Streptococcus pneumoniae–Induced Oxidative Stress in Lung Epithelial Cells Depends on Pneumococcal Autolysis and Is Reversible by Resveratrol

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Background. Streptococcus pneumoniae is the most common cause of community-acquired pneumonia worldwide. During pneumococcal pneumonia, the human airway epithelium is exposed to large amounts of H2O2 as a product of host and pathogen oxidative metabolism. Airway cells are known to be highly vulnerable to oxidant damage, but the pathophysiology of oxidative stress induced by S. pneumoniae and the role of nuclear factor erythroid 2–related factor 2 (Nrf2)–mediated antioxidant systems of the host are not well characterized.

Methods. For glutathione/glutathione disulfide analysis BEAS-2B cells, primary broncho-epithelial cells (pBEC), explanted human lung tissue and mouse lungs were infected with different S. pneumoniae strains (D39, A66, R6x, H2O2/pneumolysin/LytA–deficient mutants of R6x). Cell death was proven by LDH assay and cell viability by IL-8 ELISA. The translocation of Nrf2 and the expression of catalase were shown via Western blot. The binding of Nrf2 at the catalase promoter was analyzed by ChIP.

Results. We observed a significant induction of oxidative stress induced by S. pneumoniae in vivo, ex vivo, and in vitro. Upon stimulation, the oxidant-responsive transcription factor Nrf2 was activated, and catalase was upregulated via Nrf2. The pneumococci-induced oxidative stress was independent of S. pneumoniae–derived H2O2 and pneumolysin but depended on the pneumococcal autolysin LytA. The Nrf2 inducer resveratrol, as opposed to catalase, reversed oxidative stress in lung epithelial cells.

Conclusions. These observations indicate a H2O2-independent induction of oxidative stress in lung epithelial cells via the release of bacterial factors of S. pneumoniae. Resveratrol might be an option for prevention of acute lung injury and inflammatory responses observed in pneumococcal pneumonia.

Keywords. autolysin; glutathione; hydrogen peroxide; lung epithelial cells; Nuclear factor erythroid 2–related factor 2; pneumolysin; Streptococcus pneumoniae.
pneumococcal infection caused by S. pneumoniae, which makes this pathogen the most dangerous among all other bacteria [1, 2]. Despite the availability of effective antibiotic regimens and improvements in the management of critically ill patients, the overall mortality due to pneumococci bacteremia remains high [1, 2].

An intriguing feature of S. pneumoniae is the accumulation of large amounts of H2O2 derived from high activity of the bacterial pyruvate oxidase (SpxB) and the absence of the most important H2O2-detoxifying enzyme, catalase [1, 3]. Under physiological conditions, H2O2 is well balanced by enzymatic and nonenzymatic antioxidants such as glutathione (GSH), which is known as the most efficient antioxidant in lung epithelium [4]. Nuclear factor erythroid 2–related factor 2 (Nrf2; encoded by NFE2L2) is central to the cellular oxidant defense mechanism and belongs to the cap’n’collar family of transcription factors containing the basic leucine region [5]. Nrf2 coordinates induction of phase II detoxifying and antioxidant enzymes [5, 6]. Upon stimulation with bacteria and other oxidants, Nrf2 binds to antioxidant-response elements (AREs) in the nucleus encoding many detoxifying agents and antioxidant enzymes, such as GSH or catalase, to protect cells from oxidative stress [6–8].

Although hydrogen radical–related injury considerably contributes to deleterious lung failure, the antioxidant defense of the human lung epithelium upon infection with pneumococci remains poorly understood. Based on the knowledge that S. pneumoniae is associated with a large accumulation of H2O2 in the environment and that H2O2 is a major oxidant stressor on lung epithelial cells, we wondered whether S. pneumoniae induces oxidative stress that, in turn, activates Nrf2 in lung epithelial cells as a defense against cytotoxic damage and whether it is possible to prevent oxidative stress via pretreatment with antioxidants.

### MATERIALS AND METHODS

#### Materials

H2O2 (30%) was obtained from Roth (Karlsruhe, Germany), and catalase from bovine liver and resveratrol were both obtained from Sigma-Aldrich (Seelze, Germany). All other chemicals used were of analytical grade and obtained from commercial sources.

#### Bacterial Strains and Generation of Supernatants and Extracts

Description of the S. pneumoniae strains and the generation of bacterial supernatants and extracts are given in the Supplementary Materials.

#### Cell Culture, In Vivo, and Ex Vivo Models

Details of the cell culture and infection procedures are given in the Supplementary Materials.

#### GSH and GSH Disulfide (GSSG) Assay

The analysis of GSH and GSSG was performed using a previously described protocol [9]. The ratio of GSH to GSSG was calculated and is indicated as the percentage of the control value for in vitro and ex vivo analysis. Quantitative results were given for the in vivo measurements.

#### Interleukin 8 (IL-8) Enzyme-Linked Immunosorbent Assay (ELISA) and Lactate Dehydrogenase (LDH) Release Assay

For all in vitro GSH/GSSG experiments, pneumococci-dependent cell activation was evaluated by measuring IL-8 release, and cell death was confirmed by the absence of LDH. Stimulations were further analyzed only if the initial assay induced a significant IL-8 response without significant release of LDH in the supernatant (data not shown). The supernatants for the in vitro experiments were collected, prepared, and analyzed for IL-8 by using an ELISA (Becton Dickinson, Heidelberg, Germany) and for LDH by using the Cytotoxicity Detection Kit (Roche, Mannheim, Germany) according to the instructions of the manufacturers.

#### Chromatin Immunoprecipitation (ChIP)

BEAS-2B cells were stimulated and processed for ChIP analysis as previously described [10]. Immunoprecipitations were performed with antibodies against Nrf2 and polymerase 2 (POL2; both from Santa Cruz Biotechnology, Santa Cruz, CA). The following primer sequences for catalase promoter analysis were used: forward: 5′-CACCCAGGCTGCTCAATCA-3′; reverse: 5′-ACTTCAGGCTAGCCAATCA-3′. Quantification of relative band intensity of 3 different experiments was analyzed using the pixel values (Adobe Photoshop elements 7.0).

#### Western Blot

For Western blot analysis, cells were lysed (total protein or fractionated cytosol/nucleus), subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred to a Hybond-ECL membrane (GE Healthcare, Munich, Germany) by electrobhlotting. Membranes were exposed to antibodies to Nrf2, lamin B, YY1, or actin (all from Santa Cruz Biotechnology, Santa Cruz, CA); subsequently incubated with secondary antibodies (labeled with IRDye 800 or Cy5.5, Rockland Immunochemicals, Gilbertsville, PA); and detected by an Odyssey infrared imaging system (LI-COR, Bad Homburg, Germany).

#### Statistical Analyses

Data are shown as the mean ± standard error of the mean for at least 3 independent experiments. One-way analysis of variance was used for dose-dependent or time-dependent comparisons, and main effects of 2 groups were compared by the Newman–Keuls post hoc test. In vivo data were compared by the Mann–Whitney U test.
RESULTS

S. pneumoniae Induces Oxidative Stress

After intranasal infection of C57BL/6 mice with encapsulated wild-type pneumococci strain A66, animals were euthanized 24 hours and 48 hours after infection and GSH and GSSG levels were analyzed in homogenized mice lung tissue. There was a significant reduction of the GSH to GSSG ratio 48 hours after infection, compared with 24 hours after infection (Figure 1A). Cultured human lung tissue was infected ex vivo with 10^5–10^7 colony-forming units (CFU)/mL of wild-type pneumococci (D39) and revealed a significant reduction of the GSH to GSSG ratio in a dose-dependent manner (Figure 1B). In vitro, the human bronchial epithelial cell line BEAS-2B demonstrated likewise a significant reduction of GSH to GSSG ratio 24 hours after stimulation with 10^6 CFU/mL D39 (Figure 1C).

Figure 1. Pneumococci induce oxidative stress in the lung. A, Mice were infected intranasally with 5 × 10^6 colony-forming units (CFU) of Streptococcus pneumoniae strain A66 (A) for 24 hours and 48 hours. B–D, Explanted human lung tissue and BEAS-2B cells were treated with 10^5–10^7 CFU/mL of S. pneumoniae D39 (B and C) or R6x (D) for 24 hours. E and F, Primary bronchial epithelial cells (pBECs) were treated with 10^5–10^6 CFU/mL R6x for 24 hours (E) or with 10^6 CFU/mL R6x for 8 h, 15 h, or 20 h (F). Ratios of glutathione to glutathione disulfide (GSH/GSSG) are presented in micromoles (A) or as the percentage of control values (B–F). All graphs represent a summary of at least 3 independent experiments. In vivo data were compared by the Mann–Whitney U test. In vitro data were analyzed by 1-way analysis of variance and the Newman–Keuls post hoc test. Abbreviation: PBS, phosphate-buffered saline.
Unencapsulated mutant of *S. pneumoniae* (R6x) induced a level of oxidative stress that was similar to or minimally higher than that induced by wild-type pneumococci in BEAS-2B cells (Figure 1D) which excludes pneumococcal capsule as an inducer of oxidative stress. Therefore, only unencapsulated D39 mutants were used for further experiments. To rule out that BEAS-2B cells, which derived from a SV40-transformed cell line, behave differently from normal lung epithelium cells, primary bronchial epithelial cells (pBECs) obtained by airway brushing during bronchoscopy were stimulated with 10^5 and 10^6 CFU/mL R6x. Consistently, pBECs demonstrated a significant reduction in the GSH to GSSG ratio in a dose-dependent and time-dependent manner upon stimulation (Figure 1E and 1F).

**Nrf2 Is Activated After Stimulation With *S. pneumoniae***

Nrf2 is an essential transcription factor in the antioxidant stress response and binds to a variety of different antioxidant promoters [5–8]. Using Western blot analysis, stimulation with 10^6 CFU/mL R6x demonstrated a higher Nrf2 protein concentration in the nucleus of BEAS-2B and pBECs as a predictor of Nrf2 activation after pneumococci stimulation (Figure 2A and 2B). The Nrf2-dependent gene catalase, known as the main H_2O_2-detoxifying enzyme that *S. pneumoniae* does not possess, was further assessed. ChiP showed that nuclear Nrf2 was bound at the ARE at the catalase promoter and that POL2 was recruited, indicating gene transcription (Figure 2C; relative quantification of band intensity is given in Supplementary Figure 1A and 1B). An increased concentration of catalase protein was found...
in BEAS-B2 cells after stimulation with $10^6$ CFU/mL R6x, compared with untreated controls (Figure 2D). Interestingly, pretreatment with resveratrol, a known inducer of Nrf2, significantly reversed oxidative stress in a dose-dependent manner in BEAS-B2 cells after stimulation with $10^6$ CFU/mL R6x (Figure 2E), indicating the involvement of Nrf2 in oxidative stress caused by S. pneumoniae.

**S. pneumoniae–Induced Oxidative Stress Is Independent on H$_2$O$_2$ Release**

After stimulation for 24 hours with 0.5 mM or 1 mM H$_2$O$_2$, BEAS-2B cells showed a significant reduction of the GSH to GSSG ratio (Figure 3A), which could be reversed by simultaneous incubation of 600 U of catalase and 1 mM H$_2$O$_2$ (Figure 3B). A pyruvate oxidase–deficient pneumococcal mutant (D39ΔcpsΔspxb) that is not able to generate H$_2$O$_2$ induced a significant reduction of the GSH to GSSG ratio, similar to the H$_2$O$_2$-competent pneumococci (Figure 3C). To confirm that the release of pneumococcal-derived H$_2$O$_2$ is not the primary inductor of the observed oxidative stress, the GSH to GSSG ratio was evaluated with the H$_2$O$_2$-competent S. pneumoniae strain and simultaneous incubation of 600 U of catalase. In accordance, catalase did not reverse pneumococci-induced oxidative stress in lung cells, which indicates a minor role of H$_2$O$_2$ in the induction of oxidative stress triggered by S. pneumoniae in host cells (Figure 3D). The H$_2$O$_2$ production of the S. pneumoniae strains used in this study showed a slightly higher release of H$_2$O$_2$ in encapsulated strains (D39 and A66), compared with unencapsulated mutant strains (R6x and R6xΔlytA; data not shown).

**S. pneumoniae–Induced Oxidative Stress Is Related to Pneumococcal Autolysin**

The main pneumococcal autolysin, LytA, degrades the bacterial cell wall, resulting in bacterial lysis, which leads to the release of intracellular molecules and bacterial cell wall components.
To test whether oxidative stress is induced by extracellular or intracellular components of *S. pneumoniae*, the unencapsulated *S. pneumoniae* mutant R6x and the corresponding strain additionally deficient of LytA were compared. To examine whether R6x undergoes autolysis in our experimental setting, we measured CFU of R6x and R6xΔlytA, which were grown for 24 hours in cell-culture medium. During bacterial growth in THY, we could not observe any differences between R6x and R6xΔlytA (Supplementary Figure 2A), whereas the CFU of the 2 strains significantly differed 24 hours after incubation in cell culture medium (Supplementary Figure 2B), indicating autolysis of R6x. Stimulation of BEAS-2B cells with 10^6

Figure 4. *Streptococcus pneumoniae*-related oxidative stress induction depended on pneumococcal autolysis. BEAS-2B cells were stimulated with R6x (A), R6x deficient in the main autolysin, LytA (R6xΔlytA; B), supernatants of R6x and R6xΔlytA (C and D), or R6x deficient in pneumococcal exotoxin pneumolysin (R6xΔply; E and F) with indicated doses for 24 hours. Cell culture supernatants were collected, and interleukin 8 (IL-8) release was determined by enzyme-linked immunosorbent assay (B, D, and F). Corresponding cell pellets were harvested, and intracellular ratio of glutathione to glutathione disulfide (GSH/GSSG) was measured and calculated as the percentage of the unstimulated control value (A, C, and F). All data embody a summary from at least 3 independent experiments. The given data were statistically analyzed by 1-way analysis of variance and the Newman–Keuls post hoc test.

[11, 12].
CFU/mL. R6xΔlytA did not induce a decrease in the GSH to GSSG ratio, as was seen with R6x, indicating that oxidative stress is related to pneumococcal LytA (Figure 4A). IL-8 secretion by BEAS-2B cells was similar upon infection with R6xΔlytA and R6x, demonstrating sufficient proinflammatory cell activation with both strains (Figure 4B). Additionally, we generated bacterial supernatants (10⁶ CFU/mL in cell culture medium) of R6x and R6xΔlytA to stimulate the cells for 24 hours. While the supernatants of R6x significantly reduced the GSH to GSSG ratio (comparable to viable R6x), neither viable R6xΔlytA nor their supernatants were able to induce oxidative stress (Figure 4C). Further, we observed a stronger IL-8 release by R6x supernatants, compared with viable R6x, whereas the supernatants from R6xΔlytA induced slightly less IL-8 than supernatants from viable R6xΔlytA (Figure 4D).

Pneumolysin is a major pathogenic factor of pneumococci. After LytA-dependent release, it acts as a pore-forming exotoxin and may further stimulate host cells by means of Toll-like receptor 4 (TLR4) [13]. BEAS-2B cells were stimulated with R6xΔply, an unencapsulated S. pneumoniae mutant that does not express pneumolysin. As is apparent from that observed for pneumolysin-deficient pneumococci, suggesting that oxidative stress is unrelated to pneumolysin. Interestingly, R6xΔply demonstrated an even stronger level of IL-8 secretion by BEAS-2B cells than the parental strain R6x (Figure 4F). To exclude the possibility that the effect is based on a bacterial cell wall–related factor that might have also been released by autolysis, BEAS-2B cells were stimulated with different concentrations of pneumococcal extracts (corresponding to 10⁶–10⁸ CFU/mL). Neither R6x nor R6xΔlytA extracts were able to induce oxidative stress in the bronchial epithelial cells (Supplementary Figure 2C). Sufficient cell stimulation was verified by IL-8 release (Supplementary Figure 2D). These data indicate that the pneumococci-induced oxidative stress in lung epithelial cells depend on a hitherto unidentified intrabacterial factor, rather than a cell-wall associated factor.

**DISCUSSION**

Herein, we demonstrated that S. pneumoniae causes a strong oxidant burden through the release of bacterial components to the epithelial surface of the lung. The oxidative stress induction in airway epithelial cells depended on the autolysis of the pneumococci, but it was independent of bacterial H₂O₂ release and the pore-forming exotoxin pneumolysin. The pretreatment of cells with the Nrf2-inducer resveratrol prevented pneumococci-related oxidative stress induction.

In principle, the airway epithelium carries an effective antioxidant defense system, including GSH, vitamins C and E, uric acid, β-carotene, and enzymatic antioxidants (eg, superoxide dismutase and catalase), and enzymes associated with GSH metabolism [14]. Despite these protective mechanisms, oxidative stress in the airway epithelium is known to cause events such as cell membrane damage, lipid peroxidation, and DNA methylation, which result in extensive cellular and tissue damage contributing significantly to the worsening of lung infections [15, 16]. However, little information about oxidative stress during pneumococcal pneumonia is available.

Interestingly, it was shown that NADPH oxidase–derived reactive oxygen species (ROS) limited the survival of mice in a pneumococcal model, suggesting unbalanced oxidative stress in the airway epithelium during pneumococcal pneumonia [11]. Moreover, blood samples from patients with pneumonia demonstrated low levels and activities of antioxidants such as superoxide dismutase, GSH peroxide, and GSH, compared with healthy controls, suggesting a high burden of oxidants [17].

GSH is well known as the most efficient antioxidant in the lung, and its depletion is associated with oxidative stress in many other diseases [4]. This study revealed a significant reduction of the GSH to GSSG ratio in human and mouse lung tissue, pBECs, and BEAS-2B after infection with pneumococci, indicating a significant manifestation of oxidative stress in airway cells due to S. pneumoniae.

Recent studies identified Nrf2 as the major oxidant transcription factor regulating the antioxidant defense [5, 6]. In studies using Nrf2 knockout mice, Nrf2 was shown to protect airway cells against many injury mediators [6, 7]. However, the role of Nrf2 in pneumococcal pneumonia and other bacterial diseases is not well characterized. Therefore, we assessed the role of Nrf2, which contributes to the regulation of the oxidant defense mechanism, in airway epithelium cells after stimulation with S. pneumoniae. This study showed that, upon stimulation, Nrf2 translocated from the cytoplasm into the nucleus in bronchial epithelial cells.

The role of Nrf2 in infectious diseases is still not well understood. Intriguingly, a recent study demonstrated that GSH depletion and the subsequent activation of Nrf2 enhances the activation of the activating transcription factor 3 (ATF3), which leads to the repression of interleukin 6 (IL-6) transcription, protecting the host against the innate cytokine storm and septic shock. On the other side, the Nrf2-ATF3–mediated suppression of IL-6 also inhibits bacterial clearance and may cause a higher susceptibility to other secondary infections [18]. These observations are in line with studies showing that (1) Nrf2 was associated with faster bacterial clearance in the lung of alcohol-fed rats [19], (2) Nrf2 effectively reduced chronic lung inflammation and B-cell responses to *Haemophilus influenzae* infections [20], (3) Nrf2 was involved in the resolution of lung injury in *Staphylococcus aureus* pneumonia in mice [21], (4) Nrf2-deficient mice with *S. aureus*–induced sepsis had better survival rates than Nrf2 knockout mice [22], and (5) Nrf2 knockout mice had a higher mortality rate when exposed to sublethal doses of lipopolysaccharide [23], comprehensively.
supporting the concept that Nrf2 has a protective effect during bacterial infections.

An important feature of *S. pneumoniae* is the production of high amounts of H$_2$O$_2$ [1, 3, 24], which is one major inducer of oxidative stress in cells and tissues [15, 25]. Catalase is well known as the major detoxifying enzyme of H$_2$O$_2$ that is transcribed in lung epithelium cells but not by *S. pneumoniae* and whose expression is regulated by Nrf2 [7]. We observed pneumococci-dependent recruitment of Nrf2 to the catalase gene promoter and forced expression of catalase protein in bronchial epithelial cells. Interestingly, pretreatment of cells with catalase, to degrade H$_2$O$_2$ only managed to reverse H$_2$O$_2$-induced oxidative stress but not pneumococci-dependent reduction of the ratio of GSH to GSGS. This was in line with our observation that pneumococcal mutants deficient in H$_2$O$_2$ production demonstrated similar levels of oxidative stress as H$_2$O$_2$-competent pneumococcal strains and that strains that produced less H$_2$O$_2$ (R6x) seemed to induce even more oxidative stress than wild-type D39, suggesting only a minor role of bacterial H$_2$O$_2$ in induction of oxidative stress in airway epithelium cells.

Strikingly, the specific Nrf2 inducer resveratrol was able to reverse oxidant stress in airway epithelium cells after pneumococcal stimulation, suggesting that activation of Nrf2 is a potential new adjuvant treatment option to reduce epithelial cell damage in patients with pneumococcal pneumonia. The molecular mechanism by which resveratrol regulates the induction of antioxidant and phase II detoxifying enzymes is not completely understood. However, Nrf2 has been shown to be central in this process [26]. In a resting state, Nrf2 binds to the cytoplasmic inhibitor Kelch-like epichlorohydrin-associated protein 1 (KEAP1) [27]. Upon stimulation with resveratrol, Nrf2 dissociates from KEAP1, probably via the activation of mitogen-activated protein kinases, translocates into the nucleus, and binds to ARE, which results in transcriptional activation of genes that protect the airway cell from ROS [5, 26] (a simplified pathway shown in Figure 5). Moreover, resveratrol may also reduce oxidative stress through inhibition of NADPH oxidase, which is known as an important ROS-producing enzyme, or by the ability to scavenge free ROS [28]. Resveratrol can be found in several plants, including red grapes and broccoli. It is inexpensive, can be easily included in diets of patients, and is not known to have serious side effects [29].

Nrf2 was previously described to be activated by the ligand binding to the pattern recognition receptors TLR2 [30] and TLR4 [31]. Both receptors are well known to detect pneumococcal components. While TLR2 recognizes pneumococcal cell wall components [10], TLR4 detects the *S. pneumoniae* exotoxin pneumolysin [13]. Pneumolysin is a pore-forming toxin that, when released in low concentrations, inhibits beating of the cilia, activates the host complement system, and induces apoptosis, inflammatory host cell responses, and several other defense mechanisms for survival advantage. In high concentrations, pneumolysin causes direct tissue damage by its pore-forming ability (the effects of pneumolysin are reviewed elsewhere [32]). Further, other studies suggest that pneumolysin is a potent activator of ROS production in neutrophils via the activity of NADPH oxidase [11, 32].

To test a possible role of these pathways, we used negative mutants of *S. pneumoniae* lacking either the main autolysin LytA or pneumolysin. Interestingly, mutants impaired in autolysis did not develop oxidative stress, suggesting that oxidative stress is mainly dependent on LytA-dependent liberation of intracellular molecules and cell wall fragments after bacterial lysis. The release of pneumolysin was proven to be dependent on the activity of pneumococcal LytA [11]. However, in our study, a pneumolysin-negative mutant induced a significant oxidative stress in the airway epithelium, just like the pneumolysin competent strains, meaning that oxidative stress is apparently not related to pneumolysin.

Overall, these observations suggest that pneumococci-induced oxidative stress in lung epithelial cells seems to be related to a hitherto unknown intrabacterial factor that is released during the autolysis of *S. pneumoniae*. This factor may be considered a new virulence factor, which possibly inhibits other microorganisms and damages host tissue for more proficient colonization and development of pneumococci-associated diseases. The identification of the responsible factor is going to be a goal of future studies. Furthermore, it might be very interesting to investigate the role of Nrf2 during *S. pneumoniae* pneumonia, using Nrf2 knockout mice, and to examine the effects of resveratrol in pneumococci-infected wild-type mice.
In conclusion, these observations indicate that H₂O₂-independent induction of oxidative stress in lung epithelial cells can occur via the liberation of bacterial factors of *S. pneumoniae* and that resveratrol might be a promising option for treatment to attenuate acute lung injury and inflammatory responses observed in pneumococcal pneumonia.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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