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S-nitrosoglutathione supplementation to ovalbumin-sensitized and -challenged mice ameliorates methacholine-induced bronchoconstriction

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Foster MW, Yang Z, Potts EN, Foster WM, Que LG. S-nitrosoglutathione supplementation to ovalbumin-sensitized and -challenged mice ameliorates methacholine-induced bronchoconstriction. *Am J Physiol Lung Cell Mol Physiol* 301: L739–L744, 2011. First published July 22, 2011; doi:10.1152/ajplung.00134.2011. —S-nitrosoglutathione (GSNO) is an endogenous bronchodilator present in micromolar concentrations in airway lining fluid. Airway GSNO levels decrease in severe respiratory failure and asthma, which is attributable to increased metabolism by GSNO reductase (GSNOR). Indeed, we have found that GSNOR expression and activity correlate inversely with lung S-nitrosothiol (SNO) content and airway hyperresponsiveness (AHR) to methacholine (MCh) challenge in humans with asthmatic phenotypes (Que LG, Yang Z, Stamler JS, Lugogo NL, Kraft M. *Am J Respir Crit Care Med* 180: 226–231, 2009). Accordingly, we hypothesized that local aerosol delivery of GSNO could ameliorate AHR and inflammation in the ovalbumin-sensitized and -challenged (OVA) mouse model of allergic asthma. Anesthetized, paralyzed, and tracheotomized 6-wk-old male control and OVA C57BL/6 mice were administered a single 15-s treatment of 0–100 mM GSNO. Five minutes later, airway resistance to MCh was measured and SNOs were quantified in bronchoalveolar lavage (BAL). Duration of protection was evaluated following nose-only exposure to 10 mM GSNO for 10 min followed by measurements of airway resistance, inflammatory cells, and cytokines and chemokines at up to 4 h later. Acute delivery of GSNO aerosol protected OVA mice from MCh-induced AHR, with no benefit seen above 20 mM GSNO. The anti-bronchoconstrictive effects of GSNO aerosol delivered via nose cone were sustained for at least 4 h. However, administration of GSNO did not alter total BAL cell counts or cell differentials and had modest effects on cytokine and chemokine levels. In conclusion, in the OVA mouse model of allergic asthma, aerosolized GSNO has rapid and sustained anti-bronchoconstrictive effects but does not substantially alter airway inflammation.

bronchodilator; nitric oxide; allergic asthma; airway hyperresponsiveness

IT IS INCREASINGLY RECOGNIZED that both nitric oxide (NO) and the low-mass S-nitrosothiol S-nitrosoglutathione (GSNO) are important endogenous products of NO synthetase (NOS) activity. In the lung, NOS isoforms (NOS1–3) are expressed in a variety of cell types, including vascular endothelial cells, macrophages, and bronchoalveolar epithelial cells (3, 23). In human asthma, levels of NO in exhaled breath are increased, a phenomenon that appears to be due to upregulation of inducible NOS (NOS2) in respiratory epithelial cells (5). However, in the ovalbumin-sensitized and -challenged (OVA) mouse model of allergic asthma, neuronal NOS (NOS1) contributes the most to airway hyperresponsivity (AHR) (7) whereas NOS2 appears to have a modest anti-inflammatory role (31).

GSNO represents a more stable, circulating endogenous reservoir of NO and is recognized to function as an endogenous bronchodilator. GSNO is present in relatively high concentrations (200–500 nM), which fall to as low as 60 nM in asthma (14, 33). Decreased lung GSNO is thought to contribute to increased AHR (11) and may result from decreased airway epithelial NOS expression, decreased glutathione concentrations, or increased breakdown of GSNO. Indeed, GSNO metabolism is increased in animal models of allergic asthma (11, 32); in humans with mild to moderate asthma, we have shown increased activity and expression of the GSNO-metabolizing enzyme GSNO reductase (GSNOR) (33). Exhaled formate, a possible product of GSNO catabolism, is also increased in children with severe asthma (15). Finally, we have also shown that OVA mice deficient in GSNOR have increased lung SNO and are protected from the development of methacholine (MCh)-induced AHR (32).

In addition to its bronchodilatory activity, which is two log orders higher than that of theophylline (13), GSNO has been shown to increase ciliary beat frequency (26) and chloride currents (19), inhibit amiloride sensitive sodium transport, and modulate 5-lipoxygenase expression in airway epithelial cells (38). Inhalation of nebulized GSNO has been shown to rapidly reverse MCh-dependent contraction in an isolated perfused and ventilated lung (4) and has been shown to be well tolerated in humans (35). Indeed, aerosolized administration of GSNO has been shown to improve oxygenation in patients with cystic fibrosis who are deficient in lung GSNO (35). Replacement of endogenous GSNO in patients with cystic fibrosis was not associated with any changes in systemic hemodynamics or in bleeding.

Collectively, the growing body of in vitro and in vivo data suggests that increasing airway GSNO levels could provide benefit for the treatment of human asthma. However, the anti-bronchoconstrictive effects of aerosolized GSNO repletion have not been examined in an in vivo model. Thus we sought to examine whether GSNO would offer protection from MCh-induced AHR, identify a range of effective doses, and determine duration of action in the OVA mouse model of allergic asthma.

MATERIALS AND METHODS

Animals. C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed in polystyrene chambers with endotoxin-free absorbent bedding and were maintained on a 12-h daylight cycle. All animals were handled in accordance with the American Association for the Accreditation of Laboratory Animal Care guidelines by use of a protocol approved by the Duke University Institutional Review Board and Institutional Animal Care and Use Committee.

Ovalbumin sensitization and challenge and GSNO exposure. Mice were subjected to ovalbumin-induced allergic airway inflammation as

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previously described (24). Briefly, 6- to 9-wk-old male C57BL/6 mice were sensitized with 10 μ g ovalbumin (Grade V, Sigma, St. Louis, MO) mixed with aluminum hydroxide (alum) (Imject Alum, Pierce, Rockford, IL) via a 100- μ l total volume intraperitoneal injection on days 0 and 7. Nonsensitized control animals received an injection of alum alone. Mice were challenged with 1% ovalbumin in phosphate-buffered saline (PBS) for 1 h on days 14, 15, and 16. On day 18, pulmonary function was measured on anesthetized, tracheotomized, and mechanically ventilated mice by use of a flexiVent (Scireq, Montreal, PQ, Canada) small rodent ventilator. GSNO was synthesized by the method of Hart et al. (16). Aerosolized GSNO or PBS were delivered to mice either acutely during anesthesia and prior to measurements of pulmonary function by flexiVent, or in an awake state using a nose-only exposure chamber followed by subsequent assessments of pulmonary function. Based on the manufacturers' description, the SCIREQ ventilator generates a 2.5–3.0 mass median aerodynamic diameter (MMAD) aerosol that is polydispersed [geometric standard deviation (GSD) = 2.0]. In contrast, the nebulizer for the nose-only delivery device generates a 0.32 μ m MMAD aerosol having a GSD = 1.6–1.9, and thus ~84% of the aerosol output has a size distribution <0.6 μ m.

Lung lavage and analysis of airway inflammation. Following lung physiology measurements, mice were euthanized, a total of 4 ml of sterile PBS containing 100 μ M DTPA was instilled, and bronchoalveolar lavage (BAL) fluid was collected by gravity flow. BAL cells were collected by centrifugation at 1,000 rpm for 10 min at 4°C. Cellular content was examined on cytospin preparations stained with Diff Quick (Dade Behring, Newark, DE) and differential counts were based on morphology and staining of a minimum of 250 cells per sample.

Cytokine and chemokine analyses. Cytokines and chemokines were quantified in BAL via a multiplex fluorescent bead-based immunoassay (sensitivity: 0.3–10.5 pg/ml) (Bio-Rad, Hercules, CA). In brief, 50 μ l BAL or cytokine and chemokine standards were incubated (in duplicate) with 25 μ l of anti-mouse multicytokine beads in a 96-well plate for 16 h at 4°C. Biotinylated anti-mouse multicytokine reporter was then added to each well as a secondary or detection antibody and incubated with shaking at 37°C for 2 h. Streptavidin-phycoerythrin was then added to the well and incubated with shaking for an additional 2 h at 37°C in the dark, and the reaction was terminated by 25 μ l of stop solution. Samples were read on a Bio-Plex Workstation (Bio-Rad). A minimum of 50 beads per cytokine was analyzed per sample. Blank values were subtracted from all readings.

Photolysis-chemiluminescence detection of S-nitrosothiols. SNO content of BAL supernatant was measured by mercury-coupled photolysis-chemiluminescence technique as previously described (32). To distinguish SNO from total nitroso/nitrosyl species, samples were treated with 1 mM HgCl₂ for 5 min at 25°C to selectively cleave SNO. SNO content was calculated by subtracting the HgCl₂-treated value from the untreated value. A standard curve was generated from GSNO.

Analysis of MUC5AC expression. Total RNA was isolated from lung tissue with TRIzol (Invitrogen, Carlsbad, CA) and Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA) and quantified with Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). A total of 1 μ g of RNA was used for reverse transcription using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). Real-time PCR was performed in a Bio-Rad iCycler using SYBR green reagent (Bio-Rad) under standard conditions. Primer sequences were as follows: actin 5'-TCA AGA TCA TTG CTC CTC CTG-3' (forward), 5'-CTG CTT GCT GAT CCA CAT CTG-3' (reverse); Muc5AC 5'-GGT GTC TTT TGT GGG AGA GG-3' (forward); 5'-ATC CCA CCT CAC ATG GAG TC-3' (reverse).

Relative quantification was performed by comparative threshold cycle (C_T) analysis. Gene expression changes were expressed as fold change with actin as internal control.

Statistical analysis. Data were expressed as means $\pm$ SE. Significant differences between groups were identified by analysis of variance. Individual comparison between groups was confirmed by a Student's *t*-test or, when appropriate, a Bonferroni posttest. A two-tailed *P* value of < 0.05 was considered statistically significant.

RESULTS

Aerosolized GSNO administered acutely decreases airway hyperresponsiveness in allergic asthma. We first sought to determine whether an acute delivery of aerosolized GSNO would ameliorate AHR in the OVA model. Separate groups of OVA mice were anesthetized and tracheotomized and were treated for 15 s with aerosolized PBS or GSNO at 1, 10, 20, and 100 mM doses. Following a 5-min pause, airway resistance was measured in response to aerosol challenge with cumulative doses of MCh (Fig. 1A). Delivery of as little as 1 mM GSNO aerosol dose significantly reduced AHR by ~50% at the maximum MCh challenge dose compared with PBS (Fig. 1, B and C). This inhibitory effect on MCh sensitivity peaked at an aerosol GSNO dose of 10 mM [also the concentration tested on humans with cystic fibrosis (35)], whereas no further reduction in AHR was observed at 20 mM GSNO; and 100 mM GSNO led to increases in MCh-induced AHR compared with PBS aerosol (Fig. 1, B and C).

We examined the correlation between these dose-dependent effects of GSNO and total SNO levels (combination of low mass and protein SNO) in BAL. Although a protective effect was observed at the 1 mM GSNO aerosol dose vs. PBS alone, SNOs were not detectably different between these two groups (Fig. 1D). On the other hand, dose-dependent increases in SNO were measured in response to 10, 20, and 100 mM GSNO, despite the reversal of protection from MCh-induced AHR at the 100 mM GSNO aerosol concentration. Thus steady-state levels of BAL SNOs are not strictly correlated with efficacy of aerosolized GSNO. These results also suggest that, at low GSNO dosing levels (i.e., 1 mM), kinetics favor GSNO uptake into cells (or degradation in the airway lining fluid) with zero net change in steady-state SNOs; however, it appears that these pathways quickly become saturated at higher concentrations of GSNO, leading to a buildup of BAL SNO in the form of excess GSNO or SNO-protein that has limited biological activity or is actually counteractive.

Aerosolized GSNO has sustained antibronchoconstrictive effects. Having established that an acute delivery of GSNO can provide immediate protection from MCh-induced AHR in an anesthetized and mechanically ventilated animal, we next investigated whether these beneficial effects might be observed in a conscious animal and sustained for longer periods. Using a nose-only exposure chamber OVA mice were treated in an awake state for 10 min with 10 mM aerosolized GSNO. Subsequently pulmonary function was evaluated in separate groups of mice from 2 min to 4 h posttreatment with GSNO (Fig. 2A). Airway resistance changes in response to cumulative MCh aerosol challenge was greatly attenuated at the 2 min post-GSNO time point, and this remained in effect even to the longest time point tested (4 h; Fig. 2, B and C). In addition, at 4 h post-GSNO, BAL SNOs remained at higher activity levels than in PBS-treated OVA control mice (Fig. 2D), which suggests BAL SNOs may be important in sustaining an anti-bronchoconstrictive effect.

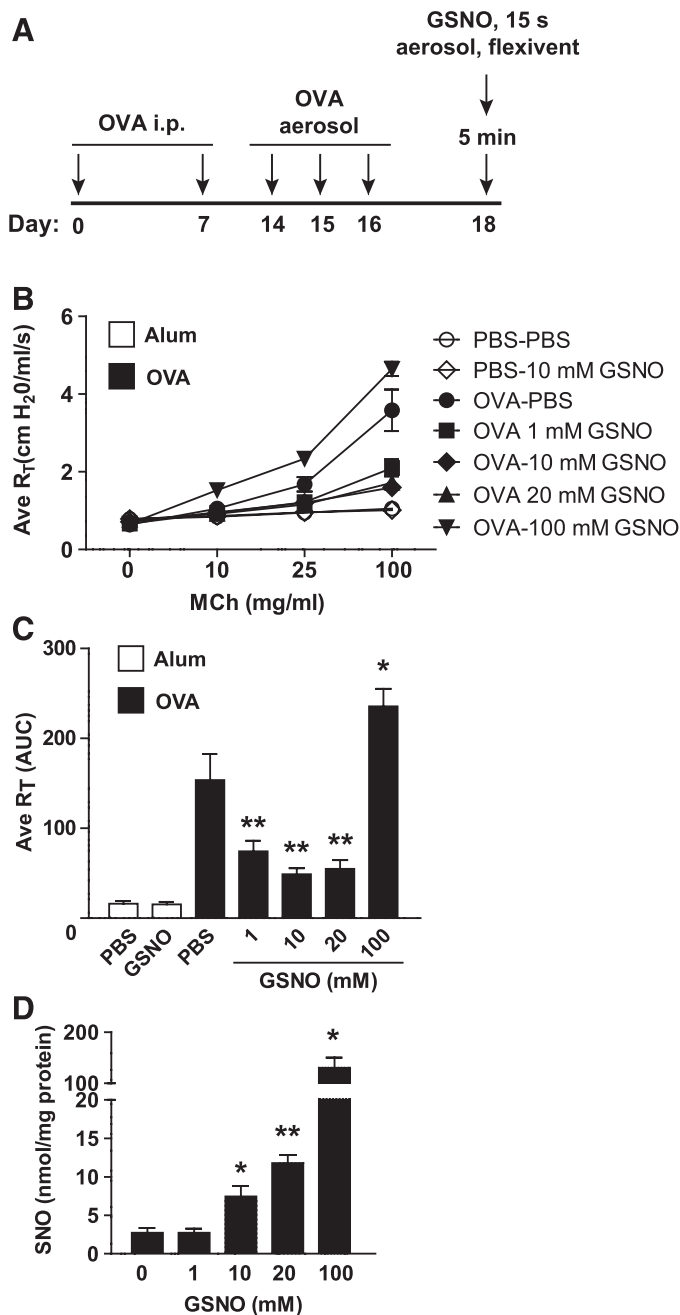


Fig. 1. Acute S-nitrosoglutathione (GSNO) repletion alters airway hyper-responsivity in the ovalbumin-sensitized and challenged (OVA) mouse. **A**: schematic showing experimental protocol. **B** and **C**: average (Ave) total pulmonary resistance (R_T) in alum control or OVA, tracheotomized, and mechanically ventilated mice was measured by flexiVent immediately after a 15-s dose of aerosolized PBS or 1–100 mM GSNO via ultrasonic nebulizer followed by methacholine (MCh) challenge. Data in **C** are area under the curve calculated using mean R_T at 0 mM MCh as a baseline within each treatment group. **D**: total S-nitrosothiols (SNOs) in bronchoalveolar lavage (BAL) from PBS- or GSNO-treated mice were quantified by mercury-coupled photolysis chemiluminescence. i.p., Intraperitoneal. Data in **B** and **C** are means $\pm$ SE ($n = 5$ –11 per group) and in **D** are means $\pm$ SE ($n = 5$ per group). * $P < 0.02$, ** $P < 0.001$.

Aerosolized GSNO has varying effects on airway inflammation. We hypothesized that the sustained activity of GSNO might be due to either a direct action on airway smooth muscle (ASM) or a reduction in proinflammatory mediators. Thus we also

evaluated the effects of aerosolized GSNO (10 mM, 2–4 h postexposure) on expression of the major respiratory tract mucin, MUC5AC, BAL cell counts and cell differentials, and BAL cytokine/chemokine levels, all of which are considered as phenotypes of allergic inflammation. At 2 or 4 h post-GSNO, we did not observe significant differences in total BAL cell counts (Fig. 3A) or relative macrophage or eosinophil cellularity vs. control (Fig. 3B), nor were total lung MUC5AC mRNA levels changed at the 4-h time point (Fig. 3C).

We quantified GSNO-dependent changes across a panel of Th1 (IFN- γ and TNF- α) and Th2 (IL-4, IL-5, IL-6, and IL-13) cytokines, chemokines (MCP-1, MIP-1 β , MIP-2), and chemoattractants (KC), which were each elevated in the OVA mouse model (Fig. 4). All of the cytokines and chemokines measured were lower 4 h post-GSNO therapy, although the largest differences were observed with the Th1 cytokines and

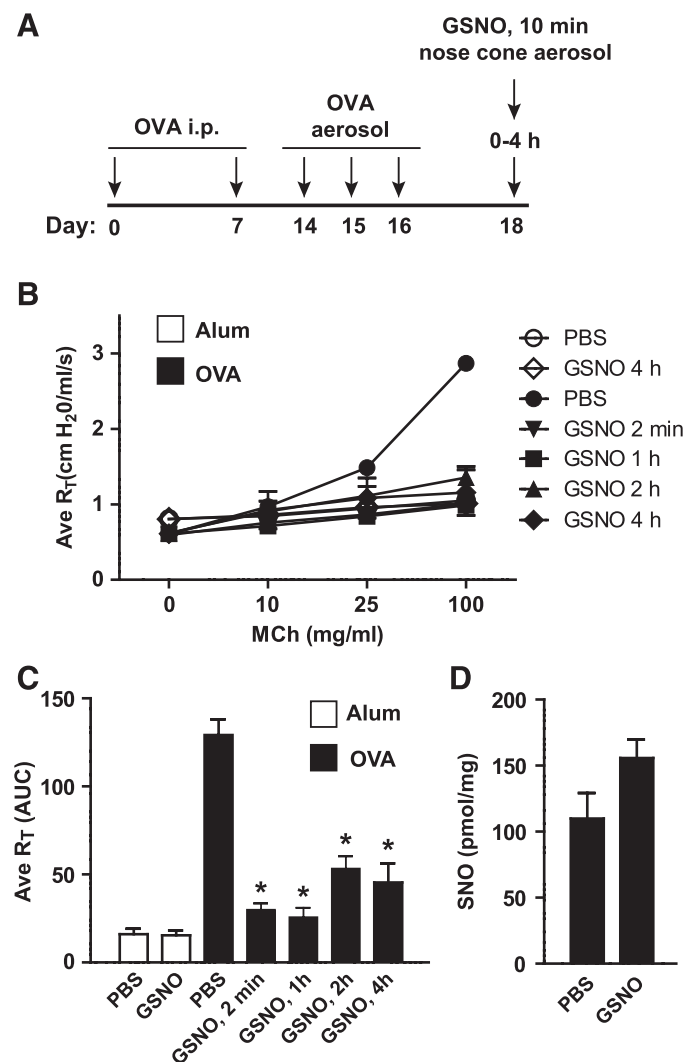
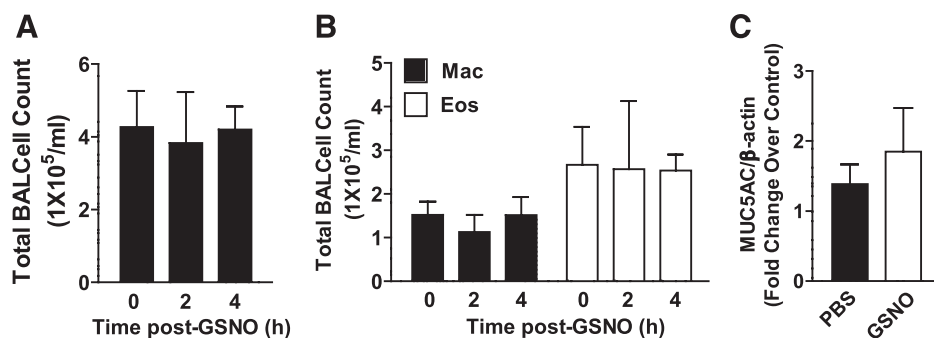


Fig. 2. Ameliorative effects of GSNO are sustained. **A**: schematic showing experimental protocol. **B** and **C**: airway resistance in alum control or OVA mice was measured by flexiVent 2 min to 4 h after nose-only inhalation of aerosolized PBS or 10 mM GSNO. Data in **C** are area under the curve (AUC) of Fig. 1B. **D**: total S-nitrosothiols in BAL from PBS- or GSNO-treated mice were quantified by mercury-coupled photolysis chemiluminescence. Data in **B** and **C** are means $\pm$ SE ($n = 8$ –11 per group), * $P < 0.001$. Data in **D** are means $\pm$ SE ($n = 3$ –4 per group).

Fig. 3. Aerosolized GSNO does not alter markers of allergic inflammation. Mac, macrophages; Eos, eosinophils. Data A, B, and C are means $\pm$ SE ($n = 4-8$ per group), $*P < 0.05$.



chemokines (Fig. 4). Thus the sustained antibronchoconstrictive effects of GSNO correlated with reduced levels of inflammatory cytokines, but not with changes in the infiltration of inflammatory cells.

DISCUSSION

GSNO has long been recognized as a potent antibronchoconstrictive agent, and GSNO deficiency as well as aberrant

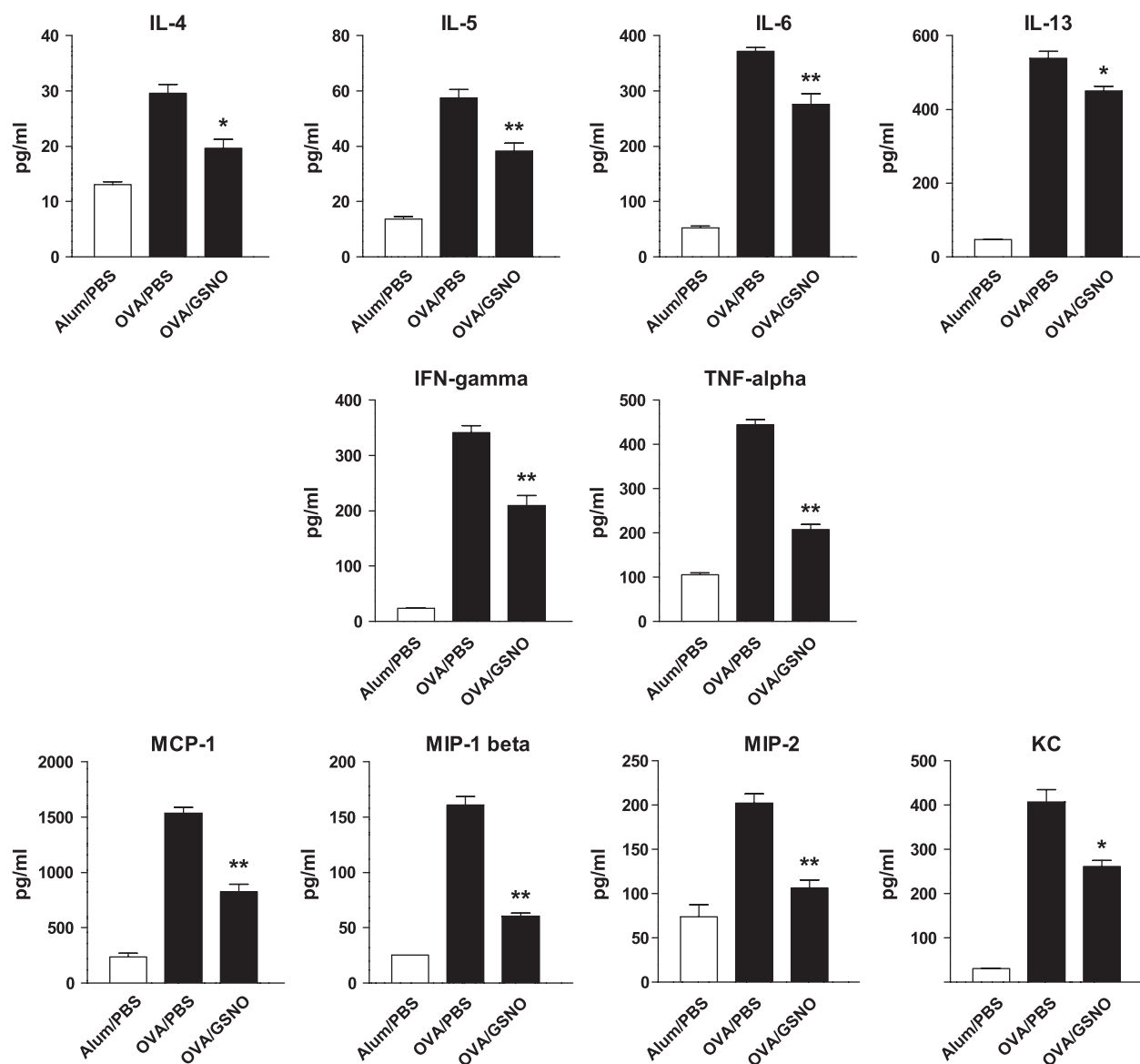


Fig. 4. GSNO therapy reduces levels of proinflammatory cytokines and chemokines. Levels of IL-4, IL-5, IL-6, IL-13, MCP-1, MIP-1B, MIP-2, and KC were measured from BAL by multiplexed immunoassay. Control data are mean $\pm$ range ($n = 2$) or mean $\pm$ SE ($n = 3$). OVA data are means $\pm$ SE ($n = 4-6$). $*P < 0.005$ and $**P < 0.0001$ (2-tailed t -test).

GSNO metabolism are correlated with human asthma. Nonetheless, the therapeutic potential of this “natural product” has not been adequately assessed *in vivo*. Here we show unequivocally that nebulized GSNO protects against airway hyperresponsivity in OVA mice when administered acutely prior to MCh challenge and that this effect can be sustained for at least 4 h following delivery of aerosolized GSNO to conscious mice. These data suggest that GSNO-based therapy has potential as an alternative to the current standard of care (e.g., short-acting β -agonists) for the acute alleviation of airway bronchoconstriction.

It is worth discussing the relevance and potential drawbacks of the OVA mouse model. This model elicits a series of responses to allergen challenge that parallel human asthma, including airway eosinophilic inflammation, airway hyperresponsiveness to nonspecific allergen challenge, IgE production, Th2 cytokine production, goblet cell metaplasia, and mucus hyperproduction (9, 24). Although the OVA model replicates the acute inflammatory and bronchoconstrictor components of asthma well, it does not adequately reproduce the chronic stage of disease since repeated allergen exposure in mice often results in tolerance rather than asthma progression. The model is also not appropriate to study eosinophilic degranulation, since mouse eosinophils do not degranulate in asthma (8). Another criticism of the model is that it lacks a direct human correlate of ovalbumin as an allergen. Although we acknowledge these limitations, the OVA model is the most frequently utilized to study airway inflammation and AHR in acute asthma and response to potential drug therapy.

We presume that the rapid-onset antibronchoconstrictive activity of aerosolized GSNO is largely the result of actions on ASM. Generally, exogenous *S*-nitrosothiols have the capacity to mediate direct effects by *S*-nitrosylation of critical protein Cys thiols or may function via decomposition to NO and subsequent activation of the NO/soluble guanylate cyclase (sGC)/protein kinase G pathway. Both of these pathways have been shown to mediate decreases in intracellular Ca^{2+} concentration and Ca^{2+} sensitivity in smooth muscle. For example, cGMP-dependent inhibition of Rho kinase has been shown to cause hypophosphorylation of the regulatory subunit of myosin phosphatase (MYPT1), leading to enhanced dephosphorylation (and decreased activation) of myosin light chain (21, 22). cGMP-dependent and -independent activation of the voltage- and Ca^{2+} -dependent K^+ (BK) channel by NO also reduces ASM bronchoconstriction (1), and *S*-nitrosylation is implicated in the NO-dependent inhibition of the ADP-ribose cyclase activity of CD38 (37), an important mediator of Ca^{2+} signaling in ASM. Although the relevance of these various pathways needs to be further investigated in the OVA mouse, it is worth noting that relaxation of ASM in humans and other species is SNO mediated and mostly unaffected by sGC inhibition (13), thus suggesting that the effects of GSNO observed here may be largely cGMP independent.

Although augmentation of airway GSNO in OVA mice results in an immediate and sustained reduction in AHR, it has little effect on BAL cell counts, eosinophilic inflammation, or mucus metaplasia (as assessed by MUC5AC expression). Note that this contrasts with models of autoimmune disease (EAE, uveitis, and Crohn's disease) in which GSNO reduces both cytokine levels and inflammation with concomitant reduction

in disease pathophysiology (12). The uncoupling of airway inflammation and AHR in the OVA model with GSNO therapy is not entirely unexpected, since we previously found that GSNO-deficient mice are protected from AHR and have elevated lung SNO content compared with WT control littermates despite no apparent changes in inflammation (32). In addition, a recent report by Olson et al. (31) likewise did not support an anti-inflammatory role for GSNO in suppressing markers of inflammation or mucus metaplasia in an allergic airway model, even though the levels of active nuclear factor kappa b (NF- κ B), a target of *S*-nitrosylation (20, 28), were reduced by GSNO in the lung. This discordance between proinflammatory cytokines and other markers of allergic inflammation may result from other compensatory effects of GSNO. It is also possible that the changes in cytokines are not sufficient to alter chemotaxis or that these changes in cytokine production occur at an earlier time point, and thus alterations in cell count may not be captured. Collectively, these data suggest that the antibronchoconstrictive activity of GSNO is largely independent of any anti-inflammatory properties.

The search for new therapies for asthma, which afflicts more than 20 million in the United States (2) at an annual cost of nearly \$50 billion (<http://www.cdc.gov/vitalsigns/asthma>), is in part driven by the problems associated with short- and long-acting β -agonists (SABAs and LABAs). Although prolonged use of SABAs is accompanied by β -adrenergic receptor (β -AR) desensitization (6, 29, 30), GSNO may actually modulate airway tone through opposition of tonic or agonist-stimulated β -AR desensitization and downregulation (36). However, it is also important to note that S(NO)-dependent modifications, including *S*-nitrosylation of sGC, can also affect desensitization of NO-based signaling (25, 34). This may explain the reversal of protection at the highest GSNO doses and emphasizes the need to examine the effects of repeat acute administration of GSNO. Furthermore, in light of the increased use of combination drugs (e.g., LABA and inhaled corticosteroids) to target airway bronchoconstriction and inflammation, the modest anti-inflammatory effects of GSNO do not detract from its potential as an asthma therapeutic. Because lung *S*-nitrosothiol levels are heterogenous in asthma, it is not far-fetched to imagine that GSNO, or other compounds that modulate SNO levels in the airway, may be used to treat those individuals with low SNO levels. Alternatively, GSNO may find use as an adjunct to β_2 -agonist therapy to prevent desensitization of the β_2 -receptor (36).

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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