NOS2 regulation of LPS-induced airway inflammation via S-nitrosylation of NF-κB p65

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Kelleher ZT, Potts EN, Brahmajothi MV, Foster MW, Auten RL, Foster WM, Marshall HE. NOS2 regulation of LPS-induced airway inflammation via S-nitrosylation of NF-κB p65. Am J Physiol Lung Cell Mol Physiol 301:L327–L333, 2011. First published July 1, 2011; doi:10.1152/ajplung.00463.2010.—Inducible nitric oxide synthase (NOS2) expression is increased in the airway epithelium of acute inflammatory disorders although the physiological impact remains unclear. We have previously shown that NOS2 inhibits NF-κB (p50-p65) activation in respiratory epithelial cells by inducing S-nitrosylation of the p65 monomer (SNOP65). In addition, we have demonstrated that mouse lung SNOP65 levels are acutely depleted in a lipopolysaccharide (LPS) model of lung injury and that augmenting SNOP65 levels before LPS treatment results in decreased airway epithelial NF-κB activation, airway inflammation, and lung injury. We now show that aerosolized LPS induces NOS2 expression in the respiratory epithelium concomitantly with an increase in lung SNOP65 levels and a decrease in airway NF-κB activity. Genetic deletion of NOS2 results in an absence of SNOP65 formation, persistent NF-κB activity in the respiratory epithelium, and prolonged airway inflammation. These results indicate that a primary function of LPS-induced NOS2 expression in the respiratory epithelium is to modulate the inflammatory response through deactivation of NF-κB via S-nitrosylation of p65, thereby counteracting the initial stimulus-coupled denitrosylation.

S-nitrosothiol; nitric oxide synthase; nuclear factor-κB; acute lung injury

NITRIC OXIDE (NO) is known to be a critical factor in the pathogenesis of inflammatory lung diseases, including acute lung injury (ALI) (27). All three nitric oxide synthase (NOS) isoforms are expressed in the lung (20), although the majority of NO production in the inflammatory state derives from inducible NOS (NOS2) (27). In animal models of ALI, a variety of insults (e.g., hyperoxia, sepsis, mechanical stretch injury) elicit an increase in airway NOS2 expression, suggesting that NOS2 serves a common role in multiple types of lung injury (19, 21, 32). A number of studies have suggested that NOS2 is predominantly proinflammatory in ALI, ostensibly because of indiscriminate generation of deleterious reactive nitrogen species (RNS) (8, 21, 32). However, the anti-inflammatory effects of NOS2 are increasingly recognized (19, 38) and likely offset any impact of RNS-mediated injury.

Protein S-nitrosylation has emerged as the predominant molecular mechanism underlying NOS2-dependent inhibition of immune response pathways (6, 31). This paradigm is best exemplified by the NF-κB transcription factor pathway, within which a number of proteins modified by S-nitrosylation have been identified (17, 25, 33). We have previously shown that cytokine-induced NOS2 expression in the respiratory epithelium and macrophages inhibits NF-κB activity via S-nitrosylation of the p65 (SNOP65) subunit of the p50-p65 heterodimer (17). S-nitrosylation of p65 inhibits p50-p65 DNA binding, leading to the reduced expression of κB-dependent inflammatory mediators, including NOS2 itself. However, evidence that NOS2 inhibits NF-κB via S-nitrosylation in animal models of inflammatory lung disease is lacking.

Recently, we established that S-nitrosothiols (SNOs) are acutely depleted in both airway fluid and lung tissue in a lipopolysaccharide (LPS) mouse model of ALI (24). We further demonstrated that the decrease in SNOs leads to denitrosylation of NF-κB p65, activation of the NF-κB in the respiratory epithelium, and initiation of the pulmonary inflammatory response. Augmentation of airway SNOs and lung tissue SNO-p65 levels by treatment with ethyl nitrite (ENO), before LPS administration, attenuates the inflammatory response and protects from the development of lung injury (24), highlighting the importance of airway SNO homeostasis and NF-κB protein denitrosylation in the pathogenesis of ALI. However, the endogenous mechanism(s) that restore lung SNO-p65 levels after LPS-induced denitrosylation have not been elucidated. In the present study, we show that LPS-induced airway epithelial NOS2 expression functions to resolve lung inflammation by mediating S-nitrosylation of NF-κB p65, thereby attenuating NF-κB activity and inhibiting continued κB-dependent proinflammatory gene expression. These findings support an anti-inflammatory role for NOS2 in the lung and provide further evidence that SNOs and protein S-nitrosylation serve to inhibit lung inflammation.

MATERIALS AND METHODS

Animal exposure. Six- to eight-week old, male C57BL/6J and NOS2 null (Nos2−/−; B6.129P2-Nos2tm1Lau) mice were purchased from Jackson Laboratories (Bar Harbor, ME). All procedures were approved by the Duke University institutional animal care and use committee. Animals were exposed to aerosolized LPS (0111:B4 Escherichia coli LPS, 4 μg/ml × 2.5 h) or PBS as previously described (14). Mice were killed by CO2 narcosis followed by thoracotomy at 1 or 6 h after completion of the aerosol treatment.

Lung samples. Whole lung lavage and lung tissue extraction were performed as previously described (24). After being harvested, lungs were frozen in liquid N2 for later preparation of tissue homogenates. Cell counting of the pooled bronchoalveolar lavage fluid (BALF) was performed with a hemocytometer, and cell differentials were determined on stained cytopsin preparations. The BALF was centrifuged at 1,500 g for 10 min to collect cells, and the protein concentration of the BALF supernatant was determined by BCA assay (Pierce Biotech-
nology, Rockford, IL). BALF supernatant was stored at −80°C for cytokine analysis.

Mouse lung extracts (MLE) were prepared by mechanical homogenization of lung tissue with a mortar and pestle in liquid N2 followed by resuspension of the powdered tissue in two volumes of hypotonic buffer [10 mM HEPES, pH 7.9, 15 mM KCl, 2 mM MgCl₂, 0.5 mM EDTA, 0.6% Nonidet P-40, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] and incubation on ice for 10 min. Nuclei were collected by centrifugation at 3,500 g for 20 min, and the supernatant (cytoplasmic extract) was removed. The nuclear pellet was resuspended in hypotonic lysis buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 25% glycerol, 2 mM MgCl₂, 0.5 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM benzamadine, and 0.5 mM PMSF) and placed on ice for 20 min to complete lysis. Nuclear debris was removed by centrifugation (15,000 g for 20 min), and the supernatant (nuclear extract) was collected. Protein concentration in the extracts was determined by BCA.

**Cytokine analysis.** A fluorescent bead immunoassay (Bio-Plex; Bio-Rad Laboratories, Hercules, CA) was used to quantify the concentrations of murine tumor necrosis factor (TNF)-α, KC, interleukin-6 (IL-6), macrophage inflammatory protein (MIP)-1α, granulocyte macrophage colony-stimulating factor (GM-CSF) in BALF. Samples were measured in duplicate (n = 5 animals/treatment group).

**Western blotting.** Equivalent protein concentration of MLE was separated by SDS-PAGE and transferred to nitrocellulose, and blots were probed with 1:50,000 of a rabbit polyclonal antibody to NOS2 (Millipore, Billerica, MA). After probing with a horseradish peroxidase-conjugated 2nd antibody (sc-2054; Santa Cruz Biotechnology, Santa Cruz, CA), immunoreactivity was detected by enhanced chemiluminescence.

**Detection of S-nitrosylated NF-κB p65.** A biotin switch assay was performed on freshly prepared MLE as previously described (24). Protein lysate (750 μg) was diluted in HEN buffer (250 mM HEPES-NaOH, pH 7.7, 1 mM EDTA, and 0.1 mM neocuproine), and free thiols were blocked by the addition of 3 mM MMTS and 1% SDS, followed by incubation at 50°C for 30 min. After acetone precipitation and resuspension in HENS buffer (HEN + 1% SDS), SNO proteins were labeled by 1 h incubation with 50 mM sodium ascorbate and 50 μg/ml biotin-HPDP (Pierce Biotechnology). Negative controls omitted ascorbate. Biotinylated proteins were precipitated by overnight incubation (4°C) with Neutravidin agarose (Pierce Biotechnology). After extensive washing, proteins were eluted from the beads by heating to 95°C in Lamelli buffer. Protein eluate and input lysate were separated by SDS-PAGE followed by Western blotting for NF-κB p65 (sc-372, rabbit polyclonal antibody; Santa Cruz Biotechnology). SNO-p65/total p65 ratios were determined by densitometry.

**NF-κB activity assay.** Nuclear protein binding to a consensus NF-κB oligonucleotide was determined using an ELISA-based assay (TransAm p65; Active Motif, Carlsbad, CA). Absorbance was read at 450 nm with samples appropriately blanked. Results were standardized to a positive control (cytokine-stimulated Jurkat cell lysate) that was provided by Active Motif.

**Immunohistochemistry.** Lungs were inflation fixed in situ at 25 cm H₂O with 4% paraformaldehyde and embedded in paraffin, and 5-μm-thick sections were prepared. After probing with a horseradish peroxidase-conjugated 2nd antibody (sc-2054; Santa Cruz Biotechnology, Santa Cruz, CA), immunoreactivity was detected by enhanced chemiluminescence.

**Data analysis.** Data are expressed as means ± SE. Significant differences between groups were identified by Student’s t-test.
RESULTS

Aerosolized LPS induces NOS2 expression in the lung airway. Both systemic and direct airway administration of LPS are known to augment NOS2 expression in the lung (4, 21). To ascertain the temporal association between LPS exposure and lung NOS2 expression in our ALI model, NOS2 levels were probed in MLE from animals treated with aerosolized PBS or LPS for 2.5 h and analyzed at 1 or 6 h postexposure. NOS2 protein was not observed in the lungs of PBS controls and was minimally detectable in LPS-treated mice at 1 h postexposure (Fig. 1A). However, prominent NOS2 induction was observed in the lung at 6 h post-LPS exposure, which was consistent with the timing of NOS2 expression seen in other LPS lung injury models (21, 38).

To determine the specific lung cell type(s) responsible for the increase in NOS2 expression in response to LPS, we performed immunochemical staining for NOS2 in mouse lung sections. NOS2 immunoreactivity was not observed in lung sections from the saline controls, consistent with low basal NOS2 expression (Fig. 1B). However, at 6 h post-LPS exposure, NOS2 expression was evident in the airway epithelium but not in inflammatory cells or endothelium. These findings are consistent with the timing of expression in cultured respiratory epithelial cells after cytokine stimulation (17) and indicate that the respiratory epithelium is the primary source of lung NOS2 activity in our aerosolized LPS model of ALI.

Airway inflammation is enhanced in NOS2−/− mice. Previous studies utilizing either NOS inhibitors or NOS2−/− mice have indicated that NOS2 can have either a pro- or anti-inflammatory role in ALI (29, 38). To determine the effect of NOS2 activity on the inflammatory response in our ALI model, cell counts and differentials were performed on BALF recovered from wild-type (WT) and NOS2−/− mice at 1 and 6 h after LPS exposure. A rapid increase in airway macrophages and neutrophils was seen in response to LPS in both WT and NOS2−/− mice at 1 h postexposure (Fig. 2). Although no difference in airway WBC counts was seen in the NOS2−/− vs. WT mice at 1 h postexposure, airway white blood cells (WBCs) remained significantly elevated in the NOS2−/− compared with WT mouse at 6 h post-LPS (Fig. 2). In addition, whereas airway inflammation appeared to have peaked in the WT mice between 1 and 6 h postexposure, airway WBC counts in the NOS2−/− mice were higher at 6 vs. 1 h postexposure, indicative of a more severe and/or sustained inflammatory state in the absence of NOS2 expression.

Aerosolized LPS is known to increase BALF levels of TNF-α, IL-1β, IL-6, KC, GM-CSF, and MIP-1β, all of which are expressed by the respiratory epithelium and function in inflammatory cell recruitment to the lung airway (24). To determine if NOS2 activity modulates the expression of these cytokines/chemokines, we quantified them in BALF from WT and NOS2−/− mice at 6 h post-LPS exposure. We found that airway levels of all of these mediators were significantly higher in the NOS2−/− mice compared with WT controls (Fig. 3).

Fig. 2. LPS-induced airway inflammation in WT and NOS2−/− mice. A: total white blood cell (WBC) in bronchoalveolar lavage fluid (BALF) were measured by cell counting. *P < 0.005. B: airway neutrophils in BALF were measured by cytostaining. PMN, polymorphonuclear neutrophils. *P < 0.01 and **P < 0.001. C: airway macrophages in BALF were measured by cytostaining. *P < 0.05. Data in A-C are means ± SE (n = 5/group).

Fig. 3. BALF cytokine/chemokine levels in WT and NOS2−/− mice. BALF cytokines and chemokines were measured by fluorescence-based immunoassay. IL, interleukin; MIP, macrophage inflammatory protein; GM-CSF, granulocyte macrophage colony-stimulating factor; TNF, tumor necrosis factor. *P < 0.01. Data are means ± SE (n = 5/group).
Cumulatively, the observed persistent influx of airway inflammatory cells and increased expression of proinflammatory cytokines/chemokines indicate that Nos2 attenuates inflammation in this model of LPS-induced lung injury.

**Prolonged NF-κB activation in Nos2−/− mice.** The transcription factor NF-κB controls the expression of numerous inflammatory mediators that function in the lung inflammatory response, including the LPS- and Nos2-responsive cytokines/chemokines TNF-α, IL-1β, IL-6, KC, GM-CSF, and MIP-1β (Fig. 3). These data, coupled with our previous finding that Nos2 modulates NF-κB activity in cultured respiratory epithelial cells and macrophages (7, 17), suggest that NF-κB might similarly be regulated by Nos2 in mouse lung. Thus we quantified NF-κB DNA binding in nuclear lysates prepared from MLE of LPS-exposed WT and Nos2−/− mice. Interestingly, basal NF-κB appeared to be lower in lungs from Nos2−/− vs. WT mice (Fig. 4A). At 1 h post-LPS exposure, both WT and Nos2−/− mouse lungs showed a significant increase in NF-κB activation compared with PBS controls (Fig. 4A), although NF-κB activity was not different between WT and Nos2−/− at this time point. At 6 vs. 1 h post-LPS, NF-κB activity appeared to be waning in the WT mice but was higher in the Nos2−/− mice; NF-κB expression was also higher in Nos2−/− vs. WT mice at 6 h post-LPS. These data suggest that airway Nos2 is a negative regulator of stimulus-coupled NF-κB activity in the lung; the onset of this effect correlates with the lag in Nos2 expression post-LPS exposure.

An increase in NF-κB activity can be the result of enhanced expression of the p65 transactivating monomer and/or nuclear translocation of p50-p65. We have previously shown that the respiratory epithelium is the predominant site of lung NF-κB activity in our LPS model of ALI (24). To determine if airway epithelial NF-κB activity is altered in Nos2−/− mice, immunofluorescence staining for NF-κB p65 was performed on fixed lung sections of WT and Nos2−/− mice 1 and 6 h post-LPS. At 1 h post-LPS, we observed a similar increase in total NF-κB p65 expression in the respiratory epithelium of both WT and Nos2−/− mice and a similar degree of NF-κB p65 nuclear translocation (Fig. 4B), consistent with the similar degree of NF-κB activation (Fig. 4A). However, Nos2−/− mice show significantly higher expression and nuclear NF-κB p65 content in the respiratory epithelium at 6 h post-LPS compared with WT mice, indicating that Nos2 modulates both NF-κB p65 expression and nuclear translocation in the respiratory epithelium in vivo.

**Decreased S-nitrosylation of NF-κB in Nos2−/− mice.** We have previously shown that Nos2-mediated S-nitrosylation of NF-κB p65 inhibits NF-κB transcriptional activity in respiratory epithelial cells and that aerosolized LPS exposure acutely decreases inhibitory S-nitrosylation of p65 in vivo (17, 24). To determine the contribution of Nos2 to lung levels of SNO-p65, we performed biotin switch assays on MLE from PBS- and LPS-treated WT and Nos2−/− mice. WT mice were characterized by a marked denitrosylation of lung NF-κB p65 at 1 h post-LPS treatment, but SNO-p65 levels were substantially increased at 6 h postexposure (Fig. 5A), consistent with the increase in airway epithelial Nos2 expression (Fig. 1). In comparison, basal lung SNO-p65 levels were low in Nos2−/−
mice, and no significant change in SNO-p65 was observed in these mice at 1 or 6 h postexposure. Collectively, these results indicate that NOS2 is a principal mediator of NF-κB p65 S-nitrosylation in mouse lung and that NOS2 mediates renitrosylation of p65 after LPS-induced lung injury.

**DISCUSSION**

The results of this study demonstrate that NOS2 functions to attenuate the inflammatory response in LPS-induced lung injury and provide a molecular mechanism for the effects of NOS2, namely S-nitrosylation of NF-κB p65 and deactivation of NF-κB in the respiratory epithelium. Our findings thus suggest that the LPS-induced increase in airway epithelial NOS2 expression serves to restore lung SNOs to homeostatic levels after their initial depletion in ALI (24).

Protein S-nitrosylation is now recognized to be integral in coordinating the immune response by regulating numerous proteins in a multitude of signaling pathways. The effect of S-nitrosylation is primarily inhibitory, resulting in the deactivation of proinflammatory pathways. For example, S-nitrosylation inhibits c-Jun NH2-terminal kinase 1 and the Toll-like receptor 4 signaling protein MyD88 (6, 31), both of which are integral in the lung response to LPS-induced injury (2, 14). Furthermore, S-nitrosylation likely serves to resolve airway inflammation, with SNO modification of β-actin and the calcium-binding protein S100A8 altering leukocyte-endothelial interaction (23, 39) and S-nitrosylation of aminophospholipid translocase enhancing macropage clearance of neutrophils (40).

It is the NF-κB transcription factor pathway, however, that appears to be most sensitive and critical to SNO regulation of the pulmonary inflammatory response. Protein S-nitrosylation inhibits NF-κB activation both at the cytoplasmic [i.e., IkBα kinase β (IKK2)] and nuclear (i.e., p50-p65) (17, 25, 33) level. In the nucleus, both subunits of the p50-p65 heterodimer are targeted for S-nitrosylation at a conserved cysteine in the Rel DNA-binding domain with modification of either monomer disrupting DNA binding and inhibiting κB-dependent transcription (17, 25). In the case of the cytoplasmic protein IKK2, S-nitrosylation basally inhibits its activity in the respiratory epithelium (33). Upon cytokine stimulation, IKK2 is rapidly denitrosylated, resulting in the phosphorylation and degradation of the inhibitory IkBα protein, nuclear translocation of the p50-p65 heterodimer, and initiation of NF-κB-dependent gene transcription. Similar to IKK2, we observe cytokine-induced denitrosylation of p65 in respiratory epithelial cells and LPS-treated mouse lung (24), implicating a common mechanism by which inhibitory protein S-nitrosylation is reversed in the NF-κB pathway. Given that NF-κB activation in the respiratory epithelium is essential in initiating the inflammatory response to LPS-induced lung injury (34), denitrosylation of NF-κB likely serves as the trigger for this response.

NOS2 expression is induced in the respiratory epithelium in inflammatory lung disease (22, 35, 37), and NOS2 is known to regulate the activity of a number of different inflammatory mediators via S-nitrosylation (13, 18, 31, 42). We have previously shown that cytokine-induced NOS2 expression in respiratory epithelial cells induces S-nitrosylation and inhibition of NF-κB p65 (17). The results of the present study extend these observations to a mouse model of lung injury supporting an anti-inflammatory role for NOS2 in the lung via inhibition of NF-κB. Of note, NOS2 deficiency was recently demonstrated to increase NF-κB activity and modestly augment allergic airway inflammation in a mouse model of asthma, although mechanistic correlation to S-nitrosylation of p65 was equivocal (30).

While our findings indicate that airway NOS2 activity inhibits inflammation in lung injury, other studies have found NOS2 to instead be proinflammatory. This discrepancy may be explained by different methods of injury, dosing, and analysis time points postinsult. For example, NOS2 deficiency appears to protect from lung injury when LPS is administered systemically as opposed to direct airway instillation (21, 38). These results may be due, in part, to cell- and site-specific NOS2 expression, since bone marrow reconstitution of NOS2-deficient mice with WT cells restores sensitivity to sepsis-induced lung injury (9). In this regard, we saw NOS2 expression only in the respiratory epithelium at 6 h post-LPS with no significant contribution from lung inflammatory cells (Fig. 1B). It is possible that, with airway-administered LPS, induction of NOS2 expression occurs initially in the respiratory epithelium followed later by inflammatory cells, whereas the reverse is true with systemic LPS.

The predominant mechanism cited for NO and, specifically, NOS2 exacerbation of lung injury is increased cell damage due to RNS production (28). However, protein nitration in ALI has been shown to be primarily dependent upon myeloperoxidase (MPO) activity and not NOS2 (5, 19). In fact, NOS2 expression is augmented in MPO−/− mouse lung with the increase in NOS2 activity shown to be protective from sepsis-induced lung injury (5). Whether this observed anti-inflammatory effect of NOS2 is mediated via lung SNOs is unclear. However, S-nitrosylation is known to inhibit NADPH oxidase (36), and we have demonstrated that augmentation of lung SNOs (via inha-
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NOS2 negative effects on airway inflammation.


