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Antioxidants preserve macrophage phagocytosis of *Pseudomonas aeruginosa* during hyperoxia

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Abstract

Pseudomonas aeruginosa (PA) is a leading cause of nosocomial pneumonia in patients receiving mechanical ventilation with hyperoxia. Exposure to supraphysiological concentrations of reactive oxygen species during hyperoxia may result in macrophage damage that reduces their ability to phagocytose PA. We tested this hypothesis in cultured macrophage-like RAW 264.7 cells and alveolar macrophages from mice exposed to hyperoxia. Exposure to hyperoxia induced a similarly impaired phagocytosis of both the mucoid and non-mucoid forms of PA in alveolar macrophages and RAW cells. Compromised PA phagocytosis was associated with cytoskeleton disorganization and actin oxidation in hyperoxic macrophages. To test whether moderate concentrations of O₂ limit the loss of phagocytic function induced by ≥ 95% O₂, mice and RAW cells were exposed to 65% O₂. Interestingly, although the resulting lung injury/cell proliferation was not significant, exposure to 65% O₂ resulted in a marked reduction in PA phagocytosis that was comparable to that of ≥95% O₂. Treatment with antioxidants, even post hyperoxic exposure, preserved actin cytoskeleton organization and phagocytosis of PA. These data suggest that hyperoxia reduces macrophage phagocytosis through effects on actin functions which can be preserved by antioxidant treatment. In addition, administration of moderate rather than higher concentrations of O₂ does not improve macrophage phagocytosis of PA.

Keywords

bacterial infection; *Pseudomonas aeruginosa*; ventilator-associated pneumonia; alveolar macrophages; actin cytoskeleton; oxidation

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INTRODUCTION

Nosocomial pneumonia (NP) is a leading cause of morbidity and mortality in the intensive care unit (ICU) [1]. According to the National Nosocomial Infection Survey, 86% of NP is associated with mechanical ventilation with hyperoxia [2]. During an average day in any American ICU, mechanical ventilation is administered to at least 40% of patients. The risk of acquiring ventilator-associated pneumonia (VAP) for ICU patients is estimated to be 1% per day [3]. A postmortem study has shown that pneumonia was present in up to 67% of patients that received mechanical ventilation [4]. Gram-negative aerobic organisms are the predominant causes of VAP. Among these, *Pseudomonas aeruginosa* (PA) is associated with 21% of the total NP cases [2].

Although PA is one of the leading virulent pathogens in ventilated patients [5], the mechanism(s) for the increased susceptibility of these individuals to infection with this organism remains unclear. The inhalation of supraphysiological oxygen concentrations for prolonged or even transient periods of time results in acute lung injury, which is characterized by the injury and death of pulmonary cells [6–11]. The role of hyperoxia as a potential cause of lung cell injury has been extensively characterized in alveolar epithelial [12–17] and endothelial cells [18, 19]. However, only a few studies have examined its impact on alveolar macrophages [20–23]. Alveolar macrophages provide the first line of defense against inhaled bacterial pathogens [24] and are exposed to 13% oxygen under normal physiological conditions [22]. Under conditions of supplemental oxygen therapy, alveolar macrophages can be exposed to $\geq 95\%$ oxygen. Alveolar macrophages isolated from hyperoxia-exposed animals and macrophages exposed to hyperoxia *in vitro* exhibit impaired chemotaxis, adherence and phagocytosis of paraffin oil-droplets and *Klebsiella* [25–29].

Prolonged exposure to hyperoxia can induce cell damage, in part, through the accumulation of increased levels of ROS, mainly superoxide ions ($O_2^{\bullet-}$) [30]. The production of ROS is normally balanced by an elaborate antioxidant defense system [31, 32]. Under hyperoxic conditions, however, excessive ROS production perturbs this balance, devastating the antioxidant protection. We hypothesized that exposure to hyperoxia results in ROS damage that impairs alveolar macrophage phagocytosis of PA and that macrophage function can be preserved by the administration of antioxidant agents that either generally enhance capacity to buffer cellular oxidant status or scavenge superoxide ions ($O_2^{\bullet-}$).

Here, we report that exposure to 95% O_2 significantly impairs macrophage phagocytosis of both the mucoid and non-mucoid forms of PA. This impairment of PA phagocytosis is correlated with a severe lung injury and cell proliferation arrest. Interestingly, exposure to 65% O_2 , a relatively moderate concentration of oxygen, also induced a significant reduction in PA phagocytosis, comparable to what observed at $\geq 95\%$ O_2 , even though the effects on lung injury and cell proliferation are negligible. One particular feature of this impairment is the disorganization of actin cytoskeleton. Administration of the antioxidants, either superoxide dismutase or procysteine, preserved macrophage phagocytosis of PA as well as actin cytoskeleton organization.

MATERIALS AND METHODS

Cell Culture and Reagents

Mouse macrophage-like RAW 264.7 cells (ATCC, Manassas, VA) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% glutamine, 100 units/ml penicillin/streptomycin (Life technologies Inc., Grand Island, NY) and maintained at 37°C

in 5% CO₂/95% room air. Hyperoxic exposure was performed in sealed humidified chambers flushed with 95% O₂/5% CO₂ or 65% O₂/5% CO₂/30%N₂ as previously described [12]. A subgroup of macrophages were treated with superoxide dismutase (SOD1) or procysteine (Cayman, Ann Arbor, MI) either pre- or post- hyperoxic exposure. The number of viable cells and cell proliferation were assessed by 3-(4,5,-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide assay (MTT) and trypan blue exclusion analyses. All the chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

Phagocytosis Assay

PAO1, a motile, piliated and nonmucoid strain of PA, was grown to log phase in Lenox medium (Becton-Dickson Inc., Sparks, MD). CF1, a mucoid clinical isolate of PA from patients with cystic fibrosis, was maintained in a synthetic medium [33]. Procedures to determine phagocytosis were performed as described with minor modifications [29]. After exposure, macrophages were incubated with PA or FITC-labeled latex minibeads (Polysciences, Warrington, PA) at $\approx$ 100:1 at 37°C for 60 min. Macrophages were then washed with chilled (4°C) Phosphate Buffered Saline (PBS). Phagocytosis of PA was visually assessed using an immunofluorescent microscope or by colony forming unit assay. To visualize the uptake of PA, macrophages were fixed with 4% paraformaldehyde for 10 minutes, washed with PBS and stained with either 5 μ g/ml of 4-6-diamidine-2-phenylindole dihydrochloride (DAPI) or Texas Red X-phalloidin (Molecular Probes, Eugene, OR) in 1% bovine serum albumin. The slides were analyzed using an epifluorescence microscope (Nikon, Melville, NY) and a BIO-RAD MRC 600 confocal scanning microscope. The internalization of PA was confirmed by z-series analysis on the confocal microscope. Uptake of PA was determined by counting at least 50 macrophages/slide in triplicate from a minimum of two independent experiments for each group. For the colony forming unit assay, macrophages were lysed in water and viable bacteria enumerated by serial dilution and culture on nutrient agar plates (Becton-Dickinson, Inc).

Exposure to Hyperoxia *in vivo* and Isolation of Alveolar Macrophages

All animal experiments were approved by the Institutional Animal Care and Use Committee of the Feinstein Institute for Medical Research. Male C57BL/6 mice (6–8 weeks), purchased from Jackson Laboratories (Bar Harbor, Maine), were exposed to >99 or 65% for 72 h as described O₂ previously and then sacrificed [7]. The lungs were lavaged with 2 separate 1-ml aliquots of sterile saline. The resulting bronchoalveolar lavage fluid (BALF) was pooled, centrifuged and the cellular pellet resuspended and plated in RPMI 1640 medium. After 3 hours, non-adherent cells were removed.

Statistical Analysis

All experiments were performed independently at least twice. The data are presented as mean $\pm$ standard error, and analyzed for statistical significance using the one-way analysis of variance (ANOVA). A *P* value of ≥ 0.05 was considered significant.

RESULTS

Hyperoxia reduces the ability of macrophages to phagocytose PA and latex beads

To test whether hyperoxia affects the ability of macrophages to phagocytose PA, RAW 264.7 cells were exposed to 95% O₂, and then incubated with PAO1, a non-mucoid strain of PA expressing green fluorescent protein. To visualize the internalization of PAO1, RAW 264.7 cells were stained with phalloidin, a reagent that labels the actin cytoskeleton, and analyzed by confocal microscopy. Exposure to 95% O₂ for 24 h reduced the uptake of

PAO1 significantly to $67 \pm 5.6\%$ of room air (RA) control (Fig. 1A, from approximately 9 bacteria/cell to 6 bacteria/cell). The uptake of PAO1 was further reduced to $33 \pm 8.6\%$ of the RA control after 48h (data not shown). The effect of hyperoxia on RAW cells to uptake of a FITC-labeled clinical mucoid isolate of PA (CF1) was also examined. The phagocytosis of CF1 was reduced to 59% of RA control in RAW 264.7 cells exposed to 95% O₂ for 24 h (Fig. 1B). To determine whether the phagocytosed PA *were viable*, the number of viable PA was determined by colony forming unit (CFU) analysis in macrophage cell lysates. Compared to the room air controls, the relative colony forming unit (RCFU) for PAO1 was moderately lower in RAW cells following exposure to 95% O₂ for 24 h (Fig. 2A). The reduction in RCFU was significantly greater after a 48 h hyperoxic exposure (Fig. 2A). Hyperoxia-reduced RCFU was also observed in RAW 264.7 cells exposed to mucoid CF1. Fig. 2B shows that similar to the results of phagocytic analysis (Fig. 1B), fewer live mucoid CF1 were detected in hyperoxia-exposed macrophages compared to those remaining at room air (RA). A comparison of the results presented in Fig. 2A and Fig. 2B indicates a significant difference in the PAO1 RCFU versus that of the mucoid CF1 strain ($90 \pm 5\%$ vs $69 \pm 3\%$, respectively; $p < 0.05$) following 24 h of hyperoxia. This difference in CFU became insignificant after a 48 h exposure to 95% O₂.

To assess whether the reduced phagocytosis (Fig. 1A–B) and RCFU of PA (Fig. 2A–B) in hyperoxic RAW 264.7 cells results from increased killing of bacteria due to higher levels of ROS generated in hyperoxic macrophages, the phagocytic activity of RAW 264.7 cells was assessed using FITC-labeled inert latex minibeads. Fig. 3 shows that RAW 264.7 cells exposed to 95% O₂ for 24 h also exhibited significantly impaired phagocytosis of latex minibeads compared with macrophages that remained at RA ($36.1 \pm 0.66\%$ vs 100, $n=6$, $p < 0.05$)

Reduced phagocytosis of *P. aeruginosa* is associated with cytoskeleton disorganization in hyperoxic macrophages

Phagocytosis is driven by a finely controlled rearrangement of the actin cytoskeleton [34]. Hyperoxia affects actin polymerization and distribution in macrophages with impaired phagocytosis of *Klebsiella pneumonia* (*K. pneumonia*) [29]. We examined the possibility that hyperoxia may reduce macrophage phagocytosis of PA through effects on actin. Phalloidin staining of RAW 264.7 cells revealed that exposure to hyperoxia resulted in dramatic cytoskeleton disorganization with increased stress fiber number and thickness, depleted cortical actin and large masses of disordered, polymerized cytoplasmic actin. (Fig. 4A). Normoxic macrophages typically exhibited the ability to form phagocytic cups around PA (Fig. 4A, inset). On the other hand, formation of phagocytic cup around PA was sparse in hyperoxic macrophages, although more actin polymerization was observed in these cells (Fig. 4B, inset). Actin polymerization/remodeling is the driving force for particle internalization during phagocytosis. To test whether actin polymerization may directly attenuate PA phagocytosis, RAW 264.7 cells were exposed to Jasplakinolide (JP), a microfilament polymerizing agent [35], stained with phalloidin and then examined for uptake of PAO1. RAW 264.7 cells exposed to 0.5 μ M and 1 μ M JP for 1 h exhibited significantly decreased phagocytosis compared to RAW 264.7 cells exposed to vehicle control (0.1% dimethyl sulfoxide) ($54 \pm 3.8\%$ for 0.5 μ M JP and $28 \pm 2.9\%$ for 1 μ M JP, Fig. 4B). At these concentrations, JP was not cytotoxic to RAW 264.7 cells (data not shown). Actin polymerization, redistribution and general disorder may therefore contribute to the impairment of PA phagocytosis.

Alveolar macrophages isolated from mice exposed to hyperoxia exhibited reduced phagocytosis of PA *ex vivo*

Alveolar macrophages (AM) play an essential role in pulmonary defense against bacterial infection [24]. To establish whether our *in vitro* observations in RAW 264.7 cells are reflective of the events in hyperoxic lungs, AM were assessed for their ability to phagocytose PAO1 *ex vivo*. AM isolated from C57BL/6 mice exposed to $\geq 99\%$ O₂ for 3 days have a markedly reduced ability for PAO1 uptake compared to the AM from mice that remained at room air ($50.89 \pm 2.03\%$, $n=6$, $p=0.0001$, Fig. 5A) and RCFU counts ($53.2 \pm 3.6\%$ vs $100 \pm 5.4\%$, $n=6$, $p<0.05$, Fig. 5B). These data indicate that AM exposed to $\geq 99\%$ O₂ *in vivo* have similarly impaired phagocytosis of PA as RAW 264.7 cells exposed to hyperoxia *in vitro*. Examination of AM viability by trypan blue exclusion analysis indicated that $> 95\%$ of alveolar macrophages isolated from mice in the hyperoxic group was viable (data not shown). These findings are comparable to those obtained from RAW 264.7 cell exposed to hyperoxia *in vitro*.

Macrophages exposed to moderate concentration of oxygen exhibited significant reduced phagocytosis of PA

Extremely high concentrations of oxygen ($\geq 95\%$ O₂) are administered to patients with severe respiratory distress. However, more often patients receive the lowest concentration of oxygen needed [36], in an effort to limit pulmonary oxygen toxicity. To test whether a reduction in supplemental oxygen concentration also limits ROS damage to pulmonary host defenses, we examined whether exposure to moderate hyperoxia (65%) O₂ can attenuate $\geq 95\%$ O₂-induced impairment of phagocytosis of PA. In contrast to exposure $\geq 95\%$ O₂, exposure to 65% O₂ does not cause significant lung damage in CB57/6L mice (Fig. 6D) or marked cell proliferation arrest in RAW 264.7 cells (Fig. 6B). Intriguingly, exposure to 65% O₂ significantly reduced PAO1 uptake by AM from hyperoxic CB57/6L mice (Fig. 6C) and hyperoxic RAW 264.7 cells (Fig. 6A). Similar results were obtained when the number of viable phagocytosed PA were determined by colony forming unit analysis in both hyperoxic alveolar macrophages and RAW 264.7 cells (data not shown). Surprisingly, the reduction in phagocytosis of PA-induced by exposure to 65% O₂, was comparable to that observed at $\geq 95\%$ O₂, if not greater (Fig. 6A). In addition, similar alteration of actin cytoskeleton occurred in macrophages exposed to 65% O₂ as those to 95% O₂ (Fig. 6A, inset).

Macrophages exposed to hyperoxia retain proinflammatory responses upon LPS stimulation

Macrophages are responsible for the production of proinflammatory mediators such as TNF- α during the initiation of the inflammatory cascade [37, 38]. To determine if the impaired phagocytosis of PA is linked to changes in secreting proinflammatory cytokines, we investigated whether exposure to hyperoxia affects TNF- α production by RAW 264.7 cells upon exposure to LPS. The level of TNF- α was not detectable in the media of room air control cells or cells treated with hyperoxia alone (Fig. 7). LPS induced substantial accumulation of TNF- α in the media of RAW 264.7 cells cultured in the room air (Fig. 7). Prior exposure to hyperoxia did not significantly alter the ability of RAW 264.7 cells to secrete TNF- α in response to LPS (Fig. 7).

Antioxidants attenuate impairment of P. aeruginosa phagocytosis and disorganization of actin cytoskeleton induced by hyperoxia

Superoxide dismutases (SOD) are antioxidant enzymes that scavenge superoxide radicals. To examine whether the reduced antibacterial function of macrophages in hyperoxia can be restored through reduction of the levels of superoxide, RAW 264.7 cells were pre-treated with SOD-1. Fig. 8 shows that pretreatment with SOD-1 preserves the uptake of PA by

RAW 264.7 cells. This effect is not due to an increase in nonspecific binding of PA to RAW cells in the presence of SOD-1, because there was no increase in PA uptake by RAW 264.7 cells that were exposed to room air and treated with SOD-1 (Fig. 8, Panel labeled with SOD/RA).

Procysteine, L-2-oxothiazolidine-4-carboxylate, is a thiol-related cysteine precursor [39, 40] that can increase intracellular levels of the antioxidant glutathione [41, 42]. To test whether it can preserve macrophage phagocytosis of PA, RAW 264.7 cells were treated with 1 mM procysteine, either before or 24 h post hyperoxic exposure. Fig. 9 shows that procysteine pre-treatment markedly enhanced macrophage ability to phagocytose PAO1 (Fig. 9A) as well as mucoid CF1 in macrophages exposed to hyperoxia for 24 h (Fig. 9B). Significant improvements in the ability to phagocytose both PAO1 and CF1 were observed in macrophages exposed to hyperoxia for 48 h ($113 \pm 9.5\%$ vs $38 \pm 7.3\%$ ($p < 0.05$) and $83 \pm 7.3\%$ vs $38 \pm 4.7\%$ ($p < 0.05$) for PAO1 and CF1, respectively). Intriguingly, when macrophages were treated with procysteine 24 h post hyperoxic exposure, the ability to phagocytose both PAO1 and CF1 was also significantly increased (Fig. 9). The degree of improvement in the phagocytosis of PA resulting from pre or post-treatment with procysteine was not significant (Fig. 9). To elucidate the mechanisms of procysteine-rescued phagocytosis of PA, we assessed the organization of the actin cytoskeleton in hyperoxic macrophages in the presence or absence of the treatment. Accompanied with the rescuing phagocytosis of PA, treatment of hyperoxic macrophages with procysteine preserved the normal organization of the actin cytoskeleton (Fig. 10C).

DISCUSSION

Our results indicate that exposure to hyperoxia in either an *in vitro* cell culture or an *in vivo* animal model system significantly impairs the ability of macrophages to phagocytose mucoid and non-mucoid strains of PA, a common cause of nosocomial pneumonia. Interestingly, exposure of 65% O₂, a moderate concentration of oxygen which does not lead to significant lung injury or marked inhibition of cell proliferation, also drastically reduces phagocytosis of PA. Exposure to hyperoxia profoundly alters actin cytoskeleton organization. The ability of macrophages to phagocytose PA in hyperoxia is preserved by antioxidant treatment, which is effective, even when administered 24h after the exposure to hyperoxia. Antioxidant treatment is associated with conservation of normal actin-cytoskeleton organization. Overall, these results suggest that hyperoxia-attenuated phagocytosis of PA is mediated by ROS effects on the actin cytoskeleton.

Reduced phagocytosis of *P. aeruginosa* is not due to decreased cell viability in macrophages exposed to hyperoxia

Exposure to hyperoxia, regardless whether the concentration of oxygen is $\geq 95\%$ or 65% O₂, induced impairment of phagocytosis of PA by macrophages. However, this impairment is not due to the loss of cell viability or cell death. First, few Trypan blue positive signals, an indicator for loss of membrane integrity/cell death, were detected in either RAW 264.7 cells exposed to hyperoxia or alveolar macrophages (AM) isolated from hyperoxic lungs ($<5\%$) at a similar level as those obtained from room air controls. This finding is consistent with previous studies which indicate that exposure to hyperoxia does not decrease viability of macrophages [21, 22, 25, 43, 44]. In addition, RAW 264.7 cells, exposure to 65% O₂, could still proliferate (Fig. 6B) even though their phagocytic ability was significantly suppressed (Fig. 6). Furthermore, the ability of hyperoxic RAW 264.7 cells to proliferate was restored when they were re-cultured in room air (data not shown), although exposure of RAW cells to $\geq 95\%$ O₂ for up to 48 hours did induce cell proliferation arrest (Fig. 6B). The blunted sensitivity to hyperoxia-induced loss of cell viability is also reflected in the proinflammatory responses [43, 44]. Results in Fig. 7 indicate that hyperoxia does not abolish the ability of

macrophages cells to release TNF- α in response to LPS. Other reports also indicate that hyperoxic macrophages retain ability to produce nitric oxide, in response to LPS and interferon- γ [29, 45]. Cell death or loss of cell viability is not therefore a likely explanation for hyperoxia-induced impairment of PA phagocytosis.

Actin cytoskeleton alterations are critical to *P. aeruginosa* phagocytosis under hyperoxic conditions

The internalization of bacteria by phagocytes is characterized by an actin-dependent extension of the plasma membrane around the particle [34]. This is followed by a secondary activities, such as the release of ROS, proteases and inflammatory cytokines from the phagocyte [46]. Oxidant-induced cell damage is thought to involve the cytoskeleton, with the actin system appearing to be most sensitive to oxidative attack [47]. Hyperoxia has been shown to reduce macrophage phagocytosis of *K. pneumonia* through effects on the actin [29]. Staining of actin cytoskeleton by phalloidin indicates that hyperoxia causes the broad spectrum of dramatic changes that we collectively describe as “actin disorganization” (Figs. 4 & 5).

Normoxic macrophages exhibited a normal cortical distribution of polymerized actin whereas hyperoxic macrophages exhibited increased levels of polymerized actin with reduced levels of cortical actin. In addition, results shown in Fig. 4 suggest that the distribution/rearrangement of acting cytoskeleton in hyperoxic macrophages does not easily accommodate phagocytic cup formation and particle engulfment. The lack of cortical actin in hyperoxic cells appears to be particularly prohibitive to the internalization of PA. The cellular location of polymerized actin appears to be critical for the impairment of phagocytosis. The large quantities of disordered polymeric actin in the central region of hyperoxic cells may also be obstructive to successful engulfment of PA. Overall, our results suggest that hyperoxia causes impairment of phagocytosis of PA by interfering with the process of directional actin polymerization/remodeling that drives bacterial internalization. Results with Jasplakinolide (Fig. 4B), an agent that increases actin polymerization, indicate that inappropriate actin polymerization may indeed be attributed to the impairment of macrophage phagocytosis of PA. Jasplakinolide has also been shown to have similar effects on uptaking *Klebsiella* [29].

The mechanism(s) by which hyperoxia induces these effects on actin remains to be fully elucidated. The actin cytoskeleton response to oxidative stress has been studied in numerous different cell types [48–50]. ROS, as well as a variety of other intracellular signals, can mediate the actin cytoskeleton reorganization in preparation for processes such as adhesion under physiological conditions [51, 52]. However, exposure to severe oxidative stress, especially those induce glutathione depletion can cause the formation of inter or intra-molecular disulfide that lead to cross-linking between actin filaments, resulting in excessive polymerization of actins and formation of more stress fibers [52]. In addition, elevated levels of ROS during hyperoxia may lead to the excessive oxidation and glutathionylation of actin's regulatory proteins [47], causing regulatory dysfunction in normal actin polymerization/remodeling. Therefore, reduction of oxidative stress may be essential for preservation of both normal actin cytoskeleton organization and phagocytic function during hyperoxia.

Antioxidant treatment preserves phagocytosis of *P. aeruginosa* during hyperoxia

Changes in the structure of the actin cytoskeleton are generally presumed to involve the oxidation of critical protein sulfhydryl residues [53, 54]. Reactive sulfhydryls are located on proteins in all cellular compartments as well as low molecular weight thiols [44]. Thiol moieties are part of the elaborate system of antioxidant defenses that respond to excessive

ROS. Procysteine, is a thiol-related compound that has been shown to be associated with increased levels of glutathione (GSH), the most important antioxidant in mammalian cells [42, 55]. Since exposure to hyperoxia reduces the intracellular level of GSH [56, 57], supplementation with procysteine was expected to enhance GSH levels and reduce ROS induced macrophage damage. Results, shown in Figs. 9&10, demonstrate that procysteine can prevent the hyperoxia-induced alteration of actin cytoskeleton and conserve the phagocytic functions in macrophages. Hyperoxia-induced changes in the actin cytoskeleton which impact phagocytosis of PA may therefore depend, in part, on the oxidation of reactive sulfhydryls. Cysteine 374 (Cys³⁷⁴) at the actin C-terminal segment is the main GSH reactive sulphhydryl [47, 52, 58]. Recent studies in fibroblasts have shown that *in vivo* actin oxidation takes place between Cys³⁷⁴ and glutathione; this modification was shown to be essential for cytoskeleton organization [52]. Inhibition of this actin glutathionylation, through either GSH depletion or expression of Cys³⁷⁴ redox insensitive mutant profoundly affected fibroblast cytoskeleton assembly. In this respect, actin glutathionylation can be viewed as a protective mechanism against further S-oxidation, permanent protein modification and ultimately irreversible cytoskeleton rearrangements. Interestingly, the oxidation of actin has been demonstrated in hyperoxic macrophages with reduced phagocytosis of *K. pneumonia* [29]. The superoxide radical is likely to significantly contribute to the reduction in phagocytosis of PA, as our results (Fig. 8) show that SOD-1 has a protective effect on antibacterial function. Even though prolonged exposure to hyperoxia does not suppress the level of SOD expression in macrophages [59], excessive levels of superoxide generated during hyperoxia may require high levels of SOD. In addition to rescuing macrophage function resulting from oxidative stress during hyperoxia, administration of antioxidants may also be useful in reducing ROS generated by bacteria themselves that are harmful to the host defense system [60, 61].

In summary, our study indicates that both moderate and high concentrations of hyperoxia impair macrophage phagocytosis of PA. The administration of moderate, rather than higher, concentrations of supplemental hyperoxia may limit pulmonary oxygen toxicity but the risk for reduced macrophage clearance of PA remains elevated. The effects of hyperoxia on macrophage phagocytosis of PA, however, can be attenuated by antioxidant supplementation. Our current effort is aimed at evaluating the efficacy of antioxidant treatments in the reduction of bacterial burden and in the ultimate improvement of the survival of infected hyperoxic animals. Successful completion of this project will no doubt highlight the possible benefits of antioxidant therapy in maintaining the phagocyte function(s) required for the prevention of nosocomial infection. This may lead to an improvement in the clinical outcomes of ventilated patients, especially those with acute respiratory distresses suffering high morbidity and mortality.

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ABBREVIATIONS

O₂ molecular Oxygen

$O_2^{\cdot-}$	superoxide ions
PA	<i>Pseudomonas. Aeruginosa</i>
<i>K. pneumonia</i>	<i>Klebsiella pneumonia</i>
ROS	reactive oxygen species
DAPI	4-6-diamidine-2-phenylindole dihydrochloride
$O_2^{\cdot-}$	superoxide ions
TNF- α	tumor necrosis factor- α
SOD-1	superoxide dismutase-1
RA	room air
CFU	colony forming units
RCFU	relative colony forming units

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Fig. 1. Reduced Uptake of *P. aeruginosa* by Macrophages in Hyperoxia

RAW 264.7 cells were exposed to 95% O₂ or to room air (RA) for 24 h, and then incubated for 1 h with either (A) PAO1, a nonmucoid strain of *P. aeruginosa* expressing green fluorescent protein, or (B) CF1, a FITC-labeled mucoid isolate of *P. aeruginosa*. RAW 264.7 cells were stained with phalloidin to visualize the macrophages in the field. At least 50 macrophages per slide, 6 slides from 3 independent experiments were counted and the numbers of bacteria/cell were graphed in the lower panels. *, p<0.05 compared to RA controls. Bar, 10 μm.

Fig. 2. Hyperoxia Reduced Viable *P. aeruginosa* in Macrophages

RAW 264.7 cells were exposed to 95% O₂ or to room air (RA) for indicated time periods, and then incubated for 1 h with either (A) PAO1, a non-mucoid strain of *P. aeruginosa* expressing green fluorescent protein, or (B) CF1, a FITC-labeled mucoid clinical isolate of *P. aeruginosa*. The number of viable phagocytosed *P. aeruginosa* was determined by colony forming unit (CFU) analysis in macrophage cell lysates. The numbers of CFU were normalized to the macrophage number and expressed as the percentage of the number obtained prior to the exposures (which was designated as 100%). *, $p < 0.05$, compared to the room air control; #, $p < 0.05$ compared to 24 h exposure to 95% O₂ and PAO1 RCFU.

Fig. 3. Reduced Uptake of Latex Beads in Hyperoxia

RAW 264.7 cells were exposed to room air (RA) or 95% O₂ for 24 h, and then incubated with FITC-labeled latex minibeads for 1 h. RAW 264.7 cells were stained with phalloidin to visualize the macrophages in the field. At least 50 macrophages per slide, 6 slides from 3 independent experiments were counted and the number of beads/macrophage were expressed in the bar graph. *, $p < 0.05$ compared to RA controls. Bar, 10 μm .

Fig. 4. Hyperoxia-Reduced Phagocytosis of *P. aeruginosa* is Associated with Disorganization of Actin Cytoskeleton and Oxidation of Actin

RAW 264.7 cells were exposed to room air (RA) or 95% O₂ for 24 h, then incubated with PAO1 for 1 h, and stained with phalloidin (A). Hyperoxic cells (O₂) exhibited altered morphology: increased stress fiber formation and thickness, disordered polymeric cytoplasmic actin (A), an absence of phagocytic cups surrounding PAO1 (A, insets) and a loss of cortical actin at margins of macrophages. Bar, 10 μm. **B.** Equal amounts of proteins were immunoprecipitated with anti-actin antibodies from RAW cells cultured in room air or 95% O₂, derivatized with DNPH. The resulting proteins were resolved on SDS-PAGE and probed with anti-DNP antibodies and HRP-conjugated secondary antibodies (left Panel). Control samples without derivatization (Der) were compared with those with derivatization as negative controls for nonspecific binding. Arrows indicate actin. Blots were then stripped and probed with anti-actin antibodies (right Panel, a representative of three independent experiments).

Fig. 5. Disorganization of Actin Cytoskeleton Reduced Phagocytosis of *P. aeruginosa*

RAW 264.7 cells were exposed to different concentrations of Jasplakinolide (JP), 0.5 or 1 μ M, for 1 h, then incubated with PAO1 for 1 h and stained with phalloidin (B). At least 50 macrophages per slide, 6 slides from 3 independent experiments, were counted and the number of PAO1/macrophage was expressed in the bar graph (A). *, $p < 0.05$, compared to medium control (Con); **, $p < 0.05$, compared to samples exposed to 0.5 μ M JP.

Macrophages treated with JP exhibited altered morphology: increased stress fiber formation and thickness, disordered polymeric cytoplasmic actin (B), and a loss of cortical actin at margins of macrophages. Bar, 10 μ m.

Fig. 6. Exposure to *in vivo* Hyperoxia Reduced Phagocytosis of *P. aeruginosa* in Alveolar Macrophages

C57BL/6 mice were exposed to >99% O₂ for 3 d (O₂) or remained in room air (RA). Alveolar macrophages were isolated from BALF, incubated for 1 h with *P. aeruginosa*, and assessed for the number of phagocytosed *P. aeruginosa*/macrophage as described in Fig. 1(A) and for live *P. aeruginosa* by CFU analysis as described in materials and methods (B). Insets in panel A illustrates the disorganized actin cytoskeleton in hyperoxic alveolar macrophages. *, $p < 0.05$, compared to the room air control ($n = 6$ in each group). Bar, 10 μm .

Fig. 7. Exposure to Moderate Hyperoxia Reduced Uptake of *P. aeruginosa* by Alveolar Macrophages and RAW 264.7 Cells Without Causing Lung Damage or Cell Proliferation Arrest C57BL/6 mice & RAW 264.7 cells were exposed to either $\geq 95\%$ or 65% O₂ or remained at room air (RA). Both (A–B) RAW 264.7 cells & (C–D) alveolar macrophages isolated from BALF were assessed for phagocytosis of *P. aeruginosa* as described in Figs.1 & 4. The number of bacteria/macrophage was presented in the bar graphs. The number of viable RAW 264.7 cells was assessed by tetrazolium compound (MTT) analysis (B) and mouse lung injury was measured by wet/dry lung ratio (D). *, $p < 0.05$, compared to the room air control; #, $p < 0.05$ compared to those exposure to 95% O₂. Bar, $10\ \mu\text{m}$.

Fig. 8. Macrophages Exposed to Prolonged Hyperoxia Respond to Endotoxin Stimulation with Production of TNF- α

RAW 264.7 cells were exposed to 95% O₂ or room air (RA) for 48h, and then incubated with 500ng/ml of Endotoxin for 18 hours prior to harvesting of supernatant and measurement of TNF- α with a TNF- α kit (R&D Systems, Minneapolis, MN). TNF- α was not detectable in the media of control cells or cells exposed to O₂ alone. Values are means $\pm$ SE of >2 separate experiments, n=4.

Fig. 9. SOD Preserves *P. aeruginosa* Uptake by Macrophages in Hyperoxia

RAW 264.7 cells were exposed to 95% O₂ for 24 h in the presence or absence of 250 U/ml superoxide dismutase-1. After hyperoxic exposure, RAW 264.7 cells were incubated with PAO1 for 1 h and stained with phalloidin to visualize the organization of the actin cytoskeleton. PAO1 was pseudocolored as white. At least 50 macrophages per slide were counted and expressed as the number of bacteria/macrophage. Macrophages treated with superoxide dismutase-1, and remained at room air were used as controls. *, $p < 0.05$, compared to the hyperoxic sample without addition of SOD1. Bar, 10 μ m.

Fig. 10. Procysteine Preserves Phagocytosis of *P. aeruginosa* in Hyperoxia

RAW 264.7 cells were exposed to 95% O₂ in the presence (Pre: added 24 h prior to; Post, added 24 h after the start of hyperoxic exposure) of 1 mM procysteine. After hyperoxic exposure, RAW 264.7 cells were incubated with either non-mucoid PAO1 (A) or mucoid CF1 (B) for 1 h, and then assayed for Colony Forming Unit. The numbers of colonies were normalized to the macrophage number and expressed as the percentage of the number (which is designated as 100%) prior to the exposures (either hyperoxia or room air). *, p < 0.05, compared to the hyperoxic control without procysteine treatments.

Fig. 11. Procysteine Inhibits Hyperoxia-Suppressed Uptake of PAO1 and Disorganization of the Actin Cytoskeleton in Macrophages

RAW 264.7 cells were exposed to room air (A), or exposed to 95% O₂ for 48 h without (B) or with addition of 1 mM procysteine after 24 h hyperoxia (C). Macrophages were then incubated with PAO1 (green) for 1 h, stained with phalloidin (red) to visualize the organization of actin cytoskeleton in the field. Bar, 10 μm.