NOS-2 in the airways. A–C: representative images from heterozygous H mice with dox for 3 wk: transverse section of bronchiole showing intense NOS-2 staining (brown) of many epithelial cells (×100) (A), transverse section of a bronchiole (×200) (B), and distal lung showing staining of a few cells (×200) (C). D: faint NOS-2 staining in some epithelial cells of the bronchiole of H mice without dox (×100). E: NOS-2 staining was not discernable in bronchiolar epithelial cells of W mice with dox for 2 wk (×100). F: IgG control shows no staining of bronchiolar epithelium in lungs of H mice with dox for 3 wk (×100).
tion and NO production in asthmatic subjects (17, 24, 35). This is desirable because the biological effects of NO are now known to be highly dependent on the cellular compartments where NO is elaborated (48). There are other sources of NO, both enzymatic and nonenzymatic, and other tissue compartments that may contribute to the increased $FE_{NO}$ in asthmatic subjects (21, 58), but, given the freely diffusible nature of NO and the high levels of NO generated by NOS-2 (nanomoles) relative to NOS-1 and NOS-3 (picomoles), we believe that these alternative sources of NO are unlikely to have much impact on airway structure and function once NOS-2 is overexpressed.

The CC10-rtTA-NOS-2 mouse has the following seven fundamental characteristics:

1) NOS-2 mRNA and protein are induced in airway epithelium, with a high degree of special selectivity, by the addition of dox (0.5 mg/ml) to drinking water (Figs. 2 and 3);
2) induction is associated with a relatively rapid (within 24 h) increase in $FE_{NO}$ that is reversed (within 48 h) by the removal of dox (Fig. 4);
3) there is a small amount of transgenic leak as detected by RT-PCR and $FE_{NO}$ increases 2.5-fold with dox (n = 13). The specific NOS-2 inhibitor 1400W significantly attenuated the effect of dox (n = 9, P values are shown).

Table 1. Cell count, differential, and total protein in bronchoalveolar lavage

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Total Cell Count, $\times 10^5$/ml</th>
<th>Macrophage, %</th>
<th>Lymphocyte, %</th>
<th>Neutrophil, %</th>
<th>Total Protein, mg/ml</th>
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<tbody>
<tr>
<td>W + dox</td>
<td>284±40</td>
<td>96.5±1.3</td>
<td>2.0±0.5</td>
<td>1.6±1.0</td>
<td>0.23±0.01</td>
</tr>
<tr>
<td>H</td>
<td>319±17</td>
<td>92.0±2.0</td>
<td>5.0±2.5</td>
<td>2.9±2.2</td>
<td>0.26±0.01</td>
</tr>
<tr>
<td>H + dox</td>
<td>328±20</td>
<td>96.0±1.0</td>
<td>2.0±0.5</td>
<td>2.0±0.5</td>
<td>0.25±0.01</td>
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Values are means ± SE; n = 10 in each group. There are no significant differences between wild-type (W) mice with doxycycline (dox), heterozygous (H) mice without dox, or H mice with dox.
(Figs. 2B and 4B), but, in the absence of dox, \(F_{\text{ENO}}\) levels are only slightly elevated compared with W mice and approximate those found in humans without asthma (Fig. 4B); 4) in the presence of dox, \(F_{\text{ENO}}\) increases and approaches values found in many asthmatic subjects (Fig. 4B); 5) NO induction results in a decline in baseline airway resistance but in no change in the dissipative or elastic properties of the lung (Fig. 5A, Table 2); 6) NO induction causes a decrease in airway responsiveness to methacholine (Fig. 6); and 7) induction of NO for up to 3 wk does not cause lung in\(flammation\) (Table 1). All experiments involved heterozygous CC10-rtTA-NOS-2 mice and comparisons to two controls, W with dox and H mice without dox. Dox has no effect on lung function in W mice (data not shown). This ensures that observations reflect NO induction and not the effects of dox or randomly inserted transgenes.

To our knowledge, this is the first report of NOS-2 overexpression in airway epithelium in vivo. NOS-2 is thought to be a major source of increased NO in exhaled air of asthmatic...
subjects for the following reasons: 1) NOS-2 mRNA, protein, and activity are dramatically induced in airway epithelial cells by inflammatory cytokines known to be increased in the airways of asthmatic subjects (9); 2) NOS-2 immunoreactivity is greater in airway epithelial cells in biopsies from asthmatic subjects than in those from nonasthmatic controls (16, 17); 3) selective inhibitors of NOS-2 decrease FeNO in asthmatic subjects (57); and 4) corticosteroids decrease FeNO and NOS-2 levels in the airways of asthmatic subjects (26). We now show that NOS-2 expression and FeNO of CC10-rtTA-NOS-2 mice with and without dox are strikingly similar to those reported for asthmatic and nonasthmatic subjects (17, 19), respectively. These data provide new evidence in support of the role of the constant-phase model of lung mechanics, or sensitization and challenge protocols.

In contrast to our original hypothesis, our studies with CC10-rtTA-NOS-2 mice show that NO has beneficial effects on airway function per se when increased to levels typically associated with asthma by NOS-2 induction in airway epithelium. Specifically, the constant-phase model of lung mechanics indicated that H mice with dox had an ~38% reduction in baseline Raw compared with controls but no change in the dissipative (Gl) or elastic (Hl) properties of the lung. Of note, we could find no significant differences in enhanced pause between mouse groups (37). Impedance data fitted to the constant-phase model allow for a distinction between central and peripheral lung events. Raw is a measure of central (large) airway resistance, and our study demonstrates that NO produced by NOS-2 induced in airway epithelial cells decreases Raw. Gl reflects changes in physical properties of tissues in the peripheral lung or regional airway heterogeneity. Acute changes in Hl are likely to reflect lung derecruitment (airway closure) and chronic changes in Hl are likely to reflect an alteration of the mechanical properties of the peripheral lung parenchyma (12, 22). Our data suggest that NO produced by NOS-2 induced in airway epithelial cells has no effect on these peripheral properties of the lung. The small amount of transgenic leak in H mice in the absence of dox had no effect on lung function. These findings and the absence of differences between groups in intraluminal content of the airways (i.e., mucus production by PAS staining, BAL cell count, and BAL total protein) suggest that the principal effect of NO on lung mechanics is the relaxation of smooth muscle surrounding the airways. The mechanism may involve activation of soluble guanylate cyclase and increased formation of cGMP (56). The decreased airway responsiveness to methacholine of H mice and some human studies (15, 36, 39). However, other studies suggest that NO has no effect on airway tone and responsiveness and that it may even enhance airway responsiveness (28, 38, 51). Some of these inconsistencies are probably related to differences in reagents (e.g., type of NO donor or NOS inhibitor), animal species, bronchoconstricting agonists, technique for measuring lung mechanics, or sensitization and challenge protocols.

Table 2. Lung mechanics

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Raw, cmH2O·ml−1·s−1</th>
<th>Gl, cmH2O·ml</th>
<th>Hl, cmH2O·ml</th>
<th>η</th>
</tr>
</thead>
<tbody>
<tr>
<td>W + dox</td>
<td>0.38±0.05</td>
<td>3.6±0.1</td>
<td>19±1.6</td>
<td>0.19±0.02</td>
</tr>
<tr>
<td>H</td>
<td>0.42±0.03</td>
<td>3.5±0.2</td>
<td>21±1.3</td>
<td>0.18±0.04</td>
</tr>
<tr>
<td>H + dox</td>
<td>0.26±0.02*</td>
<td>4.2±0.3</td>
<td>21±4.5</td>
<td>0.19±0.05</td>
</tr>
<tr>
<td>H + dox + 1400W</td>
<td>0.38±0.04</td>
<td>4.2±0.4</td>
<td>21±4.5</td>
<td>0.19±0.05</td>
</tr>
<tr>
<td>W + dox + 1400W</td>
<td>0.38±0.05</td>
<td>4.2±0.4</td>
<td>21±4.5</td>
<td>0.19±0.05</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 9 in each group. Raw, airway resistance; Gl, coefficient of tissue resistance; Hl, elastance; η, hysteresivity. *Raw of H mice given dox is significantly lower than that for other groups.
receiving dox may be due to a decrease in baseline Raw or to effects in the cholinergic pathway (8). Treatment of CC10-tTA-NOS-2 mice with dox for up to 3 wk had no discernable proinflammatory effect as assessed by lung histology, BAL cell count, and BAL total protein. In subsequent studies, histological analyses of mice at 10 wk with dox did not show discernable proinflammatory effects (data not shown). This was an unexpected finding because NO per se is thought to generate and perpetuate airway inflammation in asthmatic subjects (3). For example, NO has been shown to damage airway epithelium, increase mucus secretion, increase microvascular leak and plasma exudation into the airways (30), reduce eosinophil apoptosis, increase eosinophil recruitment to the airways (11), and skew lymphocytes toward a Th2 phenotype (4). Although our data suggest that endogenous NO produced by NOS-2 in airway epithelium per se has no proinflammatory effects, it is likely that NO can act in concert with other prophlogistic stimuli to elicit airway inflammation. The generation of peroxynitrite, a strong oxidizing agent, by the reaction of NO and superoxide anion, is an example of a potentially important interaction that may be necessary for inflammation (44, 45). It is possible that concurrent inflammation, longer periods of NO induction, or a different genetic background could modify the effects of NO on lung homeostasis in our mouse.

In summary, we have produced a novel transgenic mouse that can overexpress NOS-2 in airway epithelium and increase FENO to levels observed in asthmatic subjects. Using this model, we have shown that induction of NO may have beneficial effects on lung function by decreasing Raw and responsiveness to methacholine and that endogenous NO per se does not cause airway inflammation. These findings are contrary to some studies suggesting that endogenous production of NO is insufficient to achieve bronchodilatation (14) and that NO by itself can cause airway inflammation (4). Transgenic models controlling the expression of NOS enzymes in cellular compartments may help define the role of NO in lung homeostasis and disease.

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REFERENCES