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NADPH Oxidase and Nrf2 Regulate Gastric Aspiration–Induced Inflammation and Acute Lung Injury

Bruce A. Davidson,*,†‡ R. Robert Vethanayagam,§ Melissa J. Grimm,§ Barbara A. Mullan,*,†‡ Krishnan Raghavendran,§ Timothy S. Blackwell,‖ Michael L. Freeman,§ Vanniaraajan Ayyasamy,** Keshav K. Singh,†† Michael B. Sporn,‖‖ Kiyoshi Itagaki, §§ Carl J. Hauser, §§ Paul R. Knight,* and Brahm H. Segal,*§‖‖

Recruitment of neutrophils and release of reactive oxygen species are considered to be major pathogenic components driving acute lung injury (ALI). However, NADPH oxidase, the major source of reactive oxygen species in activated phagocytes, can paradoxically limit inflammation and injury. We hypothesized that NADPH oxidase protects against ALI by limiting neutrophilic inflammation and activating Nrf2, a transcriptional factor that induces antioxidative and cytoprotective pathways. Our objective was to delineate the roles of NADPH oxidase and Nrf2 in modulating acute lung inflammation and injury in clinically relevant models of acute gastric aspiration injury, a major cause of ALI. Acid aspiration caused increased ALI (as assessed by bronchoalveolar lavage fluid albumin concentration) in both NADPH oxidase–deficient mice and Nrf2–/– mice compared with wild-type mice. NADPH oxidase reduced airway neutrophil accumulation, but Nrf2 decreased ALI without affecting neutrophil recovery. Acid injury resulted in a 120-fold increase in mitochondrial DNA, a proinflammatory and injurious product of cellular necrosis, in cell-free bronchoalveolar lavage fluid. Pharmacologic activation of Nrf2 by the triterpenoid 1-[(2-cyano-3,12-dioxooleana-1,9(11)-dien-28-yl)limidazole limited aspiration-induced ALI in wild-type mice and reduced endothelial cell injury caused by mitochondrial extract–primed human neutrophils, leading to the conclusion that NADPH oxidase and Nrf2 have coordinated, but distinct, functions in modulating inflammation and injury. These results also point to Nrf2 as a therapeutic target to limit ALI by attenuating neutrophil–induced cellular injury. The Journal of Immunology, 2013, 190: 1714–1724.

Acute lung injury (ALI) is a syndrome that results from a number of insults that damage the alveolar–capillary wall (ACW), leading to pulmonary edema and recruitment of inflammatory cells. Acute respiratory distress syndrome (ARDS), the most severe form of ALI, is associated with a high mortality rate (1, 2). Specific insults that result in direct cell injury, such as gastric aspiration, can cause a mild self-limited illness or progress to fatal ARDS. This range of illness is likely to be modulated by both the nature of the caustic insult, underlying patient comorbidities, and host factors governing the inflammatory response (3). Prior studies have implicated neutrophils (polymorphonuclear leukocyte [neutrophil] [PMNs]) and reactive oxygen species (ROS) in the pathogenesis of ALI (4–7). Interestingly, endogenous hydrogen peroxide can have anti-inflammatory, protective effects in experimental ALI (8). Thus, although ROS can cause direct cellular injury and activate inflammatory responses, they can also mediate signaling functions that are protective. The lack of demonstrated benefit for antioxidant strategies in improving ALI (9) may reflect ROS having both exacerbating and protective effects in ALI.

NADPH oxidase generates the oxidative burst in PMNs, leading to the production of ROS (e.g., superoxide anion, hydrogen peroxide, hydroxyl anion, and hypohalous acid), and activation and release of PMN granular proteases (10–13). The critical role of NADPH oxidase in antimicrobial host defense is demonstrated by chronic granulomatous disease, an inherited disorder of NADPH oxidase characterized by severe bacterial and fungal infections and by excessive inflammation, such as Crohn-like inflammatory bowel disease (14–16). In addition to this enzyme’s critical host defense function, NADPH oxidase also regulates inflammation. In studies of...
lung inflammation induced by microbial-derived products, NADPH oxidase restrained lung inflammation by activation of Nrf2, a redox-sensitive antioxidative and anti-inflammatory transcription factor (17).

Pathogen recognition receptors (e.g., TLRs) sample microbial motifs and initiate signaling that may result in NADPH oxidase activation. In addition to inhaled Ags and microbes, the lung is exposed to caustic irritants through inhalation and gastric acid aspiration. Common innate recognition pathways are activated by microbial products and released intracellular products of cell injury, termed damage-associated molecular patterns (DAMPs) (18, 19). Seen in this light, ALI can result both from the direct insult and inflammation-induced injury primed by DAMPs.

This study uses clinically relevant models of aspiration-induced ALI to investigate mechanisms that modulate both inflammation and injury. There is a broad range of conditions that predispose to gastric aspiration–induced ALI (e.g., general anesthesia, alcohol and narcotic abuse, and neurologic disorders). Gastric aspiration results in a spectrum of potential outcomes ranging from being asymptomatic, producing a rapidly resolved pneumonitis, or progression to a severe and sustained ALI. ARDS is seen in 10–25% of witnessed gastric aspiration events (20–23) and carries a mortality rate of 35–60% (22, 24, 25). Our laboratory has established gastric aspiration–induced ALI/ARDS models in mice and rats that have remarkable fidelity with human ALI in terms of histopathology, ACW permeability, severe hypoxemia, reduction in surfactant activity, lung compliance, and lung vital capacity (26–30). In these models, the ALI/ARDS picture is manifest when the contents of the aspirate contain both acidic and small gastric food particle components. When acid alone is instilled into the lungs, a two-phase injury results. The initial injury phase (within 1 h of acid exposure) is primarily due to the acid’s direct caustic effects on pulmonary tissue (31), whereas the second injury phase (beginning at 3 h and peaking at 4–6 h postexposure) results from recruited PMNs (32). When sterile small gastric particles are instilled into the lungs in a nonacidic vehicle, the initial direct caustic tissue damage is not seen, but the acute PMN inflammatory phase is manifest within the same time frame as with acid-induced injury. In both cases, the inflammatory phase quickly resolves within 24 h. The combination of acid (caustic agent) and gastric particles (a noncaustic proinflammatory agent) produces a synergistic lung injury (i.e., greater than the additive effect of each single insult) that is sustained for ≥48 h in mice and rats (27, 30). This synergistic lung injury is likely to be a major factor predisposing to ARDS following gastric aspiration. Together, these results point to both the aspiration insult (e.g., pH, particle concentration) and host factors that modulate the inflammatory response influencing the severity of and recovery from ALI. Because NADPH oxidase and Nrf2 regulate inflammation and oxidative stress, it was reasoned that both pathways would have specific roles in modulating ALI following aspiration insults.

Our previous results demonstrated that NADPH oxidase–deficient (p4phox−/−) mice had increased and sustained pulmonary PMN accumulation, capillary leak, and proinflammatory cytokines compared with wild-type (WT) mice following acid aspiration (33). These were paradoxical results because NADPH oxidase–generated ROS would be expected to increase lung damage. We hypothesized that NADPH oxidase would reduce aspiration-induced ALI both by limiting neutrophilic inflammation and activating Nrf2. In the current study, the roles of NADPH oxidase and Nrf2 in modulating acute lung inflammation and injury in response to clinically relevant models of aspiration were evaluated. NADPH oxidase and Nrf2 limited acid aspiration-induced ALI, but through distinct mechanisms. The major effect of NADPH oxidase was to reduce PMN alveolitis, whereas the major effect of Nrf2 was to decrease ALI without significantly affecting PMN airway accumulation. Together, these results challenge the notion that ROS have exclusively an injurious effect in driving ALI and point to a more complex role for NADPH oxidase–derived ROS in modulating inflammation and injury following caustic insult.

Materials and Methods

Mouse knockout models

Mice with a targeted disruption of the p4phox gene have a defective NADPH oxidase, rendering phagocytes incapable of generating measurable superoxide (34). p4phox−/− mice were derived from C57BL/6 and 129 intercrosses and backcrossed 14 generations in the C57BL/6 lineage. Nrf2−/− mice were generated from C57BL/6 and 129 intercrosses as described (35) and backcrossed nine generations in the C57BL/6 lineage. Nrf2−/− mice were kindly provided through a Material Transfer Agreement from Jefferson Chan, MD (University of Southern California School of Medicine, Irvine, CA). Knockout mice were expanded by homozygous mating at the Roswell Park Cancer Institute. Male mice were used in all experiments (8–12 wk old) and were maintained under specific pathogen-free conditions. All procedures performed on animals were approved by the Institutional Animal Care and Use Committee at the Roswell Park Cancer Institute and complied with the U.S. Department of Health and Human Services’ Guide for the Care and Use of Laboratory Animals.

Gastric aspiration injury models

The gastric aspiration injury models in this study used an intratracheal (i.t.) instillation of one of the following injury vehicles: normal saline, pH ≈5.3 (vehicle control); saline plus HCl, pH 1.25 (acid); 7.5 mg/ml gastric particles (part.), pH ≈5.3; or saline plus HCl plus 7.5 mg/ml gastric particles (acid+part.), pH 1.25, as previously described (30, 33). A ventral midline tracheostomy was performed following induction of isoflurane anesthesia, and 3.6 ml/kg injury vehicle plus 0.2 ml air was instilled with a 1-ml syringe through a 22-gauge catheter inserted in the trachea (33). Mice were fluid-resuscitated with 1 ml normal saline injected s.c. into the ventral neck and allowed to recover in room air with food and water ad libitum. Particle results were derived from gastric filtrates, as previously described (28, 30). The particle preparation procedure removes gastric enzymes and bile salts and results in a mixture of gastric particles that ranged in size from 2–30 μm in diameter. The animals were sacrificed at 5 h postinjury vehicle instillation, the time of the peak synergistic inflammatory injury phase.

Bronchoalveolar lavage and lung processing

Mice were anesthetized with isoflurane, exsanguinated by transecting the inferior vena cava, and the lung vasculature was flushed with 5 ml saline injected into the right ventricle. A 22-gauge cannula was secured in the trachea, and bronchoalveolar lavage (BAL) was performed with five 1-ml saline instillations (33). The lung lobes were collected, flash frozen in liquid N2, and stored at −80°C. For histological analysis, tracheas were cannulated and lungs fixed by insufflating with 10% neutral buffered formalin for 24 h. Lungs were then paraffin-embedded, cut in 5-μm sections, and stained with H&E.

The recovered BAL was centrifuged at 1500 × g for 3 min at 4°C. The pelleted cells were counted by a Coulter counter (Beckman Coulter, Fullerton, CA), and the white cell differential was assessed using a Diff-Quik staining kit (Baxter, Miami, FL). Cell-free BAL supernatants were stored at −80°C until further analysis. BAL albumin concentration ([albumin]) was determined by ELISA (Bethyl Laboratories, Montgomery, TX). BAL TNF-α bioactivity was assessed by a cytotoxicity assay using WEHI 164 subclone 13 cells, as previously described (26). Other cytokines were measured by ELISA using capture and detection Abs and recombinant cytokine standards from R&D Systems (Minneapolis, MN).

NADPH oxidase activity was assessed by quantitation of GADPH (a cytoplasmic protein) and TBP (a nuclear protein) were almost entirely restricted to the cytoplasmic and nuclear fractions of lung homogenates, respectively (data not available).
shown). Proteins from cytoplasmic fractions were quantified using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Samples (40 μg protein/well) were loaded onto a 10% acrylamide gel, electrophoresed, and transferred to a polyvinylidene difluoride membrane. The membrane was incubated with goat anti-NAD(P)H quinone oxidoreductase 1 (NQO1; Immunex, San Diego, CA) or rabbit anti-actin (Sigma-Aldrich, St. Louis, MO) followed by alkaline phosphatase-conjugated donkey anti-goat or goat anti-rabbit secondary Abs (Santa Cruz Biotechnology, Santa Cruz, CA). Proteins were visualized with the ECL system (GE Healthcare, Piscataway, NJ) according to the manufacturer’s protocol, and fluorescence was detected using the Storm fluorescence scanner (GE Healthcare). Signal intensities were measured using ImageQuant software (GE Healthcare), and NQO1 expression was quantitated by fluorescence normalized to β-actin expression in each sample.

Treatment with 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole

1-[2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im) is a semisynthetic triterpenoid that directly induces Nrf2 activity (36). CDDO-Im was dissolved in PBS plus 10% DMSO plus 1% cremophor-EL. In experiments using CDDO-Im treatment, mice were injected i.p. with 100 μl 2 mg/ml CDDO-Im (0.2 mg/mouse), or vehicle, 5–10 min post–acid+part. instillation.

Extraction and quantitation of mitochondrial DNA from cell-free BAL

Total DNA was isolated from 400 μl cell-free BAL using the QIAamp DNA Mini kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. DNA was eluted in a 40-μl volume, and 5 μl was used for PCR reactions. Primers for mouse mitochondrial DNA (mtDNA) 16S rRNA (forward 5′-CTGAGAGAGACGCACGGCAGA-3′ and reverse 5′-GCTCTCGCTCATCCACCTG-3′) and mouse nuclear DNA β2-microglobulin (forward 5′-ATGGGAAAGCCGGAGCACATGCT-3′ and reverse 5′-GACCATC-TCAGTGGGGTGTAAT-3′) were synthesized (Invitrogen, Carlsbad, CA). Primer sequences have no significant homology with DNA found in any bacterial species published on the National Center for Biotechnology Information. Purified mtDNA was extracted from mouse liver mitochondria (Mitochondria Isolation Kit for Tissue; Pierce Chemical, Rockford, IL), which was used for generation of standard curves. No protein contamination was detected in the purified mtDNA, and nuclear DNA was minimal. Quantitative real time-PCR was performed in triplicate for absolute quantification of BAL mtDNA using SYBR Green ER Master mix (Invtrogen) in a 7900HT Fast Real Time PCR system (Applied Biosystems, Bedford, MA). Samples that produced no PCR products after 40 cycles were considered undetectable.

Assessment of mitochondrial DAMP-activated PMNs effects on vitro endothelial cell permeability

EA.hy926 cells, derived by the fusion of HUVECs with the continuous human lung carcinoma cell line A549, were obtained from Dr. Cora-Jean C. Edgell (University of North Carolina at Chapel Hill, Chapel Hill, NC) and maintained in DMEM medium with 10% PBS and penicillin and streptomycin in a 5% CO2 at 37°C (37). Transendothelial electrical resistance was measured in EA.hy926 monolayers by seeding 2 to 3 × 105 cells onto cytoseine and fibronectin–pretreated 8W10E+ cultureware (40 electrodes/well) and incubating at 37°C, 5% CO2, in an electric cell-substrate impedance-sensing system (Applied BioPhysics, Troy, NY) (38, 39). This system measures changes in impedance of the monolayer to alternating current flow. The microcurrents used have no detectable effect on the cells. Confluence was determined by capacitance at 64 kHz, coming to a plateau (∼10 nF) after an overnight incubation. In this system, impedance decreases and capacitance increases as permeability increases. Endothelial cell permeability change was assessed as capacitance change at 64 kHz (38, 40). Capacitance values in each well were normalized to capacitance at the start of treatments. Values from each microelectrode were pooled at discrete time points and plotted versus time as the mean ± SD (n = 2 wells).

Mitochondria were isolated from 200 mg mouse liver (Mitochondria Isolation Kit for Tissue; Pierce Chemical), suspended in 1 ml HBSS plus protease inhibitor mixture, and sonicated on ice (VCX130 Vibra-Cell; Sonics & Materials, Newton, CT) for 10 cycles of 30 s on and 30 s off at 50% amplitude, and the disrupted mitochondria were centrifuged at 12,000 × g for 10 min at 4°C, and the supernatant was used as a crude mitochondrial DAMP (MDT) solution. PMNs from healthy human donors were isolated from heparinized blood using PMN isolation medium (Thermo Fisher Scientific, Hudson, NH) (40). At the time of permeability measurement, the medium in each well was replaced with 200 μl medium containing various dilutions of MDT or 20 μg/ml Cpi (Invivogen, San Diego, CA) ± 2 × 105 PMNs ≤ 100 nM CDDO-Im and incubated at 37°C in 5% CO2. The endothelial cell layer capacitance was evaluated over 20 h. Studies performed using human PMNs were approved by the Institutional Review Board of the Beth Israel Deaconess Medical Center and conducted in accordance with the guidelines of the World Medical Association’s Declaration of Helsinki. Written informed consent was received from participants prior to inclusion in the study.

Survival analysis

Survival analysis of the WT and p47phox−/− mouse strains as a function of the different modes of aspiration was assessed by the Kaplan–Meier method, with all data censored by 48 h. The comparison between the two survival curves for WT and p47phox−/− mice at different gastric particle concentrations was made using the Mantel–Cox log-rank test.

Statistical analysis

Descriptive statistics were expressed as mean ± SEM. Comparisons between genotypes of continuous variables were assessed by Student t test with Bonferroni correction for multiple comparisons after confirmation of normal distribution by Shapiro-Wilk testing. If data were not normally distributed, the Mann–Whitney Wilcoxon rank-sum test was used to determine statistical significance. Prism for Mac OS X version 5.0c (GraphPad, San Diego, CA) software was used for the statistical analyses mentioned above.

To evaluate synergy between pulmonary exposure to acid and gastric particles, a two-way ANOVA using a generalized linear regression fit model was performed using JMP v.7.0.1 (SAS Institute, Cary, NC) statistic software. Synergy was defined as an interaction between variables (e.g., acid+part.) resulting in an effect (e.g., albumin leak) that is significantly greater than additive. The primary analysis consisted of fitting a standard 2 × 2 factorial ANOVA model with factors for acid (no, yes) and particulate (no, yes) for each genetic mouse strain (i.e., WT, p47phox−/−; or Nrf2−/−). Each factor in the model was coded as a 0/1 indicator variable such that it could be reparameterized in a generalized linear regression fit model. The full model consisted of the main effects of the two factors (i.e., acid, particulate) and their interaction (i.e., acid × particulate), where the interactions within the regression model were given as products of indicator variables corresponding to the main effects. The equation fitted for each time point was as follows:

\[ y = A + (B \times b) + (C \times c) + (D \times b \times c) \]

with y = measured parameter (i.e., BAL [albumin]), A = intercept, B = acid effect, b = acid indicating variable (0 = no acid, 1 = acid), C = particulate effect, c = indicating variable (0 = no particulate, 1 = particulate), and D = acid/particulate interaction effect. The fit model was performed without polynomial centering to accentuate the analysis for interaction, as opposed to the main effects of the two factors. Data presented in the figures that are statistically significant for synergistic interaction (more than additive) are indicated with the ◊ symbol over the appropriate group in the figure.

Results

NADPH oxidase limits aspiration-induced ALI

Different models of gastric aspiration that mimic what is observed in the clinic were used to understand mechanisms of aspiration-induced ALI. Aspiration of acid alone results in direct caustic injury, whereas aspiration of particles in a nonacidic vehicle causes foreign body–induced inflammation. Challenge with acid plus gastric particles resulted in inflammation-mediated synergistic lung injury results (i.e., an interaction that produces injury greater than the injuries produced by acid or particles alone), as assessed by extravasation of albumin into the airspaces, as well as blood oxygenation (i.e., PaO2/FiO2) (27–30). Histopathological analysis at 5 h after challenge demonstrated distinct patterns of lung inflammation and injury in WT mice as a function of the aspiration vehicle contents. Acid alone resulted in direct epithelial injury with denuded bronchial epithelium and necrotic cells and debris within the bronchial and bronchiolar airspace with moderate PMN infiltrate; gastric particles alone provoked generalized PMN infiltration as well as focal areas of dense PMN inflammation.
without evidence of necrotic cells; and combined acid and gastric particles led to a similar PMN infiltration pattern as seen in part. injury, but also resulted in focal areas of bronchial epithelial cell necrosis (Fig. 1). These results establish that the nature of the aspirate is linked to the extent of acute lung inflammation and injury.

Using these distinct clinically relevant modes of aspiration, this study sought to understand host factors that modulate acute lung inflammation and injury. Our previous results demonstrated that NADPH oxidase–deficient (p47<sup>phox</sup><sup>−/−</sup>) mice developed significantly greater alveolar PMN leukocytosis and BAL [albumin] (a marker of loss of ACW integrity) compared with WT mice (33). Based on these findings, a comprehensive evaluation of the role NADPH oxidase plays in modulating direct caustic injury (acid) versus foreign body–induced PMN inflammation (part.) and combined insult (acid+part.) was conducted. The analysis was focused on an early time point (5 h) after aspiration based on previous findings that PMN-dependent ALI begins at 3 to 4 h and peaks at 4–6 h following the initial caustic insult (32).

The p47<sup>phox</sup><sup>−/−</sup> mice were more susceptible to acid+part.–induced mortality compared with similarly treated WT mice (Fig. 2, Supplemental Fig. 1), strongly supporting a beneficial role of NADPH oxidase in mitigating injury. Administration of acid alone or particles alone did not result in mortality in either genotype. To test the hypothesis that NADPH oxidase plays a role in the synergistic lung injury induced by gastric acid and particle aspiration, BAL [albumin] was measured from WT and p47<sup>phox</sup><sup>−/−</sup> mice that were injured by pulmonary instillation of saline, acid, part., or acid+part. and sacrificed 5 h later. The particle concentration in the part. and acid+part. injury models was limited to 7.5 mg/ml so a nonlethal lung injury could be studied.

Consistent with our previous study (33), the acid pulmonary insult produced an increase (>2.5-fold) in albumin leakage in the p47<sup>phox</sup><sup>−/−</sup> genotype compared with the WT mice (12,317 ± 3,581 compared with 3,929 ± 994 µg/ml; p < 0.025), pointing to a protective role for NADPH oxidase in acid aspiration. The p47<sup>phox</sup><sup>−/−</sup> genotype did not have a significant effect on BAL [albumin] in the saline (vehicle control), part., or acid+part. injury groups. Also consistent with previous results (30), WT mice demonstrated a synergistic increase in lung injury (assessed by albumin leakage into the airspaces) that resulted from the combined injuries, as determined by two-way ANOVA (p < 0.03 for in-

![FIGURE 1](http://www.jimmunol.org/)
teraction) (Fig. 3A). However, the synergistic increase in lung injury did not occur in the p47phox−/− mice (p > 0.96). The synergistic increase in BAL [albumin] in the WT mice was 165% more than what would be expected if the vehicle instillation, acid, and particulate main effects were additive. Using the same calculation, the aspiration injury in p47phox−/− mice resulted in only a 2% increase over the additive effects, showing that NADPH oxidase is required for synergistic ALI. Together, these results demonstrate a protective role for NADPH oxidase in enhancing survival following acid+part. challenge and in limiting acid aspiration-induced ALI.

NADPH oxidase limits PMN accumulation into the airspaces following aspiration

There was substantial discordance in the accumulation of PMNs into the airspaces compared with the BAL [albumin] (Fig. 3B). When injured with acid-alone, the p47phox−/− mice had a 2.7-fold increase in the number of PMNs in the BAL compared with WT mice (2.2 ± 0.4 × 10^6 compared with 8.0 ± 2.4 × 10^5 PMNs; p < 0.013). The p47phox−/− mice experienced a >5.8-fold increase in BAL PMNs compared with WT mice after part. injury (2.7 ± 0.5 × 10^6 compared with 4.7 ± 1.2 × 10^5 PMNs; p < 0.004), and a >3.8-fold increase over WT mice after acid+part. injury (2.4 ± 0.4 × 10^5 compared with 6.2 ± 1.1 × 10^5 PMNs; p < 0.0008).

Paradoxically, accumulation of PMNs into the airspaces did not correlate with the BAL [albumin] caused by the different injury models in either mouse genotype. To assess injury relative to inflammation, the ratio of BAL [albumin]/PMN recovery was analyzed as a function of genotype and aspiration contents, a higher ratio indicating increased ACW dysfunction per recovered PMN. The BAL [albumin]/PMN ratio was greatest for acid and lowest for part. aspiration in both genotypes (Fig. 3C). These results are consistent with similar aspiration experiments in rats (data not shown). The p47phox−/− mice also exhibited a lower BAL [albumin]/PMN ratio following part. and acid+part. aspirations compared with similarly treated WT mice (Fig. 3C). These findings point to NADPH oxidase limiting the degree of PMN alveolitis while increasing the injury per recovered PMN.

FIGURE 2. Effect of particle concentration on survival of WT and p47phox−/− mice following acid+part. administration. Acid+part. injury was instilled, i.t., into the lungs of WT and p47phox−/− mice at the indicated [particle]. Experiments were designed to sacrifice the mice at 5, 24, or 48 h postinjury. The data were censored at the designated sacrifice time points; therefore, 48 h was the maximum time of censor. WT (filled circle with solid line) and p47phox−/− (filled square with dotted line) survival displayed as mean ± SEM. For WT mice: n = 59 (0), 13 (5), 68 (7.5), 8 (10), and 10 (40 mg/ml). For p47phox−/− mice: n = 92 (0), 20 (5), 97 (7.5), 16 (10), and 40 (40 mg/ml). Data were assembled from 50 independent experiments in addition, 45 p47phox−/− mice had 40 mg/ml part. alone instilled, i.t., into the lungs, and none died. Individual Kaplan–Meier plots are displayed in Supplemental Fig. 1. *p < 0.05 for comparison between genotypes at the same particle concentration by Mantel–Cox log-rank test.

FIGURE 3. Effect of NADPH oxidase on acid and gastric particulate aspiration-induced synergistic lung injury and inflammation. Saline, acid, part., or acid+part. was instilled into the lungs, i.t., of WT and p47phox−/− mice. BAL was performed 5 h later, and the recovered BAL was assessed for: albumin concentration ([albumin]) as an indicator of lung injury (white inset bars within the acid+part. data represent predicted values if injury was additive) (A); PMN alveolitis (B); and ratio of BAL [albumin] to number of recovered PMNs, an indicator of the degree of lung injury/PMN (C). n = 6–9 for each group from seven independent experiments. *p < 0.05 for comparisons indicated by the brackets, /p < 0.05 for synergistic interaction of acid and particulate components within the indicated genotype (WT or p47phox−/−) by two-way ANOVA.

NADPH oxidase modulates cytokine responses in gastric aspiration

BAL was analyzed for concentrations of the acute proinflammatory cytokines TNF-α and IL-1β (Fig. 4A, 4B). Part. injury led to a robust TNF-α response that was blunted when the gastric particulate injury vehicle was acidified (i.e., acid+part.) in the WT mice (525 ± 95 and 194 ± 50 pg/ml, respectively; p < 0.008) and, to a much greater degree, in p47phox−/− mice (1059 ± 150 and 57 ± 15 pg/ml, respectively; p < 0.0001). BAL [TNF-α] was 2-fold greater in p47phox−/− compared with WT mice in response to part. (p < 0.0085), but was lower in p47phox−/− compared with WT mice following acid injury (p = 0.022) and acid+part. injury (p = 0.018). Thus, paradoxically, there was an inverse relationship between BAL [TNF-α] (Fig. 4A) and BAL [albumin] per recovered BAL PMN in both genotypes (Fig. 3C).

In contrast to BAL [TNF-α] data, BAL [IL-1β] levels followed the expected paradigm of proinflammatory cytokines with regard to the loss of ACW integrity (Fig. 4B). Acid+part. aspiration resulted in an increase in BAL [IL-1β] relative to part.-induced albumin BAL levels in both WT (6932 ± 1232 and 201 ± 51 pg/
The three aspiration injuries.

and 450

6

b

difference in BAL [IL-1β] compared with acid+part. in both WT (406 ± 69 and 43 pg/ml, respectively; p < 0.0001) and p47phox−/− mice (244 ± 42 and 52 ± 7 pg/ml, respectively; p < 0.0003). No significant differences in BAL concentrations occurred between the genotypes with regards to other inflammation-associated cytokines tested (IFN-γ, IL-6, IL-10, IL-12, IL-17, MCP-1, and MIP-1α). These results point to NADPH oxidase-modulating airway levels of proinflammatory cytokines as a function of the type of aspiration insult.

Nr2−/− mice have increased lung injury compared with WT mice following acid aspiration, but have a distinct phenotype from NADPH oxidase–deficient mice

Nr2 is a transcription factor that is activated by intracellular oxidants to upregulate expression of numerous antioxidant and cytotoxic genes (41). Because NADPH oxidase had a protective role following acid aspiration, we hypothesized that oxidant-induced activation of Nr2 might be a mechanism by which NADPH oxidase limits ALI. To test this hypothesis, saline, acid, part., or acid+part. was instilled i.t., in WT and Nr2−/− mice, followed by sacrifice at 5 h. Similar to p47phox−/− mice, acid produced an increase (2-fold) in BAL albumin levels in Nr2−/− compared with WT mice (8227 ± 761 and 3929 ± 994 µg/ml, respectively; p < 0.005) (Fig. 5A). Interestingly, there was no difference in BAL [albumin] between the Nr2−/− and WT mice following the part. or acid+part. insults. In contrast to p47phox−/− mice, synergistic lung injury occurred in Nr2−/− mice subjected to acid+part., with the increase in BAL [albumin] being 126% more than what would be expected if the vehicle instillation, acid, and particulate main effects were additive.

Nr2 has no effect on PMN accumulation into the airspaces following gastric aspiration

Nr2−/− mice had levels of PMN inflammation that were similar to WT mice following all gastric aspiration insults (Fig. 5B). These findings are in contrast to the increased BAL PMN leukocytosis observed in p47phox−/− mice after acid, part., and acid+part. administration (Fig. 3B). In addition, Nr2−/− mice had greater BAL [albumin]/PMN ratio (a reflection of injury per recovered PMN) compared with WT mice following acid aspiration (0.24 ± 0.06 and 0.11 ± 0.03 pg/ml/PMN, respectively; p = 0.02), reflecting greater injury per recovered PMN. (Fig. 5C). Together, these results demonstrate that although both NADPH oxidase and Nr2 limit acid aspiration-induced ALI, they do so through distinct mechanisms. The major effect of NADPH oxidase is to reduce BAL PMN alveolitis, whereas the major effect of Nr2 is to decrease lung injury without significantly affecting airway PMN accumulation.

NADPH oxidase is required for full Nr2 activation following acid aspiration

Our prior studies showed that NADPH oxidase limited zymosan-induced acute lung inflammation, in part, through activation of Nr2 (17). We therefore postulated that NADPH oxidase would also be required for Nr2 activation following acid insult. To assess Nr2 activation in injured lungs, protein levels of NQO1 were examined in cytoplasmic extracts of lung homogenates of saline- and acid-injured WT, p47phox−/−, and Nr2−/− mice, the latter serving as a specificity control for Nr2-independent NQO1 expression (Fig. 6). There was a small increase in NQO1 levels in WT lungs following acid injury compared with the saline-injured group (3.54 ± 0.19 × 104 and 2.82 ± 0.27 × 104 NQO1/β-actin density units, respectively; p < 0.025). NQO1 levels were reduced in lungs of p47phox−/− compared with WT mice following both

ml, respectively; p < 0.0001) and p47phox−/− mice (7993 ± 1673 and 450 ± 104 pg/ml, respectively; p < 0.0005). There was no difference in BAL [IL-1β] between the two genotypes for any of the three aspiration injuries.

Based on the increased BAL PMN leukocytosis in p47phox−/− following aspiration injury (Fig. 3B), BAL [keratinocyte chemotactic attractant (KC)] and [MIP-2], the major PMN chemotactic chemokines, were evaluated (Fig. 4C, 4D). There was a small increase in BAL [MIP-2] in p47phox−/− compared with WT mice injured with acid+part. (51.7 ± 7.0 and 31.1 ± 0.5 pg/ml, respectively; p < 0.015). As with the BAL [TNF-α], part. caused greater BAL [KC] compared with acid+part. in both WT (406 ± 44 and 81 ± 16 pg/ml, respectively; p < 0.0001) and p47phox−/− mice (477 ± 88 and 134 ± 43 pg/ml, respectively; p < 0.003). A similar effect was observed with BAL [MIP-2] in WT (166 ± 28 and 31 ± 0.5 pg/ml, respectively; p < 0.0001) and p47phox−/− mice (244 ± 42 and 52 ± 7 pg/ml, respectively; p < 0.0003). No significant differences in BAL concentrations occurred between the genotypes with regards to other inflammation-associated cytokines tested (IFN-γ, IL-6, IL-10, IL-12, IL-17, MCP-1, and MIP-1α). These results point to NADPH oxidase-modulating airway levels of proinflammatory cytokines as a function of the type of aspiration insult.

FIGURE 4. Effect of NADPH oxidase on acid and gastric particulate aspiration-induced pulmonary proinflammatory cytokine and CXC chemokine production. Cell-free BAL recovered from WT and p47phox−/− mice 5 h following pulmonary injury, as described in Fig. 3, were analyzed for: TNF-α by WEHI bioassy (A); IL-1β by ELISA (B); KC by ELISA (C); and MIP-2 by ELISA (D). n = 6–9 for each group from seven independent experiments. *p < 0.05, †p < 0.1 for comparisons indicated by the brackets.

Nr2−/− mice have increased lung injury compared with WT mice following acid aspiration, but have a distinct phenotype from NADPH oxidase–deficient mice

Nr2 is a transcription factor that is activated by intracellular oxidants to upregulate expression of numerous antioxidant and cytotoxic genes (41). Because NADPH oxidase had a protective role following acid aspiration, we hypothesized that oxidant-induced activation of Nr2 might be a mechanism by which NADPH oxidase limits ALI. To test this hypothesis, saline, acid, part., or acid+part. was instilled i.t., in WT and Nr2−/− mice, followed by sacrifice at 5 h. Similar to p47phox−/− mice, acid produced an increase (2-fold) in BAL albumin levels in Nr2−/− compared with WT mice (8227 ± 761 and 3929 ± 994 µg/ml, respectively; p < 0.005) (Fig. 5A). Interestingly, there was no difference in BAL [albumin] between the Nr2−/− and WT mice following the part. or acid+part. insults. In contrast to p47phox−/− mice, synergistic lung injury occurred in Nr2−/− mice subjected to acid+part., with the increase in BAL [albumin] being 126% more than what would be expected if the vehicle instillation, acid, and particulate main effects were additive.

Nr2 has no effect on PMN accumulation into the airspaces following gastric aspiration

Nr2−/− mice had levels of PMN inflammation that were similar to WT mice following all gastric aspiration insults (Fig. 5B). These findings are in contrast to the increased BAL PMN leukocytosis observed in p47phox−/− mice after acid, part., and acid+part. administration (Fig. 3B). In addition, Nr2−/− mice had greater BAL [albumin]/PMN ratio (a reflection of injury per recovered PMN) compared with WT mice following acid aspiration (0.24 ± 0.06 and 0.11 ± 0.03 pg/ml/PMN, respectively; p = 0.02), reflecting greater injury per recovered PMN. (Fig. 5C). Together, these results demonstrate that although both NADPH oxidase and Nr2 limit acid aspiration-induced ALI, they do so through distinct mechanisms. The major effect of NADPH oxidase is to reduce BAL PMN alveolitis, whereas the major effect of Nr2 is to decrease lung injury without significantly affecting airway PMN accumulation.

NADPH oxidase is required for full Nr2 activation following acid aspiration

Our prior studies showed that NADPH oxidase limited zymosan-induced acute lung inflammation, in part, through activation of Nr2 (17). We therefore postulated that NADPH oxidase would also be required for Nr2 activation following acid insult. To assess Nr2 activation in injured lungs, protein levels of NQO1 were examined in cytoplasmic extracts of lung homogenates of saline- and acid-injured WT, p47phox−/−, and Nr2−/− mice, the latter serving as a specificity control for Nr2-independent NQO1 expression (Fig. 6). There was a small increase in NQO1 levels in WT lungs following acid injury compared with the saline-injured group (3.54 ± 0.19 × 104 and 2.82 ± 0.27 × 104 NQO1/β-actin density units, respectively; p < 0.025). NQO1 levels were reduced in lungs of p47phox−/− compared with WT mice following both
saline and acid administration, and, in contrast to WT mice, acid challenge did not increase NQO1 levels in the lungs of p47phox−/− mice. Lung NQO1 protein expression was lowest in Nrf2−/− mice. Although NADPH oxidase and Nrf2 have distinct functions in modulating inflammation, these results support a model in which NADPH oxidase–induced Nrf2 activation is one of a number of pathways that protects against inflammation-mediated injury.

**CDDO-Im, an Nrf2 activator, is protective in aspiration-induced ALI**

CDDO-Im is a triterpenoid that potently activates Nrf2 (36). Triterpenoids directly interact with thiol groups of the Keap1 redox sensor, leading to its dissociation from Nrf2 (42). We therefore asked whether CDDO-Im could be used therapeutically in aspiration-induced ALI. WT mice had acid+part. instilled into the lungs, i.t., followed by i.p. CDDO-Im (0.2 mg/mouse) or vehicle 15 min later. Acid+part. was selected because it results in the greatest increase in injury and PMN recruitment. The 15-min time point was chosen to simulate administering a therapeutic agent early following a witnessed aspiration event. CDDO-Im treatment had no effect on BAL albumin levels at 5 h post–acid+part., but

led to a 4-fold decrease in albumin leak at 24 h postinjury (4.2 ± 1.1 and 16.8 ± 6.6 mg/ml, respectively; p < 0.02) (Fig. 7A). Macrophage BAL recovery was similar between treatment groups. Paradoxically, although CDDO-Im did not affect BAL PMN numbers at 5 h, it led to an ~60% increase in BAL PMN recovery at 24 h (8.4 ± 0.8 × 10^6 and 5.3 ± 0.7 × 10^5 PMNs in CDDO-Im– and placebo-treated mice, respectively; p < 0.03) (Fig. 7B). At 24 h post–acid+part., CDDO-Im led to a lower BAL [albumin]/PMN ratio compared with placebo (0.007 ± 0.001 and 0.041 ± 0.021 µg/ml/PMN, respectively; p < 0.016) (Fig. 7C). NQO1 protein expression was increased in lung homogenates of mice that received CDDO-Im at both 5 and 24 h (Fig. 7D). Thus, despite leading to an increase in airway PMN accumulation, CDDO-Im reduced ALI following acid+part. aspiration.

**Acid aspiration causes rapid release of MTDs**

Histopathological assessment of the pulmonary injury 5 h after installment of the gastric aspirate indicated that the acid-containing aspirates caused necrosis of pulmonary epithelial cells, whereas the part.-only injury did not (Fig. 1). Zhang et al. (19) showed that MTDs released systemically following traumatic injury activate PMNs and induce PMN-mediated lung injury. We hypothesized that MTDs released following acid insult may prime PMN-mediated ALI. Instillation of acid, i.t., in WT mice resulted in a 120-fold increase in recovery of mtDNA (an MTD) (measured by quantitative PCR of the mitochondrial 16S rRNA gene) in cell-free BAL (Fig. 8A). At 5 and 60 min postaspiration, the mean percentage of PMNs in the BAL was <5% of the total cells recovered (data not shown). Thus, the recovered mtDNA at these early time points likely represents direct acid-induced cell disruption rather than inflammation-induced injury. mtDNA was not detected in serum (data not shown), suggesting restriction of the direct cell injury to the airspace tissues of the lungs.

**CDDO-Im limits endothelial cell permeability induced by MTDs and activated PMNs**

Based on our in vivo findings that aspiration injury leads to the rapid release of mtDNA and that CDDO-Im reduced ALI, an in vitro approach was used to dissect the roles of MTDs and
unstimulated versus activated PMNs in causing endothelial cell injury and the protective effect of CDDO-Im. A confluent endothelial cell monolayer was exposed to isolated human PMNs and an MTD extract derived from lysed mitochondria isolated from rat liver, and a biocapacitance system was used to monitor real-time changes in endothelial permeability changes as previously described (43). The combination of PMNs and MTDs increased endothelial cell permeability, and the injury was dependent on the MTD concentration (Fig. 8B). Addition of CDDO-Im reduced endothelial cell injury caused by MTDs and PMNs, whereas unstimulated PMNs did not result in increased endothelial cell permeability compared with medium (Fig. 8C). MTDs in the absence of PMNs

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resulted in increased endothelial cell permeability, which was reduced by CDDO-Im (Fig. 8D). The MTD extract contains mitochondrial DNA (a ligand of TLR-9), formylated peptides, and likely other products that can prime innate immune responses and cause cellular injury (19). To investigate the effect of CDDO-Im on PMN-mediated injury as distinct from direct endothelial injury caused by MTDs, we used TLR-9–primed PMNs. In prior studies, CpG sequences (a pure TLR-9 ligand) did not directly affect endothelial cell permeability, but stimulated neutrophil injury to endothelial cells (43). Addition of CDDO-Im attenuated endothelial cell injury caused by CpG-primed PMNs (Fig. 8E). Together, these results show that CDDO-Im can mitigate endothelial cell injury induced directly by MTDs and by TLR-9–activated PMNs. Our proposed model for the interaction of NADPH oxidase and Nrf2 in modulating inflammation and injury following gastric acid challenge is summarized in Fig. 9.

Discussion

These studies demonstrate important interactions between NADPH oxidase and Nrf2 as host factors that modulate inflammatory stress and different components involved in aspiration pneumonitis and injury. Acid aspiration is a caustic insult that leads to damage and necrosis of alveolar cells and release of DAMPs that activate innate immune responses. DAMP-primed PMNs are recruited to the lung and amplify injury; this inflammation-induced injury that follows the initial insult can lead to sustained ALI and ARDS. In PMNs, NADPH oxidase is rapidly activated by infectious threat and other stimuli and is the major source of ROS generation that targets microbes. The immediate effect of ROS generation from NADPH oxidase is expected to augment injury to cells and extracellular matrix. NADPH oxidase activation in neutrophils is also linked to generation of neutrophil extracellular traps (13, 44, 45), which contain serine proteases and other antimicrobial products that are potentially injurious. Although neutrophil extracellular trap neutrophils can exacerbate ALI (46), studies on the specific role of proteases in aspiration-induced ALI have produced different results (32, 47, 48). Paradoxically, NADPH oxidase–deficient mice have augmented lung inflammation and capillary leak after acid challenge, pointing to NADPH oxidase also mediating protective responses that limit injury. Our results support a model in which NADPH oxidase limits injury through a number of pathways. First, NADPH oxidase limits the extent of PMN alveolitis. Second, NADPH oxidase–derived ROS are associated with activation of Nrf2, a redox-sensitive transcriptional factor that induces antioxidant and cytoprotective responses.

BAL PMN numbers were reduced in WT compared with p47phox−/− mice following all of the tested aspiration models. NADPH oxidase stimulation of PMN apoptosis (49) and macrophage-mediated clearance of apoptotic PMNs (efferocytosis) (50, 51) may be pathways that dampen PMN inflammation. In contrast to NADPH oxidase, the major effect of Nrf2 was to limit ALI without modulating PMN alveolitis. Nrf2 is activated by oxidative stress and induces numerous cytoprotective pathways, such as phase 2 antioxidant enzymes and peroxisome proliferator-activated receptor-γ (52, 53), which can limit organ injury. Together, these results point to distinct roles for NADPH oxidase and Nrf2 in modulating inflammation and injury as a function of the nature of the insult. Because NADPH oxidase and Nrf2 are expressed in several lineages, a limitation of these experiments is that they do not provide knowledge regarding in which specific cell lineages NADPH oxidase and Nrf2 activity modulate inflammation and injury.

Circulating MTDs that are released following cellular injury can activate PMNs by ligation of specific pathogen-recognition receptors and elicit PMN-mediated lung capillary leak in a rat model that mimics sepsis-induced ARDS (19). mtDNA was isolated in cell-free BAL from acid-injured mice, leading to the speculation that MTDs released following acid-induced cellular necrosis may activate phagocytes and augment lung injury beyond the immediate cellular damage caused by acid instillation. The Nrf2 electrophile activator, CDDO-Im, was protective in acid+part.-induced ALI. CDDO-Im was administered 15 min after aspiration to simulate administering a therapeutic following witnessed aspiration. The reduction in ALI was not observed at 5 h after aspiration, but was apparent at 24 h. These results suggest that Nrf2 activation induces cytoprotective pathways that do not affect the immediate injury from gastric aspiration, but likely modulate inflammation-induced ALI. Paradoxically, CDDO-Im limited aspiration-induced ALI while increasing BAL PMN recovery (Fig. 7B), a finding consistent with CDDO-Im reducing the capacity of recruited PMNs to cause tissue injury (Fig. 7C). CDDO-Im could act at the level of recruited PMNs, lung stromal cells (e.g., endothelial and epithelial cells), or both. A limitation of these studies is that CDDO-Im can affect a number of pathways, and therefore, its protective effect may be Nrf2 independent. Consistent with these in vivo results, CDDO-Im attenuated endothelial cell injury caused by MTDs and activated PMNs (Fig. 8). These in vivo and in vitro results suggest that targeting Nrf2 can limit inflammation-induced injury.

Nrf2 regulates the expression of numerous genes in vivo, most of which have cytoprotective and antioxidant functions that mitigate cellular stress induced by electrophiles and free radicals (41). The reactive oxidants H2O2 and hypochlorous acid activate Nrf2 in both macrophages and airway epithelial cells in vitro (54–57). In prior studies, both acid and acid+part. aspiration result in reduced pulmonary antioxidant reserve capacity (58). These findings, along
with the current study results, lead us to postulate that activation of Nrf2 may limit ALI, in part, by mitigating inflammation-induced oxidative stress.

Although both p47^{phox-/-} mice and Nrf2^{+/--} mice had ≈2-fold greater ALI following acid aspiration compared with similarly treated WT mice (Figs. 3A and 5A), there were important phenotypic differences between the two knockout mice. NADPH oxidase limited BAL PMN inflammation (Fig. 3B), but Nrf2 did not affect PMN recovery (Fig. 5B). Indeed, NADPH oxidase activation had either a neutral or aggravating effect on ALI when ALI was normalized to BAL PMN recovery (Fig. 3C). In contrast, Nrf2^{+/--} mice had a greater BAL [albumin]/PMN ratio compared with WT mice following acid aspiration, supporting the conclusion that Nrf2 is involved in decreasing the injury produced by recruited PMNs. This finding may reflect decreases in ROS scavenging or other cytoprotective mechanisms in Nrf2-deficient PMNs (41, 59). A change in antioxidant capacity would be predicted to produce a greater injury with decreased PMN numbers. Additionally, NADPH oxidase, but not Nrf2, was required for the synergistic lung injury resulting from the combination of acid and gastric particle aspiration. These results clearly indicate that NADPH oxidase and Nrf2 activation have distinct roles in modulating aspiration-induced lung inflammation and ALI.

Airway cytokine and chemokine levels were most strongly related to the pathogenic model of aspiration (Fig. 4). BAL IL-1β levels correlated with the degree of lung injury and were generally similar between WT and p47^{phox-/-} mice (Fig. 4B). Prior studies have produced different results regarding the role of NADPH oxidase in inflammasome activation required for caspase-regulated IL-1β production (60–62). BAL TNF-α levels inversely correlated with injury, with the highest levels observed after part (the least injurious aspiration insult). TNF-α can enhance PMN recruitment by activation of adhesion molecules (63); however, in the part. model, these recruited PMNs appeared to have a lower injury potential compared with other aspiration insults (Fig. 3C). These results are consistent with our prior studies showing reduced BAL TNF-α levels following acid+part. compared with part. alone (29, 30) and suggest that caustic cellular injury may blunt certain cytokine responses.

Although NADPH oxidase is the major source of ROS in activated PMNs, other sources of ROS may also alter inflammatory responses following gastric aspiration. Superoxide anion generated by xanthine oxidase can mediate both antibacterial host defense (64) and ALI (65) in NADPH oxidase–deficient mice. In addition, the p47^{phox} subunit may also have NADPH oxidase–independent effects on inflammation and injury. Prior studies have demonstrated similar phenotypic expression of lung inflammation and injury in both p47^{phox-/-} and X-linked gp91^{phox-/-} deficient mice (17, 66). These consistent findings from two different mouse models support a critical role of NADPH oxidase in control of inflammation as opposed to an individual NADPH oxidase protein subunit functioning independently of NADPH oxidase or an artifact introduced during generation of one of the knockout colonies.

ROS can exacerbate ALI through several mechanisms, including direct cellular injury, NF-κB activation, and activation of injurious inflammatory responses (67–69). NADPH oxidase activation leads to rapid generation of ROS and activation of PMN granule proteases responsible for killing invading pathogens (10). Although initial actions are expected to be injurious, this study demonstrates that NADPH oxidase can also counterbalance these early proinflammatory and injurious events. This outcome may also explain the numerous unsuccessful studies using antioxidant approaches to limit inflammation in several diseases, including ALI and sepsis (9). We anticipate that recognition of the mechanisms involved in NADPH oxidase activation leading to the stimulation of protective pathways may result in the development of novel therapeutic approaches in gastric aspiration, as well as other ALI etiologies.

Disclosures
The authors have no financial interests of conflict.

References


