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In Vivo evidence of free radical generation in the mouse lung after exposure to *Pseudomonas Aeruginosa* bacterium: An ESR spin-trapping investigation

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Abstract

In the *Pseudomonas aeruginosa*-induced rodent pneumonia model, it is thought that free radicals are significantly associated with the disease pathogenesis. However, until now there has been no direct evidence of free radical generation *in vivo*. Here we used electron spin resonance (ESR) and *in vivo* spin-trapping with α -(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone to investigate free radical production in a murine model. We detected and identified generation of lipid-derived free radicals *in vivo* ($a^N = 14.86 \pm 0.03$ G and $a^H_\beta = 2.48 \pm 0.09$ G). To further investigate the mechanism of lipid radical production, we used modulating agents and knockout mice. We found that with GdCl₃ (phagocytic toxicant), NADPH oxidase knockout mice (Nox2^{-/-}), allopurinol (xanthine oxidase inhibitor), and Desferal (metal chelator), generation of lipid radicals was decreased; histopathological and biological markers of acute lung injury were noticeably improved. Our study demonstrates that lipid-derived free radical formation is mediated by NADPH oxidase and xanthine oxidase activation, and that metal-catalyzed hydroxyl radical-like species play important roles in lung injury caused by *Pseudomonas aeruginosa*.

Keywords

Free radicals; mice; *Pseudomonas aeruginosa* pneumonia; NADPH-oxidase; Xanthine oxidase

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is a common Gram negative bacterium that is invasive and toxigenic, produces respiratory infections in patients with abnormal host defenses, and is an essential nosocomial pathogen [1]. Frequently, lung infections caused by this opportunistic pathogen can present as a spectrum of clinical symptoms from a rapidly fatal pneumonia in immuno-compromised patients with AIDS to a life-threatening infection when the host has a low level of immunity as in recipients of cancer chemotherapy, bone marrow or lung transplants. Even patients with prolonged intubation of the airway in the intensive care unit are often colonized with *P. aeruginosa* [2–4].

Murine models of acute and chronic lung infection with *P. aeruginosa* have played a major role in the search for the molecular mechanisms underlying the pathogen virulence and host defense. In the last decade there has been accumulating evidence that *P. aeruginosa* infection may result in acute lung injury primarily by inducing the release of host-derived mediators responsible for the influx of phagocytes in the lung; in their antibacterial action, the phagocytes may release reactive oxygen species and reactive nitrogen species [4, 5]. Several lines of evidence suggest that animals challenged with *P. aeruginosa* undergo increases in membrane lipid peroxidation, protein oxidation and DNA damage that were positively associated with indices of lung injury and neutrophil infiltration [4, 5]. The role of reactive oxygen species in inducing injury to the lung and other tissues as a result of the *P. aeruginosa*-induced inflammatory response has been reported by other investigators, but direct evidence for their generation has been lacking.

Free radicals such as superoxide, nitric oxide, and peroxynitrite are thought to play important roles in the pathogenesis of acute lung injury because SOD (or its chemical mimics) [6], nitric oxide synthase inhibitors [7], and *N*-acetylcysteine all inhibit LPS-induced damage [8, 9]. Therefore, lung damage is thought to be caused by these reactive species either directly or indirectly.

P. aeruginosa also has LPS as one of its components. Most Gram-negative endotoxins (LPS) are potent immune stimulants through their interactions with TLR4 [4]. LPS isolated from *P. aeruginosa* has been shown to possess immunogenic potential but is less potent than LPS from other Gram-negative bacteria.

Recently, it has been proposed that production of free radicals by various bacterial products such as pyocyanin, LPS, and exotoxin A and by overactive immune responses of the host play vital roles in lung injury induced by *P. aeruginosa*. Production of lipid-derived free radicals in the acute respiratory distress syndrome (ARDS) model induced by intratracheal instillation of LPS was demonstrated in our previous studies, where we also confirmed that activation of NADPH oxidase from infiltrated phagocytes is critical and plays an important role in free radical generation by LPS [10].

Since LPS uses only one bacterial component, here we have used *Pseudomonas aeruginosa* as a source of bacterial infection with the goal of finding out where additional treatments such as anti-oxidant therapy, anti-protease therapy, etc. may be of use in supplementing antibiotic therapy. In this work we have used the ESR spin-trapping technique with α -(4-pyridyl-1-oxide)-*N*-tert-butyl nitron (POBN) to show that lipid-derived free radicals are generated during lung injury in the *Pseudomonas aeruginosa*-induced pneumonia model.

MATERIALS AND METHODS

Materials

2,2'-dipyridyl (Abbott Laboratories, North Chicago, IL), Desferal, pentobarbital, uric acid, and allopurinol (Sigma, St. Louis, MO), and modified Wright's stain kit (Fisher Chemicals, Pittsburgh, PA) were used as received. The spin trap α -(4-pyridyl-1-oxide)-*N*-tert-butyl nitron (POBN) was purchased from Alexis, San Francisco, CA.

Animals and treatments

Adult male C57BL/6 mice weighing about 20 g (8 weeks) were used in this study. Mice were anesthetized by pentobarbital (40 mg/kg), and pneumonia was induced by intratracheal instillation of 2×10^7 cells of *P. aeruginosa* suspended in 0.05 ml saline. Twenty-three hours after *P. aeruginosa* instillation, mice were anesthetized by pentobarbital (30 mg/kg) and were injected with POBN intraperitoneally (6 mmol/kg). One hour later, POBN-treated rats

were sacrificed, and lipid extracts of the lungs were measured for radical adduct content. In all *in vivo* and control experiments, sample preparations were performed *in situ* with fresh lung tissue. Control mice received 0.05 ml saline. Another group of mice was pretreated with GdCl₃ (7 mg/kg, intravenously, 24 h before *P. aeruginosa* instillation), allopurinol (2 mg/kg, intraperitoneally, 24 h and 2 h before *P. aeruginosa* instillation), or Desferal (50 mg/kg, intraperitoneally, 24 h and 2 h before *P. aeruginosa* instillation).

NADPH-oxidase knockout mice (Nox2^{-/-}) were obtained from the Jackson Laboratory (Bar Harbor, ME). Age-matched mice of the C57BL/6 strain that **possessed** normal NADPH-oxidase activity served as control animals for NADPH-oxidase deficiency experiments.

Both knockout mice and control mice were group-housed in a temperature-controlled room at 23–25°C with a 12/24 light/dark cycle and allowed free access to food and acidic water. The studies adhered to the National Institutes of Health guidelines for the care and handling of experimental animals. All animal studies were approved by the Institutional Review Board.

***In vivo* ESR studies**

Mice were treated with *P. aeruginosa* or saline as shown in Animals and treatments. The lungs were homogenized in 2.5 ml of 2:1 chloroform: methanol, 0.5 ml of 30 mM 2,2'-dipyridyl, 2 ml of 1.2 mM ultrapure phenol, and 2 ml of deionized water using a homogenizer (Fisher Scientific Power Gen 125) in an ice bath. The 2,2'-dipyridyl was used to inhibit *ex vivo* ferrous-dependent reactions. The phenol was used as an antioxidant to protect from *ex vivo* oxidation. In the case of pre-treatment with Desferal (DFO), we used Desferal (100 mg/kg ip) instead of 2,2'-dipyridyl (DP) to protect against artifactual production of free radicals, because using both DP and DFO simultaneously sometimes increases free radical production artifactually, presumably via electron transfer between the DP-iron complex and the DFO-iron complex.

To the homogenate obtained above, 16 ml of 2:1 chloroform:methanol was added and the resulting sample was shaken, then centrifuged at 2,000 rpm for 10 min (Beckman TJ-6) as described in [11, 12]. The chloroform layer was isolated and dried by passing through a sodium sulfate column. The solvent was evaporated to 0.5 ml of solution by bubbling with N₂. Sample handling lasted approximately 90 min for all experiments.

Immediately after solvent evaporation, ESR spectra were recorded at room temperature using a quartz flat cell in a Bruker EMX EPR spectrometer equipped with a super high-Q cavity. Spectra were recorded on an IBM-compatible computer interfaced with the spectrometer with instrument settings of 9.79 GHz, 20.2 mW microwave power, 100 kHz modulation frequency, 1,300 ms conversion time, and 655 ms time constant. The ESR spectra were simulated with a computer optimization procedure [13].

Histopathology

Control or treated lung tissue was removed 24 h after intratracheal instillation of *P. aeruginosa* and fixed-inflated to 20 cm H₂O pressure with 10% formalin. After fixation, all lobes of the lung were cut sagittally through the center of each lobe. Slices of each pulmonary lobe 2–3 mm thick were embedded in paraffin. Tissue sections of 3 µm thickness were stained with hematoxylin-eosin.

Broncho-alveolar lavage fluid (BAL fluid) and cell counts

As described previously [14], three injections and aspirations with 1 ml of sterile ice-cold saline containing 1 mM EDTA were used to collect the BAL fluid in the mice. The lavage

fluid was injected gently and then aspirated three times by syringe. Cells counts from fresh BAL fluid were determined by using a hemocytometer, and differential cell counts were performed by cytopsin (Thermo Shandon) on 500 cells from BAL fluid with a modified Wright's stain.

Preparation of *Pseudomonas aeruginosa*

P. aeruginosa laboratory-type strain PAO1 was used in our experiment. *P. aeruginosa* was cultured in tryptic soy broth (Difco) at 37°C for 18 h. The cells were washed 4 times in sterile PBS and resuspended in PBS at a concentration of 2×10^7 colony forming units (CFU). A 50 μ l inoculum of a bacterial suspension of the laboratory strain of *P. aeruginosa* PAO1 was used for intratracheal instillation. 50 μ l of PBS was instilled into the trachea of control mice. BAL fluid or homogenized tissue was cultured in tryptic soy agar and viable CFU counted 24 h later.

Statistical analysis

The statistical significance of the difference was determined by an unpaired Student's t test. Data are expressed as means $\pm$ SD. Differences between groups were considered statistically significant at the level of $p < 0.01$.

Results

Detection of free radicals in mouse lungs instilled with *Pseudomonas aeruginosa*

The technique of spin trapping involves the addition of a primary free radical across the double bond of a diamagnetic compound (spin trap) to form a radical adduct more stable than the primary free radical. This technique involves the indirect detection of primary free radicals that cannot be directly observed by conventional ESR due to low steady-state concentrations and/or very short relaxation times, which lead to very broad lines [15]. It is known that the greatest limitation of organic extraction is that only non-polar radical adducts that are soluble in chloroform, such as the POBN, lipid-derived radicals, can be detected. In addition, the radical adduct must be stable enough to survive not only the biological environment in which it was made but also homogenization of the tissue and the time required for solvent extraction and evaporation which, under our experimental conditions, was approximately 90 min. We chose POBN as our spin trap because it readily traps lipid radicals that are stable in lipid extraction of tissue for the requisite amount of time.

Twenty-four hours after administration of *P. aeruginosa* (2×10^7 cell count) and 90 min after sample preparation, a stable six-line ESR spectrum could be reproducibly detected in the lung extract of POBN-injected mice (Fig. 1A). The instillation of saline instead of *P. aeruginosa* resulted in a much weaker signal (Fig. 1B). Without the spin trap, neither *P. aeruginosa* nor saline instillation yielded a detectable spectrum. The increase in signal intensity of the POBN radical adduct in *P. aeruginosa*-treated lungs compared to saline-treated lungs was statistically significant (*P. aeruginosa* infection 20.7 ± 8.7 mm, control 6.5 ± 1.4 mm, $p < 0.01$).

To evaluate the possibility of *ex vivo* free radical generation, we performed a series of control experiments. In the lung extract from a mouse that had been treated with *P. aeruginosa* intratracheal instillation and then homogenized with POBN *ex vivo*, we detected a much smaller signal than that formed *in vivo* (Fig. 1C). In the extract from a mouse treated with POBN and then homogenized with *P. aeruginosa ex vivo*, the signal was also quite weak (Fig. 1D). In a system where both POBN and *P. aeruginosa* were added *ex vivo* to non-treated lungs and then homogenized, there was no detectable ESR spectrum of any radical adduct (Fig. 1E). The *ex vivo* concentration of POBN was chosen to be 5 mM on the

basis of the concentrations in blood, heart and liver reported by Liu *et al.* [16]. The *ex vivo* concentration of *P. aeruginosa* (2×10^7) was chosen to be high enough to cause inflammation. In the system where both POBN and *P. aeruginosa* (2×10^7 counts) were added *in vitro* to a solution not containing lung tissue and then homogenized, there was no detectable ESR spectrum of any radical adduct (Fig. 1F). These experiments indicate that the radical adduct formation detected in lipid extracts of lung was not produced *ex vivo*.

In order to study the implications for pseudomonal endotoxin, we used antibiotic-inactivated or boiled bacteria for intratracheal instillation (Fig. 2). Fig. 2A shows a spectrum from lung lipid extract of a mouse treated with *P. aeruginosa* and POBN for ease of comparison. Inactivated bacteria decreased the ESR signal by over 50% but did not eliminate it. These results imply that pseudomonal endotoxin produced nearly 50% of the lipid-derived free radical in the absence of viable bacteria (Figs. 2B, 2C). The small POBN spin adduct signal on Fig. 2D is from a control mouse that had not been treated with *P. aeruginosa*.

Computer simulation of the POBN radical adduct spectrum and confirmation of *in vivo* generation of free radicals

When the ESR spectrum was simulated using a computer program developed in this laboratory [13], the hyperfine coupling constants for the POBN radical adducts were $a^N = 14.86 \pm 0.03$ G and $a^H_\beta = 2.48 \pm 0.09$ G. To ascertain whether the POBN radical adduct detected was derived from lipid, we compared the hyperfine coupling constants with literature values (Table 1). There were only minor variations in hyperfine coupling constants between the *P. aeruginosa*-induced radical adducts and other radical adducts of lipid-derived free radicals identified as probably polyunsaturated fatty acid-derived (Table 1).

Based upon its hyperfine coupling constants, the radical responsible for the 4-POBN radical adduct is not hydroxyl or any other oxygen-centered radical adduct. The radical adduct of the six-line spectrum in Fig. 1A was derived from an endogenous source (e.g., lipids). Although the coupling constants of 4-POBN radical adducts are relatively independent of the structure of the trapped radical and, therefore, cannot be used to definitively identify the free radical intermediate we have detected, we provisionally assign it as a carbon-centered, PUFA-derived radical. We previously reported the detection and identification of a chloroform-soluble, 4-POBN radical adduct following LPS instillation in the rat lung [10]. The assignment of these species was based on the hyperfine coupling constants ($a^N = 14.94$ G and $a^H = 2.42$ G) of authentic ethyl and pentyl radical adducts of 4-POBN previously obtained *in vitro* [11] [17]. The production of carbon-centered, lipid radical adducts as a result of enhanced lipid peroxidation *in vivo* is the most realistic assignment of the six-line spectrum shown in Fig. 1A as supported by MS data [17,18].

The hydroxyl radical can initiate lipid peroxidation by abstracting hydrogen from lipid molecules. To investigate whether hydroxyl radical was produced in the lungs of *P. aeruginosa*-treated mice, the hydroxyl radical scavenger dimethyl sulfoxide (DMSO) was administered to mice with *P. aeruginosa* because it is well known that a reaction between DMSO and hydroxyl radical will yield $\cdot\text{CH}_3$ which, in the presence of O_2 , is converted to $\cdot\text{OCH}_3$ [17]. The $\cdot\text{CH}_3$ and $\cdot\text{OCH}_3$ are then detected as POBN adducts. The amount of POBN lipid radical did not appear to be increased by the presence of DMSO, and experiments with ^{13}C -labeled DMSO did not change the appearance of the 6-line ESR signal shown in Fig. 1. Either this approach failed to detect the hydroxyl radical, or another species is responsible for the initiation of lipid peroxidation (Fig. 1A).

Histopathological analysis of lung instilled with *P. aeruginosa*

Murine models of acute and chronic lung infection with *P. aeruginosa* have been used to study the molecular mechanisms underlying the pathogen virulence and host defense [18]. Lung morphology and histopathology by electron and light microscopy has been reported for animal models and humans with *P. aeruginosa* infection. [5, 19]. Our experimental protocol showed that twenty-four hours after intratracheal instillation of *P. aeruginosa*, inflammatory responses were confirmed by histological analysis of neutrophil infiltration and increasing cell counts of broncho-alveolar lavage fluid (BAL fluid). In the BAL fluid of mice treated intratracheally with *P. aeruginosa*, neutrophil counts increased significantly ($p < 0.001$) over those of the control group, but alveolar macrophages did not increase (Fig. 3). These data suggest that in this model *P. aeruginosa* caused severe lung inflammation in the form of neutrophil alveolitis.

Effect of GdCl_3 treatment to evaluate the role of phagocytes

As GdCl_3 is well known to decrease phagocyte activity [20], we evaluated its inhibitory effect on the production of free radicals in the lungs of mice treated intratracheally with *P. aeruginosa*. When GdCl_3 was administered to mice 24 h before *P. aeruginosa* instillation, the production of free radicals in this system decreased by 78.5% while the hyperfine coupling constants ($a^N = 14.94 \pm 0.07$ G and $a^H_\beta = 2.42 \pm 0.06$ G) were unchanged (Fig. 4). At the same time, the levels of neutrophils and macrophages in the bronchoalveolar lavage fluid also decreased significantly (data not shown). Parallel to changes in the neutrophil population, two lung-injury parameters, wet weight/dry weight ratio ($p < 0.01$) and the protein concentration in the BAL fluid ($p < 0.01$), were considerably decreased by GdCl_3 pre-treatment (Table 2). In the histopathological study, GdCl_3 pre-treatment resulted in a remarkable decrease in lung injury, significantly inhibiting diffuse alveolar damage including interstitial edema, infiltration with neutrophils and monocytes, parenchymal hemorrhage, collapse of air space, and fibrin exudation into alveolar space (Fig. 5). This inhibition applied not only to lung injury parameters and histopathological findings but also to free radical production in the lung. These results confirm that free radical production in this system depends on phagocytes, probably by infiltrating neutrophils.

We evaluated the clearance of *P. aeruginosa* in this model with or without the administration of GdCl_3 . The counts of *P. aeruginosa* in the mouse lung 24 h after bacterial inoculation are shown in Table 3. In this study, four different mice were used for bacteria counts at each time point. Because we observed an increase in bacterial yield with GdCl_3 treatment ($p < 0.001$) at the same time that symptoms decreased, these data suggest that lung injury in this model results from phagocytic cell infiltration, not from the bacteria itself (Table 3).

Effect of modulating agents and knockout mice on lipid-derived free radical production and histopathological data

In order to analyze the mechanism of lipid-derived free radical in detail in this system, we examined the effects of a xanthine oxidase inhibitor (allopurinol), a metal chelater (Desferal) and NADPH oxidase knockout mice ($\text{Nox2}^{-/-}$).

In the NADPH oxidase knockout ($\text{Nox2}^{-/-}$) mouse model, the production of lipid-derived free radical was significantly decreased and lung injury was mitigated (Figs. 4 and 5). Superoxide or superoxide-derived free radicals are probably important in the production of lipid-derived free radicals. All control experiments were performed using a control strain (age-matched C57BL/6) for NADPH oxidase knockout mice ($\text{Nox2}^{-/-}$). Free radical production, bronchoalveolar lavage cell counts, and histopathological findings in the control strain were similar to those in our previous experiments (Figs. 1, 3, 5).

We used Desferal to determine whether hydroxyl radical and/or other iron-dependent species were involved in the production of lipid-derived free radicals in this model since Desferal readily chelates iron to form the redox-inert Fe^{3+} complex, which prevents $\cdot\text{OH}$ radical formation via the Fenton reaction [21]. When mice were pretreated with Desferal 2 h and 24 h before *P. aeruginosa* inoculation, the production of lipid-derived free radicals was decreased by 55% and histopathological findings were noticeably improved (Figs. 4 and 5). We measured the bacterial counts of *P. aeruginosa* with and without Desferal 24 h after administration of *P. aeruginosa* and found no difference (data not shown).

Allopurinol, a competitive inhibitor of xanthine oxidase [22], also inhibited free radical production and lung injury seen in histological examinations (Figs. 4 and 5). Xanthine oxidase activity is a new pathway in the production of lipid-derived free radicals in *P. aeruginosa* treated mice; the activity was measured as described previously [18,19]. Its activity in lung homogenate was significantly increased after *Pseudomonas* infection (xanthine oxidase activity: Control 0.002 ± 0.001 , *Pseudomonas* infection 0.082 ± 0.005 nmol/min/ml). In the histopathological study, allopurinol was quite effective in limiting *Pseudomonas*-mediated lung injury (Fig. 5).

From these data, we have demonstrated that the mechanism of lung injury from lipid-derived free radical production involves two enzymatic pathways: NADPH-oxidase and xanthine oxidase.

Discussion

We have provided ESR evidence that free radicals are being generated *in vivo* in the lung and are dependent on *Pseudomonas* endotoxin. Based on ESR spectral simulations, we identified the radical adducts as carbon-centered, lipid-derived. We suggest that the carbon-centered radicals detected are probably an intermediate of enhanced lipid peroxidation in the lung caused by *P. aeruginosa in vivo* [5, 23]. and our previous *in vitro* studies [10, 11]. In fact, we have confirmed by various control experiments that the POBN-radical adduct was formed *in vivo* and not during sample collection or handling. Although the major difficulty of the spin-trapping technique *in vivo* is the mere detection of a radical adduct, other factors must be considered when spin traps are administered *in vivo*. For example, it is not known whether the background ESR spectrum detected in the lipid extract of the control animal after the injection of POBN was formed during sample handling or *in vivo* prior to sample collection. Based on our previous studies with HPLC/MS, we propose that they are radical adducts of ambient levels of endogenous radicals [17, 24, 25].

Since free radicals in biological systems are characterized by their high reactivity, short lifetimes, and low concentrations, the type of spin trap used is an important factor in determining how informative and sensitive the spin-trapping technique may be for a given free radical. A number of our previously reported spin-trapping investigations have used the nitron spin trap α -(4-pyridyl-1-oxide)-N-tert-butyl nitron (POBN) due to its relative hydrophilicity, low toxicity, solubility and stability leading to reproducible results (10–12, 37). The detection of a free radical generated *in vivo* is only possible if the spin trap and free radical concentrations are high enough and the rate of spin trapping occurs rapidly. Due to the formation of relatively unreactive radical adducts, POBN might be expected to protect the lung tissue from the effects of free radicals. However, under the conditions employed here, it is considered unlikely that POBN will react with all but a very small fraction of radicals generated (due to competing reactions of radicals with biomolecules) and therefore it is unlikely that the small concentration of the spin trapping agent (attributable to *in vivo* pharmacokinetics) will have a significant effect on toxicity due to radical scavenging.

Histopathological analysis of the lung tissue instilled with *P. aeruginosa* and the inflammatory response through cell counts of bronchoalveolar lavage were followed as indices of lung injury and were assessed to determine if radical generation preceded or was associated with changes in the lungs. Although *P. aeruginosa* did not significantly enhance alveolar macrophage numbers in bronchoalveolar lavage, it significantly increased total cell and neutrophil counts. These data demonstrate that free radical generation was associated with severe lung inflammation as a result of neutrophil infiltration caused by *P. aeruginosa*. This is consistent with other studies which showed increased oxidative stress in patients or experimental animals with pneumonia [4, 5]. It should be noted that the role of specific virulence factors, some lung enzyme activities, pulmonary oxidant-antioxidant status, etc. in *P. aeruginosa* infected animals have been extensively studied [4, 5] and were not examined in the present study.

Lipid radicals can cause tissue injury via protein damage [26, 27] where protein is oxidized as a result of the free radical chain reaction of lipid peroxidation. When we examined the radical mechanism using GdCl_3 as a phagocytic toxicant [28] and NADPH oxidase knockout mice ($\text{Nox2}^{-/-}$), we found a significant decrease of the lipid-derived radical production and improved histo-pathological parameters in the lungs. With the use of GdCl_3 , we also demonstrated that the participation of alveolar macrophages is indispensable for free radical generation, neutrophilic inflammation, and lung injury. The inhibition of neutrophilic infiltration by GdCl_3 , which is probably the result of enzyme blockage and factors related to neutrophil migration and adhesion that are released from macrophages, has been reported previously in LPS- and ozone-induced lung injury [10, 29]. The production of reactive oxygen species by the phagocyte multicomponent NADPH oxidase system is well known as a critical component of antimicrobial host defense [30, 31]. Therefore, we concluded that NADPH oxidase activation from phagocytes plays an essential role in the generation of free radicals in mice with pneumonia caused by *P. aeruginosa*.

Recently, additional homologs of NADPH oxidase have been discovered and suggested to have specific involvement in respiratory and cardiovascular disease [32, 33]. *In vitro* studies have demonstrated that a wide range of inflammatory factors upregulate the expression of $\text{Nox2}^{-/-}$, and superoxide auto-augments the formation of superoxide through an upregulation of NADPH-oxidase activity in pulmonary artery endothelial cells [34]. Other reports have described the presence of NADPH oxidases in different types of cells with different functions [33]. For example, the oxidase in neutrophils releases large amounts of superoxide in bursts, whereas the vascular NADPH oxidases continuously produce low levels of superoxide [33]. In this study the data from genetically modified mice that lack an active catalytic subunit of NADPH oxidase, $\text{Nox2}^{-/-}$, provide evidence for the involvement of the $\text{Nox2}^{-/-}$ protein component of the NADPH oxidase system. The xanthine/xanthine oxidase system has been shown to upregulate the expression of $\text{Nox2}^{-/-}$, although the xanthine oxidase inhibitor allopurinol had no effect [33, 34]. However, the present study does not exclude or imply the role of other cytosolic NADPH oxidases [30–32].

The contribution of xanthine oxidase has been reported by Wright *et al.* in cytokine-induced acute lung injury in rats [35]. Kahl *et al.* reported that LPS increased xanthine oxidase activity of plasma in an LPS-induced sepsis model [36]. LPS is also produced by *Pseudomonas*, a Gram negative bacterium. In *Pseudomonas* pneumonia, LPS and other bacterial extracts induced many kinds of cytokines, which have been thought to increase the xanthine oxidase activity. However, this increase has not been experimentally confirmed.

We have recently reported the importance of xanthine oxidase activity in the pathogenesis of lung injury caused by super-antigens [37]. We have shown that when 4-amino-6-hydroxypyrazolo(3,4-d)-pyrimidine (an allopurinol derivative) was used as an antagonist of

xanthine oxidase, survival rate and histopathological findings of lung injury were significantly improved. In view of this, we have evaluated here the efficacy of allopurinol in free radical production and histological findings. We found an inhibitory effect of allopurinol on radical production and a beneficial effect of allopurinol for histopathological findings. However, these experiments did not address the potential role of xanthine oxidoreductase activity on free radical generation in humans since much lower activity has been reported in a variety of human tissues relative to other species including rats and mice [38–41]. Other studies have demonstrated that xanthine oxidase and xanthine dehydrogenase are interconvertible forms of the same enzyme, and some cytokines or hypoxia upregulated their generation at the translational and post-translational levels [42–44]. Tumor necrosis factor, (TNF- α), interleukin-1, and IFN- γ were also shown to induce their activity in epithelial cells [42]. All these findings indicate that xanthine oxidase/dehydrogenase is regulated in a cell-specific manner and by inflammatory cytokines and complex physiological and pathological events. However, we do not have data to confirm or reject these findings.

Desferal is a chelating agent for iron and other metals. Most stored iron is in the ferric state (Fe³⁺), the release of which can create a pool of Fe which, in turn, can participate in the Fenton reaction to form hydroxyl radical. Desferal readily chelates iron to form a redox-inert Fe³⁺ complex and prevent hydroxyl radical formation. Here Desferal had a protective effect on lung injury most likely by Fe³⁺ chelation, thereby inhibiting lipid peroxidation and free radical formation [45]. In addition, iron is necessary for bacterial growth. Although several papers have shown that metal-chelating agents inhibit bacterial growth in *P. aeruginosa* [46], there has been no direct evidence that bacterial growth is inhibited by Desferal. In our study, bacterial counts of *P. aeruginosa* with and without Desferal 24 h after administration of *P. aeruginosa* were not changed, thus supporting the conclusion that Desferal inhibits free radical production by inhibiting the Fenton reaction. It has been reported that alterations in proinflammatory cytokines, adhesion molecules, and chemotactic gradients play an important role in the accumulation of neutrophils during lung inflammation, and Desferal has been shown to interfere with the adhesion functions of activated neutrophils. [47, 48]. In addition, the effect of Desferal on the POBN radical adduct detected by ESR as a result of *P. Aeruginosa* treatment may not reflect the role of free iron in lipid peroxidation but rather the chain-breaking antioxidant character of Desferal [49–51]. These data suggest that there may be other mechanisms that account for its protective effects in addition to the inhibition of iron's catalytic generation of hydroxyl radical. Literature data imply that products of *P. Aeruginosa* may also lead to oxidant-mediated tissue injury. For example, pyocyanin produced by many strains of *P. aeruginosa* has been shown to undergo cellular redox cycling, to interact synergistically with iron bound to another *P. Aeruginosa* secretory product, the siderophore pyochelin, to damage pulmonary endothelial cells through production of •OH [52]. ESR spin-trapping evidence that pyocyanine and protease-cleaved Fe-transferrin act synergistically to enhance endothelial cell injury via formation of •OH has also been demonstrated in vitro [53].

The experiments from our in vivo study do not provide evidence of hydroxyl radical generation. The hyperfine coupling constants of the radical adduct that we did observe are not consistent with a POBN/OH adduct detected in either aqueous solution or the nonpolar solvent benzene [54, 55] (Table 1). Furthermore, the POBN oxygen-centered radical detected in a lipid extract of skin (assigned to OOH) also had coupling constants closer to those from POBN/OH than to carbon-centered adducts (Table 1). Therefore, we conclude that our radical adduct is a carbon-centered adduct, as its coupling constants are very similar to those of other POBN-carbon-centered adducts (Table 1).

There are other potential mechanisms by which free radical generation may be triggered in the lungs of mice infected with *P. aeruginosa* in addition to NADPH-oxidase and xanthine-

oxidase involvement. One potential common inflammatory mechanism is participation of reactive nitrogen species such as nitric oxide and peroxynitrite that lead to the nitration of cellular lipids, proteins and nucleotides. Though we did not assess markers of reactive nitrogen species, this is clearly an important focus for subsequent studies of the mechanisms of free radical generation in the *P. aeruginosa* mouse model of pneumonia.

In conclusion, the present investigation has shown (i) that lipid-derived radicals are generated in the lungs of mice with pneumonia by *P. aeruginosa*; (ii) that NADPH and xanthine oxidase are required for the generation of these radicals; and (iii) that metal-catalyzed, hydroxyl-like species play an essential role in lung injury caused by *Pseudomonas aeruginosa*. Taken together, our results imply that NADPH-oxidase, xanthine-oxidase and ferric iron work synergistically to generate free radical metabolites in lung inflammation caused by *P. aeruginosa*. In this respect, the primary role of macrophage toxicants, xanthine oxidase inhibitors, and iron chelators in inhibiting free radical-initiated peroxidative tissue injury is established to a great degree. Thus, this report suggests that enzyme inhibitors might be useful for the development of therapeutic agents in supportive therapy not only for pseudomonal infection but also for lung injury in general.

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Fig. 1. ESR spectrum in mouse lung treated with intratracheal *P. aeruginosa* instillation
 (A) Representative ESR spectrum of POBN radical adduct(s) detected in lipid extracts of lung 24 h after intratracheal instillation of *P. aeruginosa* and 1 h after intraperitoneal administration of POBN. (B) Same as in (A), but mice were not given *P. aeruginosa*. (C) ESR spectra of mouse lungs treated with intratracheal *P. aeruginosa* instillation and *ex vivo* addition of POBN. (D) ESR spectra of non-treated mouse lungs 1 h after intraperitoneal administration of POBN and *ex vivo* addition of *P. aeruginosa*. (E) ESR spectra of non-treated mouse lungs with *ex vivo* addition of POBN and *P. aeruginosa*. (F) ESR spectra of *in vitro* addition of POBN and *P. aeruginosa* without mouse lung.

Fig. 2. LPS implication for free radical production in mouse lung treated with intratracheal *Pseudomonas aeruginosa* (PA) instillation

(A) ESR spectrum of POBN radical adduct(s) detected in lipid extracts of lung 24 h after intratracheal instillation of *P. aeruginosa* and 1 h after intraperitoneal administration of POBN. (B) ESR spectrum of POBN radical adduct(s) from gentamycin-treated PA (incubated with gentamycin for 2 h at 37°C) was added instead of non-treated PA. (C) ESR spectrum of POBN radical adduct(s) when boiled PA (PA was boiled for 1 h at 100°C) was instilled instead of non-treated PA. (D) ESR spectrum of POBN radical adduct(s) from saline control group.

BALF cell counts

Fig. 3. Cell counts in BALF of *P. aeruginosa*-treated and control mice

Bronchoalveolar lavage (BAL) of mouse lung treated with or without intratracheal *P. aeruginosa* (PA) instillation was performed with saline containing 1 mM EDTA. Cell counts from BAL were determined by using the hemocytometer method, and differential counts were performed on 400 cells from BALF smears stained with a modified Wright's stain. Results are expressed as means $\pm$ SD of three independent experiments (n=3).

Fig. 4. Inhibition of free radical generation in mouse lung treated with intratracheal *P. aeruginosa* (PA) instillation using GdCl₃, NADPH oxidase KO mice, allopurinol (ALP), and Desferal (DFO)

ESR signal intensity of radical adducts detected in lipid extract from lungs of GdCl₃-pretreated mice (5 mg/kg iv), NADPH knockout mice (Nox2^{-/-}), allopurinol-pretreated mice (2 mg/kg ip), and Desferal-treated mice (100 mg/kg ip) 24 h after intratracheal instillation of *P. aeruginosa* and 1 h after intraperitoneal administration of POBN. Results are expressed as a percentage of the control mice and are the means $\pm$ SD of four independent experiments (n=4).

Fig. 5. Effect of modulating agents/KO mice on *P. aeruginosa* (PA)-infected lung in histological findings

Representative histopathological findings from control lungs and lungs of PA treated mice, NADPH knockout mice, allopurinol-pretreated mice, and Desferal-pretreated mice 24 h after intratracheal instillation of *P. aeruginosa* (n=4). Hematoxylin-eosin stain was used (original magnification, $\times 200$).

Table 1

Hyperfine Coupling Constants of POBN radical adducts

Radical (system)	aN	aβH	Solvent	Reference
Pentyl (in vitro, lung extract)	15.24±0.03	2.44±0.03	CHCl ₃ /MeOH	11
Ethyl (in vitro, lung extract)	15.27±0.01	2.47±0.03	CHCl ₃ /MeOH	11
•C (arachidonic acid-derived)	15.12±0.01	2.35±0.01	CHCl ₃ /MeOH	11
•C (linoleic acid-derived)	15.04±0.01	2.31±0.01	CHCl ₃ /MeOH	11
•C (LPS, lung lipid extract)	14.94±0.07	2.42±0.06	CHCl ₃ /MeOH	10
•C (PA, lung lipid extract)	14.86±0.03	2.48±0.09	CHCl ₃ /MeOH	this work
•C (PPIX, light, skin extract)	14.8	2.6	CHCl ₃ /MeOH	54
•OOH (PPIX, light-skin extract)	13.8	1.8	CHCl ₃ /MeOH	54
•OH (in vitro)	14.4	1.8	Benzene	55
•OH (in vitro)	14.9	1.6	Aqueous	55

Table 2GdCl₃ pre-treatment effect on the lung wet weight/dry weight ratios and the lavage protein concentrations

	Lung wet weight/dry weight ratio	* Protein (mg/ml)
Control	4.69 ± 0.28	0.26 ± 0.01
PA-IT	5.87 ± 0.06 ^{\$}	2.17 ± 0.22 [†]
GdCl ₃ + PA-IT	4.95 ± 0.07 ^{\$\$}	0.96 ± 0.11 ^{††}

* Protein concentrations were measured by the bicinchoninic acid protein assay.

^{\$} p < 0.001 versus control group,

^{\$\$} p < 0.01 versus PA-IT group,

[†] p < 0.001 versus control group,

^{††} p < 0.01 versus PA-IT group

Table 3GdCl₃ pre-treatment effect on bacteria counts by colony-forming method (n = 4, mean ± SD)

	Bacterial Counts	
	BAL (10 ⁴ cfu/ml)	Tissue (10 ⁵ cfu/ml)
PA-IT	0.19 ± 0.11	0.46 ± 0.16
GdCl ₃ + PA-IT	1.76 ± 0.69 [§]	5.76 ± 0.64 [‡]

* Bronchoalveolar lavage (BAL) and tissue homogenate were used for counting by colony-forming methods.

[§] $p < 0.05$ versus PA-IT group,

[‡] $p < 0.001$ versus PA-IT group