NAD(P)H quinone oxidoreductase 1 regulates neutrophil elastase-induced mucus cell metaplasia

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Meyer ML, Potts-Kant EN, Ghio AJ, Fischer BM, Foster WM, Voynow JA. NAD(P)H quinone oxidoreductase 1 regulates neutrophil elastase-induced mucus cell metaplasia. Am J Physiol Lung Cell Mol Physiol 303: L181–L188, 2012. First published June 1, 2012; doi:10.1152/ajplung.00084.2012.—Mucous cell metaplasia (MCM) is increased expression of a major respiratory tract mucin, MUC5AC. Neutrophil elastase (NE) upregulates MUC5AC in primary airway epithelial cells by generating reactive oxygen species, and this response is due in part to upregulation of NADPH quinone oxidoreductase 1 (NQO1) activity. Delivery of NE directly to the airway triggers inflammation and MCM and increases synthesis and secretion of MUC5AC protein from airway epithelial cells. We hypothesized that NE-induced MCM is mediated in vivo by NQO1. Male wild-type and Nqo1-null mice (C57BL/6 background) were exposed to human NE (50 μg) or vehicle via oropharyngeal aspiration on days 1, 4, and 7. On days 8 and 11, lung tissues and bronchoalveolar lavage (BAL) samples were obtained and evaluated for MCM, inflammation, and oxidative stress. MCM, inflammation, and production of specific cytokines, granulocyte-macrophage colony-stimulating factor, macrophage inflammatory protein-2, interleukin-4, and interleukin-5 were diminished in NE-treated Nqo1-null mice compared with NE-treated wild-type mice. However, in contrast to the role of NQO1 in vitro, we demonstrate that NE-treated Nqo1-null mice had greater levels of BAL and lung tissue lipid carbonyls and greater BAL iron on day 11, all consistent with increased oxidative stress. NQO1 is required for NE-induced inflammation and MCM. This model system demonstrates that NE-induced MCM directly correlates with inflammation, but not with oxidative stress.

neutrophil elastase; inflammation

MUCOUS CELL METAPLASIA (MCM) (34, 40, 52), neutrophil-predominant airway inflammation (33, 36), and mucus obstruction are pathological features of chronic inflammatory airway diseases, like cystic fibrosis, chronic bronchitis, and severe exacerbations of asthma. These features contribute to the significant morbidity and mortality of these diseases due to mucus plugging and recurrent infections (10). Neutrophil elastase (NE), a serine protease released by neutrophils, is found in high concentrations in the airway surface liquid of patients with chronic inflammatory airway diseases (31, 45). NE impairs mucociliary clearance and stimulates mucin production (15, 25, 49) and secretion (24, 28, 35). We and others have reported that NE upregulates MUC5AC expression in primary human airway epithelial cells and in vivo induces MCM of the airway in mice directly challenged with NE (7, 48). In primary human airway epithelial cultures, MUC5AC expression is upregulated by NE via a pathway involving the generation of reactive oxygen species (ROS) (17). However, the intracellular mechanism of NE-induced MCM is not fully elucidated.

We and others have reported that NE stimulates normal airway epithelial cells to produce ROS via mitochondrial sources (3), DUOX activation (41), release of iron from extracellular proteins and increased iron uptake (16, 17), and NAPDH quinone oxidoreductase 1 (NQO1) activity (56). NQO1 is a reducing enzyme found in the normal human respiratory epithelium (42). NQO1 catalyzes an obligate two-electron reduction of quinones to hydroquinones, utilizing either NADH or NADPH as a reducing cofactor (14). Depending on the type of hydroquinone that is produced, it can possess antioxidant or bioactivation properties (9). Importantly, NE-regulated oxidative stress and MUC5AC gene expression are dependent on NQO1 (56). NQO1 confers pulmonary susceptibility to ozone by translating oxidative stress to airway inflammation (47). In this report, we tested the hypothesis that NE-induced MCM is mediated by NQO1 via the generation of ROS and airway inflammation.

MATERIALS AND METHODS

Animals. C57BL/6J mice were obtained commercially (Jackson Laboratories, Bar Harbor, ME). Nqo1-deficient mice (on a C57BL/6 background) were obtained from a breeding colony that was established at Duke University from breeding pairs that were generously provided by Dr. Frank Gonzalez at the National Cancer Institute (Bethesda, MD). Male C57BL/6J or Nqo1-null mice were used at 6–8 wk of age (25–30 g). The animals were housed in plastic shoebox-type cages suspended over absorbent bedding and were maintained on a 12-h diurnal cycle. Food and water were provided ad libitum. The study protocol was approved by the Institutional Animal Care and Use Committee at Duke University Medical Center and conformed to the principles for laboratory animal research outlined by the Animal Welfare Act and the Department of Health, Education, and Welfare Guidelines for the Experimental Use of Animals.

NE exposures. Animals received NE or control vehicle diluted in PBS by oropharyngeal aspiration (18) on days 1, 4, and 7 (Fig. 1). Immediately after inhalational anesthesia with isoflurane (IsoFlo from Abbott Laboratories and Open-Circuit Gas Anesthesia System from Stoelting), animals were suspended by their upper incisors on a 60° incline board, and a liquid volume of human NE [50 μg (43.75 units)/40 μl PBS, 42.37 μM; specific activity 875 U/mg protein; Elastin Products, Owensville, MO] or control vehicle in PBS was delivered to the distal part of the oropharynx with the animal’s tongue extended. With the tongue extended, the animal was unable to swallow, and the liquid volume was aspirated in the respiratory tract. Mice were killed by inhalational exposure to 100% CO2 gas at 1 and 4 days...
flamatory protein-2, granulocyte monocyte colony stimulating factor, interferon-γ, IL-9, IL-13, IL-6, and tumor necrosis factor-α. Briefly, BAL samples (50 μl) and standard concentrations of the respective cytokines (50 μl) were placed in duplicate in wells in a 96-well microtiter plate. Samples were incubated with 25 μl of anti-mouse multicytokine beads, specific for mouse cytokines, and mixed on a plate shaker for 18 h at 4°C. Biotin anti-mouse multicytokine reporter was added to each well as a secondary/detection antibody and incubated at 37°C for 2 h while shaking. Streptavidin phycoerythrin (25 μl) was added to wells and incubated with shaking for 2 h, at 37°C, in the dark. Finally, the addition of stop solution (25 μl) terminated the reactions. Samples and standards were read using a Luminex instrument (Bio-Plex Workstation; Bio-Rad, Hercules, CA) in which a minimum of 50 beads/cytokine for each sample was analyzed. Blank values were subtracted from all readings.

Glutathione. Levels of total (GSH) and oxidized glutathione (GSSG) were quantified in the BAL by using a spectrophotometric assay (Glutathione Assay Kit; Cayman Chemical, Ann Arbor, MI) that quantitates GSH following reaction with DTNB (5,5'-dithio-bis-2-nitrobenzoic acid) to yield yellow-colored TNB (5-thio-2-nitrobenzoic acid). The rate of TNB production is directly proportional to the concentration of GSH in the sample. BAL (50 μl) and standards were analyzed in duplicate on a microtiter plate according to manufacturer's instructions. To determine GSSG concentrations, BAL samples were pretreated with 1 M solution of 2-vinylpyridine (Sigma-Aldrich, St. Louis, MO) in ethanol to derivatize existing GSH in the sample, preventing reaction with DTNB and, therefore, allowing only quantitation of GSSG. Derivatization is performed according to the manufacturer's instructions before spectrophotometric assay. The Cayman kit software computes that each molecule of GSSG is equivalent to two GSH molecules to derive the concentration of GSSG. We calculated the reduced glutathione concentration by subtracting the GSSG concentration from the GSH concentration.

BAL measures of iron. Equal volumes of BAL and 6 N HCl/20% trichloroacetic acid were mixed together. This was hydrolyzed at 70°C for 18 h and then centrifuged at 20,000 g for 10 min. Iron concentrations were determined in the supernatants using inductively coupled plasma optical emission spectroscopy (model Optima 4300D, Perkin Elmer, Norwalk, CT) operated at a wavelength of 238.204 nm (16, 19).

Lipid carbonyl analyses. BAL and snap-frozen lung tissue were collected for lipid carbonyl analysis by evaluation of thiobarbituric acid (TBA) reactive products as a measure of lipid peroxidation (32, 37). Samples were stored at −70°C until assayed. TBA-reactive products were assayed in 1.0 ml of BAL by adding 1.0 ml of 1% (wt/vol) TBA plus 1.0 ml of 2.8% (wt/vol) trichloroacetic acid. This was heated at 100°C for 10 min, cooled, and centrifuged, and the concentration of the resulting chromophore was determined by its absorbance at 532 nm. Lung tissue (100 mg; wet weight) was homogenized in 1.15% KCl (1.0 g/9.0 ml). To 0.2 ml of homogenate, 0.2 ml sodium dodecyl sulfate and 1.5 ml 20% acetic acid were added. The pH was adjusted to 3.5, 1.5 ml of 0.8% TBA was added, the volume was adjusted to 4.0 ml, and the reaction mixture was heated to 95°C for 60 min. One milliliter of distilled water and 5.0 ml n-butanol-pyridine (15:1 vol/vol) were added. After centrifugation for 10 min, absorbance of the organic layer was measured at 532 nm.

Statistics. Statistical analysis was performed using Statistik Software (Analytical Software, Tallahassee, FL). All data were analyzed using the Kruskal-Wallis one-way nonparametric ANOVA and post hoc comparisons by the Wilcoxon rank sum test (44). Data are presented as means ± SE. Significant differences between groups were defined as P < 0.05.

RESULTS

NE-induced MCM was attenuated in NQO1-null mice. After treatment with NE via aspiration on days 1, 4, and 7, MCM, as detected by AB/PAS staining, was observed in both wild-type (WT) and Nqo1-null mice on days 8 and 11 (Fig. 2A). MCM
was quantified by histological mucus index (Fig. 2B) and revealed significant attenuation of MCM in Nqo1-null mice compared with WT mice, especially on day 11.

NE-induced inflammation was blunted in Nqo1-null mice. NE provoked neutrophilic and eosinophilic airway inflammation in both WT and Nqo1-null mice, which was significantly diminished in Nqo1-null mice (Fig. 3). Subsequent to NE treatment, total BAL leukocyte counts in both WT and Nqo1-null mice were greater than those of control vehicle-treated animals on both days 8 and 11 (Fig. 3A). This increase in total leukocyte counts decreased over time. The percentage of neutrophils (polymorphonuclear leukocyte) increased post-NE treatment in both mice, with a peak on day 8. The increase of BAL neutrophils post-NE was significantly diminished in Nqo1-null mice (Fig. 3B). NE treatment also induced an increased percentage of eosinophils, which peaked at a later time point, on day 11 (Fig. 3C). The eosinophilic inflammation was also diminished in Nqo1-null mice at the same time points.

Cytokine analyses of the BAL revealed that NE treatment significantly increased granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage inflammatory protein-2 (MIP-2), IL-4, and IL-5 (Fig. 3, B and C). Consistent with the altered inflammatory cell profile in Nqo1-null mice, all four of these cytokines were diminished in the NE-treated Nqo1-null mice compared with NE-treated WT mice. There were no significant differences between NE-treated WT and NE-treated Nqo1-null mice in other Th2 cytokines, IL-10 or IL-13, nor other proinflammatory cytokines, including keratinocyte-derived chemokine, TNF-α, or IL-1β. Decreased neutrophilic and eosinophilic inflammation in Nqo1-null mice post-NE treatment may be explained in part by decreased levels of GM-CSF and MIP-2, and decreased levels of IL-4 and IL-5, respectively.

In the absence of NQO1, NE-triggered oxidative stress is intensified. We evaluated whether NQO1 is required for NE-generated ROS by measuring BAL GSH and GSSG, iron, and lipid carbonyls. On day 8, we noted that both WT and Nqo1-null NE-treated animals had increased red blood cells in the BAL, which cleared by day 11. Red blood cells contribute a significant load of glutathione and, therefore, confound glutathione measures contributed by cells in the airway and alveolar compartments. Therefore, we analyzed the ratio of reduced glutathione to GSSG on day 11. NE treatment slightly decreased the reduced glutathione-to-GSSG ratios in both WT and Nqo1-null mice (Fig. 4), consistent with NE-induced oxidative stress, although NE-induced changes did not meet statistical significance. NE-stimulated reduced glutathione-to-GSSG levels were not altered by the presence or absence of NQO1, suggesting that glutathione levels may reflect compensatory mechanisms following an oxidative stress.

Our laboratory has previously shown that NE increases extracellular free iron, which is taken up by cells to induce oxidative stress. We measured non-heme iron, both Fe2+ and Fe3+, in the BAL by inductively coupled plasma optical emission spectroscopy (Fig. 5). After NE treatment, both WT and Nqo1-null mice had greater BAL iron levels than vehicle-treated mice. However, on day 11, NE-treated Nqo1-null mice had significantly greater...
levels of BAL iron than NE-treated WT mice, reflecting a source of increased oxidative stress.

Finally, to obtain a global measure of oxidative stress, BAL and lung tissue lipid carbonyls were determined in Nqo1-null and WT mice post-control vehicle or NE. We found that, post-NE treatment, lipid carbonyls were increased in BAL and in murine lung in Nqo1-null mice, starting on day 8 and reaching significantly increased levels compared with WT mice on day 11 (Fig. 6). These results suggest that there is greater oxidative stress post-NE treatment in the absence of NQO1, and that there is discordance between oxidative stress and inflammation in these animal models.

**DISCUSSION**

**NQO1 regulates NE-induced inflammation and MCM.** We have previously demonstrated that NE upregulates MUC5AC in primary airway epithelial cells by generating ROS, in part via NQO1 (56). In this study, we show that NQO1 is necessary for NE-induced MCM and inflammation in the murine airway. After treatment with NE, the development of MCM, measured by a histological mucus index, was attenuated in Nqo1-null mice compared with WT mice. In concordance with the MCM, neutrophilic and eosinophilic airway inflammation and associated BAL cytokines, GM-CSF, MIP-2, IL-4, and IL-5, induced by NE were relatively diminished in Nqo1-null mice compared with WT mice. Although significant differences in the BAL chemokine/cytokine data support the correlation with reduced neutrophilic and eosinophilic inflammation in Nqo1-null mice post-NE, there may be other factors, such as altered expression of cell adhesion molecules, that also control the magnitude of inflammation post-NE in Nqo1-null mice. Our results support a strong correlation between neutrophilic/eosinophilic inflammation and MCM. In contrast to the in vitro evidence that ROS mediate NE-induced MUC5AC expression, our results demonstrate that mucous cell remodeling does not directly correlate with the generation of ROS.

**NQO1 and oxidative stress.** In contrast to the in vitro evidence in primary airway epithelial cells that NE generates ROS via NQO1 (56), loss of NQO1 resulted in increased oxidative stress in vivo, as established by increased BAL iron and increased BAL and lung lipid carbonyls. One measure of oxidative stress, the ratio of reduced glutathione to GSSG, did not vary between WT and Nqo1-null mice, following either sham or NE treatment. These results may reflect the complex interplay of the presence or absence of NQO1 on glutathione synthesis and the capacity for glutathione reduction (51).
**MAPK signaling (1).** Furthermore, compared with WT mice, Nqo1-null mice express lower levels of cytokines and chemokines in response to LPS exposure (21). Thus Nqo1-null mice have blunted innate immune responses to inflammatory mediators. Although the BAL chemokine/chemoattractant levels are consistent with decreased neutrophilic and eosinophilic inflammation in Nqo1-null mice post-NE treatment, there may be other factors, such as altered expression of cell adhesion molecules, that also control the magnitude of inflammation post-NE in Nqo1-null mice. Consistent with the results following NE exposure, our laboratory has previously reported that Nqo1-null mice are protected from ozone-induced inflammation and airway hyperresponsiveness (47). Protection from ozone-induced inflammation was unexpected in Nqo1-null mice, since the presence of NQO1 should sustain antioxidant capacity in the cells and, therefore, inhibit oxidative stress-mediated inflammation. To date, the mechanism underlying NQO1-regulated inflammation and NF-κB activation is not understood.

**Inflammation stimulates MCM.** Several in vivo models of MCM are associated with an inflammatory stimulus. The triggering inflammatory stimuli include a broad spectrum of insults, such as respiratory viral infections (22, 50), air pollutants such as ozone (11), and tobacco smoke (4, 30), foreign bodies (26), lipopolysaccharide (55), allergen-driven inflammation, including IL-13 (2, 54), IL-4 (46), IL-9 (27), or TNF-α (8) cytokine exposure, and proteases [NE (48) and aspergillus proteinases (23)]. Importantly, several reports link inhibition of inflammation with mitigation of MCM. The transcription factor, NK2 homeobox 1 (NKKX2–1) is suppressed in asthmatic patients with mucous cell hypertrophy (29), and, when overexpressed in inducible transgenic mouse models, NKKX2–1 suppresses allergen-induced inflammation, Th2 cytokine production, and MCM via the downregulation of the transcription factor, Sam pointed domain Ets-like factor (29). Nasal inhalation of LPS stimulates both neutrophilic inflammation and MCM in rats: a phosphodiesterase-4 inhibitor decreases both LPS-induced inflammation and MCM (43). Azithromycin attenuates inflammation and MCM in a murine model of allergic asthma (5), and fluticasone propionate prevents ozone-induced rat nasal inflammation and MCM (20). Thus this report identifies an important new mechanism in the development of MCM: that the activity level of a host factor, such as NQO1, modulates pulmonary inflammation and mucous cell remodeling.

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**Disclosures**

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

**Author Contributions**

ROLE OF NQO1 IN NE-INDUCED MUCUS CELL METAPLASIA

M.L.M. drafted manuscript; M.L.M., B.M.F., W.M.F., and J.A.V. edited and revised manuscript; M.L.M., B.M.F., W.M.F., and J.A.V. approved final version of manuscript.

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