

## Ascorbic Acid Attenuates Hyperoxia-Compromised Host Defense against Pulmonary Bacterial Infection

Vivek S. Patel<sup>1</sup>, Vaishali Sampat<sup>1</sup>, Michael Graham Espey<sup>2</sup>, Ravikumar Sitapara<sup>1</sup>, Haichao Wang<sup>3</sup>, Xiaojing Yang<sup>1</sup>, Charles R. Ashby Jr.<sup>1</sup>, Douglas D. Thomas<sup>4</sup>, and Lin L. Mantell<sup>1,3</sup>

<sup>1</sup>Department of Pharmaceutical Sciences, St. John's University College of Pharmacy and Health Sciences, Queens, New York; <sup>2</sup>National Cancer Institute, Bethesda, Maryland; <sup>3</sup>The Feinstein Institute for Medical Research, North Shore—Long Island Jewish Health System, Manhasset, New York; and <sup>4</sup>Department of Medicinal Chemistry and Pharmacognosy, University of Illinois at Chicago, Chicago, Illinois

ORCID IDs: 0000-0001-8583-1548 (V.S.P.); 0000-0003-2366-6254 (R.S.).

### Abstract

Supraphysiological concentrations of oxygen (hyperoxia) can compromise host defense and increase susceptibility to bacterial infections, causing ventilator-associated pneumonia. The phagocytic activity of macrophages is impaired by hyperoxia-induced increases in the levels of reactive oxygen species (ROS) and extracellular high-mobility group box protein B1 (HMGB1). Ascorbic acid (AA), an essential nutrient and antioxidant, has been shown to be beneficial in various animal models of ROS-mediated diseases. The aim of this study was to determine whether AA could attenuate hyperoxia-compromised host defense and improve macrophage functions against bacterial infections. C57BL/6 male mice were exposed to hyperoxia ( $\geq 98\%$  O<sub>2</sub>, 48 h), followed by intratracheal inoculation with *Pseudomonas aeruginosa*, and simultaneous intraperitoneal administration of AA. AA (50 mg/kg) significantly improved bacterial clearance in the lungs and airways, and significantly reduced HMGB1 accumulation in the airways. The incubation of RAW 264.7 cells (a macrophage-like cell line) with AA (0–1,000  $\mu$ M) before hyperoxic exposure (95% O<sub>2</sub>) stabilized the phagocytic activity of macrophages in a concentration-dependent manner. The AA-enhanced macrophage function was associated with significantly decreased production of

intracellular ROS and accumulation of extracellular HMGB1. These data suggest that AA supplementation can prevent or attenuate the development of ventilator-associated pneumonia in patients receiving oxygen support.

**Keywords:** hyperoxia; ascorbic acid; pneumonia; high-mobility group box 1

### Clinical Relevance

Patients receiving oxygen therapy are highly susceptible to pulmonary infections, which may lead to ventilator-associated pneumonia (VAP). Data in this study suggest that ascorbic acid (AA) levels can be significantly reduced in patients receiving oxygen therapy, and AA supplementation can prevent or attenuate the development of VAP in these patients. Thus, our findings demonstrate that boosting the host defense with a simple and inexpensive antioxidant, ascorbate, may help to reduce mortality and morbidity in patients receiving oxygen support.

Supraphysiological concentrations of oxygen (hyperoxia) are routinely used to treat patients with respiratory distress and those undergoing surgery (1–5). However, oxygen toxicity from prolonged exposure to

hyperoxia can contribute to the complications, such as ventilator-associated pneumonia (VAP) and acute lung injury, observed in these patients (6–12). Prolonged exposure to hyperoxia can compromise the

host defense against bacterial infections by impairing the efficacy of alveolar macrophages to migrate, phagocytose, kill, and clear bacteria (7, 13–18). Hyperoxia-induced deleterious effects are mainly

(Received in original form September 30, 2015; accepted in final form April 14, 2016)

This work was supported by National Heart, Lung, and Blood Institute grant HL093708 (L.L.M.) and a grant from St. John's University.

Author Contributions: Conception and design—V.S.P., V.S., M.G.E., H.W., L.L.M.; analysis and interpretation—V.S.P., V.S., M.G.E., R.S., X.Y., C.R.A., D.D.T., and L.L.M.; drafting the manuscript for important intellectual content—V.S.P., V.S., M.G.E., R.S., H.W., X.Y., C.R.A., D.D.T., and L.L.M.

Correspondence and requests for reprints should be addressed to Lin L. Mantell, M.D., Ph.D., Department of Pharmaceutical Sciences, Saint John's University College of Pharmacy and Health Sciences, 128 Saint Albert Hall, 8000 Utopia Parkway, Queens, NY 11439. E-mail: lmantell@nshs.edu

This article has an online supplement, which is accessible from this issue's table of contents at [www.atsjournals.org](http://www.atsjournals.org)

Am J Respir Cell Mol Biol Vol 55, Iss 4, pp 511–520, Oct 2016

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Originally Published in Press as DOI: 10.1165/rcmb.2015-0310OC on April 27, 2016

Internet address: [www.atsjournals.org](http://www.atsjournals.org)

attributable to an increase in the levels of reactive oxygen species (ROS), resulting in oxidative stress, which ultimately surmount the antioxidant defense mechanisms (19, 20). ROS can cause lipid peroxidation, protein, and DNA oxidation, and alter signal transduction pathways, leading to cell damage (21–25). Exposure of cultured macrophages to hyperoxia induces actin oxidation and subsequent cytoskeleton disorganization, resulting in a decrease in the ability to phagocytose bacteria (16, 18). Treatment of hyperoxia-exposed macrophages with antioxidants, such as superoxide dismutase and procysteine, can preserve actin cytoskeleton organization and increase the phagocytosis of bacteria (18, 26). Hyperoxia-exposed cells overexpressing antioxidant enzyme, manganese superoxide dismutase, exhibit reduced bacterial adherence, increased phagocytic activity (26, 27), and attenuated ROS-induced damage (28). These aforementioned findings suggest that antioxidants may be useful in attenuating ROS-induced cellular damage and impairment of macrophage functions.

Ascorbic acid (AA), an essential nutrient, is a well known reductant and scavenger of intracellular ROS (29–32). AA can donate two electrons that are lost sequentially, and is considered to be an efficacious antioxidant, as it reduces a reactive free radical with the formation of a less reactive compound (30, 33, 34).

Recent studies in our laboratory have demonstrated that high-mobility group box protein 1 (HMGB1), a late mediator of inflammation (35, 36), can significantly attenuate the phagocytic function of macrophages in airways (7, 37). In mice exposed to hyperoxia, elevated levels of airway HMGB1 are associated with an increase in both the lung bacterial burden and lung damage. Neutralizing or inhibiting HMGB1 by monoclonal antibodies or compounds, such as 3-(2,4-dimethoxybenzylidene)-anabaseine dihydrochloride (GTS)-21, respectively, in these mice, significantly decreases lung injury and hyperoxia-compromised bacterial clearance (7, 38).

In this study, we tested the hypothesis that AA supplementation can attenuate hyperoxia-compromised host defense by scavenging hyperoxia-induced excessive intracellular ROS and reducing HMGB1 levels in the airways. We show that AA significantly: (1) preserved macrophage

phagocytic function and host defense against bacterial infections, leading to improved survival; (2) decreased intracellular oxidative stress in cultured macrophages by replenishing the intracellular levels of AA; and (3) reduced extracellular HMGB1 levels in both *in vitro* and *in vivo* model systems. Some of the results presented in this study have been previously reported in the form of an abstract (39).

## Materials and Methods

Refer to the online supplement for detailed methods.

### Animal Studies

C57BL/6 mice (male, 8–12 wk old; The Jackson Laboratory, Bar Harbor, ME) were used in this study, in accordance with the Institutional Animal Care and Use Committees of St. John's University (Queens, NY). Mice were randomized to receive either sodium L-ascorbate (50 mg/kg dissolved in saline; Sigma-Aldrich, St. Louis, MO) or saline (200  $\mu$ l), administered by intraperitoneal injection every 12 hours, starting 24 hours after the onset of hyperoxic exposure. After 48 hours of hyperoxic exposure, mice were anesthetized with sodium pentobarbital (60–70 mg/kg intraperitoneal) and inoculated with  $1 \times 10^7$  CFUs of *Pseudomonas aeruginosa* PAO1 (PA) by making an approximately 1-cm incision on the neck to expose the trachea, and kept at room air (21% O<sub>2</sub>). Mice were either killed 18 hours later using intraperitoneal sodium pentobarbital (120 mg/kg) to obtain bronchoalveolar lavage fluids (BALFs) and lung tissues, or observed for survival after bacterial inoculation, as described previously (7).

### Measurement of Phagocytic Activity, HMGB1 Levels, and Immunofluorescence Analyses

RAW 264.7 cells were seeded in 24-well plates and allowed to adhere overnight at 37°C. Cells were exposed to hyperoxia (95% O<sub>2</sub>) for 24 hours (7) while in media supplemented with AA (0–1,000  $\mu$ M). The phagocytic activity was assessed by counting 200 consecutive individual macrophages per well in duplicates, as previously described (17, 18). The levels of HMGB1 in the culture media of RAW 264.7 cells and BALF samples obtained from mice were measured by Western blot analysis, as described previously (7).

HMGB1 and NF- $\kappa$ B localization was observed, as mentioned previously (17).

### Assay for Oxidative Stress

Oxidative stress was measured by assessing oxidation–reduction potential (ORP) using the RedoxSYS Diagnostic System (Luoxis Diagnostics, Inc., Englewood, CO). Cultured macrophages were exposed to hyperoxia and treated with AA, as indicated, and cell lysates were prepared. ORP of samples (30  $\mu$ l) was measured at room temperature, using the protocol provided by the manufacturer.

### Measurement of Intracellular Ascorbate

After the treatments of cultured RAW 264.7 macrophages in six-well plates, the cells were lysed as described previously (40), except that the adherent cell monolayer was rinsed with ice-cold PBS and the resulting lysate was centrifuged at  $13,000 \times g$  for 10 minutes at 3°C. Supernatants were analyzed by HPLC and intracellular concentrations of AA were calculated using the equation described previously (40, 41), with minor modifications. Samples (50  $\mu$ l) were injected and separation was performed on Agilent HC-C<sub>18</sub> (2) column (5  $\mu$ m,  $4.6 \times 1.50$  mm; Agilent Technologies, Santa Clara, CA). AA was detected by its ultraviolet absorption at a wavelength of 260 nm on a Waters 486 tunable absorbance detector (Waters, Milford, MA). At a flow rate of 1 ml/min, AA was eluted at roughly 3 minutes.

### Statistical Analyses

The data are presented as the mean ( $\pm$ SEM) of at least two independent experiments. The data were analyzed using Student's unpaired *t* test or ANOVA. The Kaplan-Meier survival curve was analyzed using the log-rank (Mantel-Cox) test. A *P* value of less than or equal to 0.05 was considered statistically significant. The *post hoc* analyses were conducted using Dunnett's or Sidak's multiple comparison test.

## Results

### AA Significantly Increases Hyperoxia-Compromised Bacterial Clearance

To determine whether AA can improve hyperoxia-compromised host defense against PA pneumonia, bacterial counts were assessed in the airways and lung tissues in a mouse model of VAP, as described

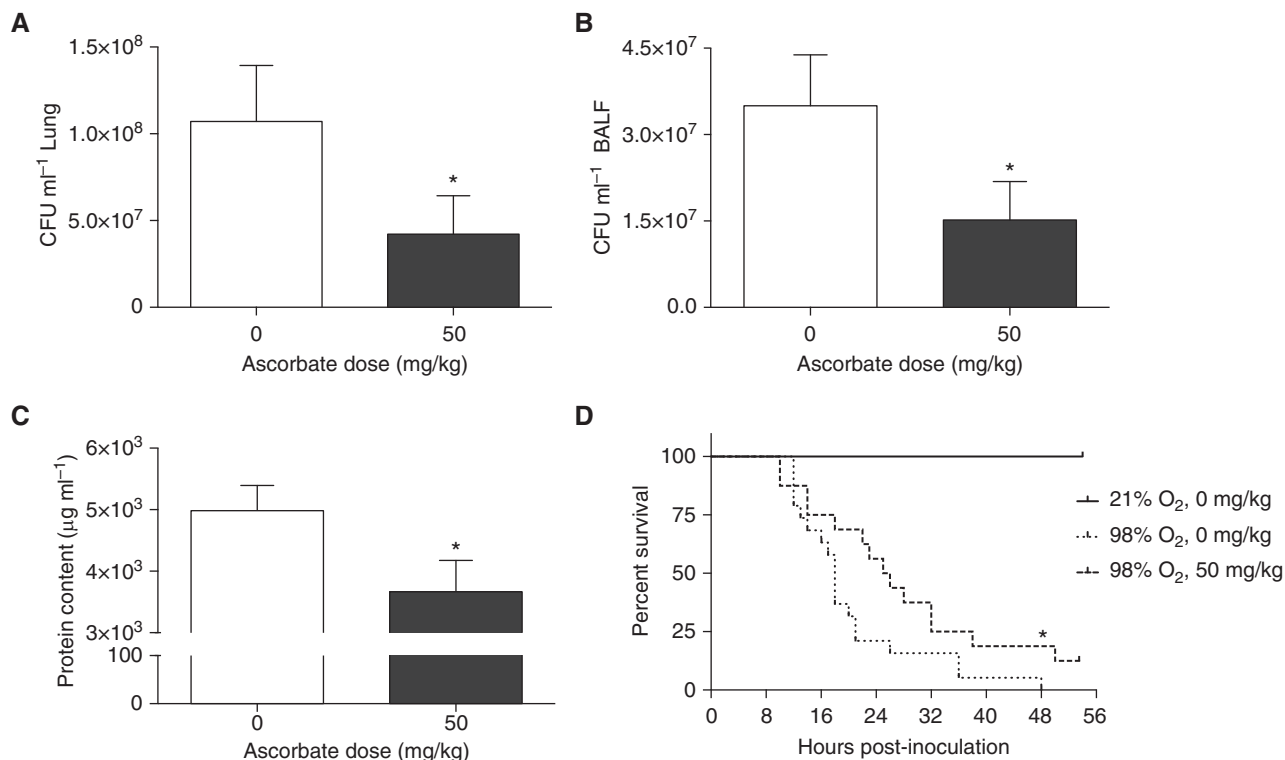
previously (7). These mice were treated with either 50 mg/kg of AA or saline (control), as described in the MATERIALS AND METHODS. Bacterial counts in the lungs of AA-treated mice were significantly less than that of saline-treated mice ( $0.4 \pm 0.2 \times 10^8$  versus  $1.1 \pm 0.3 \times 10^8$ /lung;  $t = 1.66$ , degrees of freedom [ $df$ ] = 42,  $P = 0.05$ ; Figure 1A). Similar results were obtained in the airways ( $1.5 \pm 0.6 \times 10^7$  versus  $3.5 \pm 0.9 \times 10^7$ /ml BALF;  $t = 1.78$ ,  $df = 41$ ,  $P < 0.05$ ; Figure 1B). In addition, the total protein content in BALF, an important marker of lung injury, was significantly lower in AA-treated mice compared with the controls ( $3,666 \pm 508.3$  versus  $4,983 \pm 408.9$   $\mu\text{g/ml}$  BALF of saline-treated mice;  $t = 2.02$ ,  $df = 42$ ,  $P < 0.05$ ; Figure 1C). Moreover, a significant decrease in animal mortality was observed in the AA-treated group (87.5 versus 100% in the saline-treated group; chi-square = 4.07,  $df = 1$ ,  $P < 0.05$ ; Figure 1D). These results indicate that AA improves bacterial clearance and acute lung injury, which

results in an increase in survival in this mouse model of VAP. Interestingly, treatment of mice without prior exposure to hyperoxia with the same dose of AA did not significantly alter bacterial counts in either lung tissues or airways compared with that of saline-treated mice (lungs:  $7.0 \pm 5.4 \times 10^7$  versus  $6.0 \pm 4.7 \times 10^6$ ;  $t = 1.19$ ,  $df = 14$ ,  $P = 0.25$ ; BALF:  $1.1 \pm 0.7 \times 10^7$  versus  $8.6 \pm 6.0 \times 10^5$ /ml;  $t = 1.34$ ,  $df = 13$ ,  $P = 0.20$ ; not shown in figures). Similar results were obtained for the total protein content in the airways ( $3,917 \pm 843.5$  versus  $3,568 \pm 597.5$   $\mu\text{g/ml}$  BALF of saline controls). These data suggest that the beneficial effects of AA in enhancing innate immunity against bacterial infections are limited to mice with hyperoxia-compromised host defense.

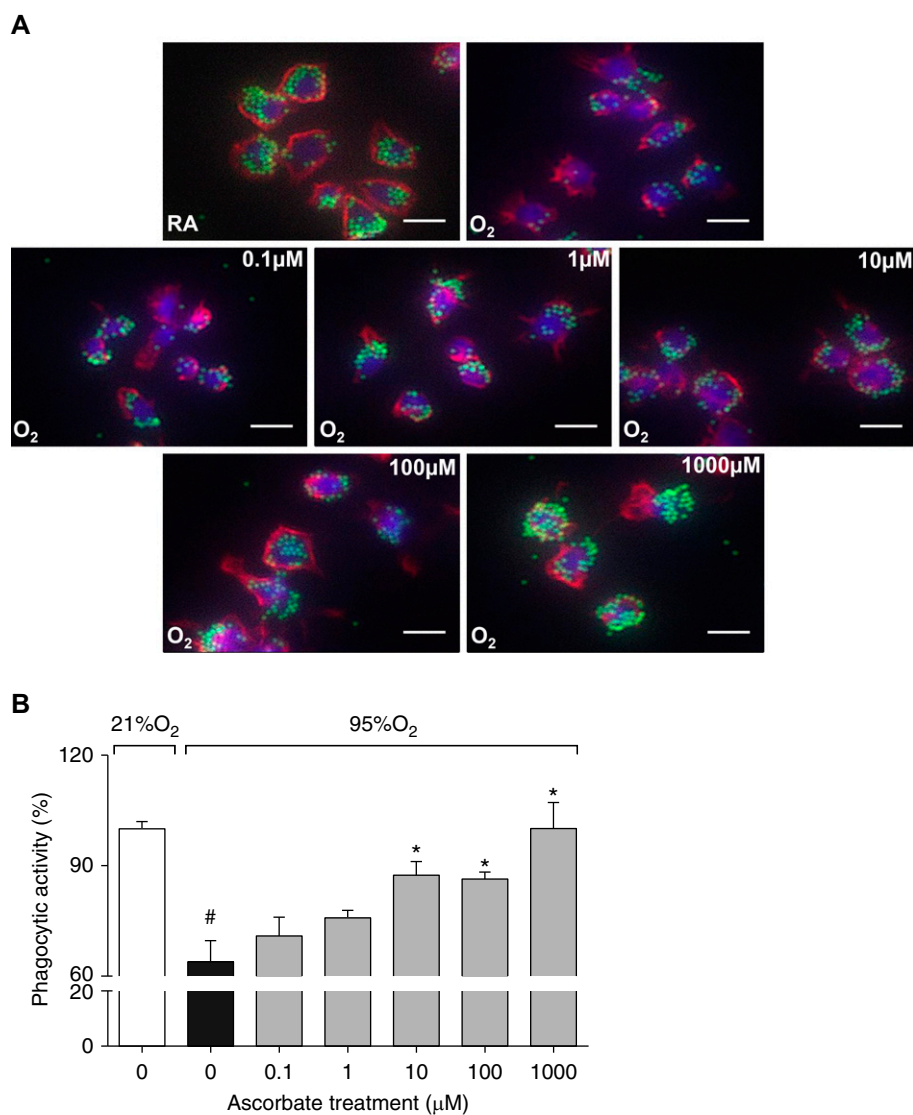
#### AA Significantly Attenuates the Impairment in Macrophage Phagocytosis Induced by Hyperoxia

Hyperoxia-compromised macrophage functions play a critical role in the reduced

host defense against bacterial infections (7). To determine the cellular mechanisms in AA-improved host defense, RAW 264.7 cells were exposed to hyperoxia in the presence of a series of AA concentrations (0–1,000  $\mu\text{M}$ ). Consistent with previous observations (7, 13, 16, 18, 26), macrophages exposed to hyperoxia in the absence of AA showed a significant decrease in phagocytosis, as indicated by a significant decrease in the number of phagocytosed beads compared with macrophages that remained in room air (21%  $\text{O}_2$  control; 63.9 versus 100%; F-statistic ( $F_{(6,35)}$ ) = 10.18,  $P < 0.0001$ ; Figures 2A and 2B). Phagocytosis in hyperoxic macrophages cultured in AA-supplemented media was significantly increased compared with those exposed to hyperoxia in the absence of AA, indicated by higher numbers of phagocytosed beads at 10, 100, and 1,000  $\mu\text{M}$  (87.4, 86.4 and 100.1, respectively, versus 63.9%;  $F_{(6,35)} = 10.18$ ,  $P < 0.01$ ; Figures 2A and 2B). These results suggest that AA can enhance innate



**Figure 1.** Ascorbic acid (AA) decreases hyperoxia-compromised bacterial clearance in mice. Male C57BL/6 mice, exposed to 98%  $\text{O}_2$  or higher for 48 hours, were inoculated with *Pseudomonas aeruginosa* (PA;  $1 \times 10^7$  CFU) intratracheally. Mice were randomized to receive either AA (50 mg/kg) or saline intraperitoneally. Bronchoalveolar lavage fluid (BALF) and lung tissues were harvested 18 hours after infection. Number of viable bacteria in lungs (A) and BALF (B) were determined by plating serial dilutions of either homogenized lung tissues or BALF, respectively. The total protein content in BALF (C) was measured using the bicinchoninic acid assay ( $n = 21$  mice per group). In another set of experiments, mice were observed for survival after inoculation with PA ( $n = 13$ –18 mice per group) (D). Data represent the mean  $\pm$  SEM of at least four independent experiments. \* $P \leq 0.05$  compared with control mice.



**Figure 2.** AA attenuates hyperoxia-compromised macrophage phagocytic function. RAW 264.7 cells either remained at room air (RA; 21% O<sub>2</sub>) or were exposed to 95% O<sub>2</sub> for 24 hours in the presence of AA (0–1,000 μM). Cells were then incubated with FITC-labeled latex mini-beads for 1 hour and stained with phalloidin and 4',6-diamidino-2-phenylindole (DAPI) to visualize the actin cytoskeleton and nuclei, respectively. Representative immunofluorescent images of RAW 264.7 cells (*red*, F-actin cytoskeleton; *green*, latex mini-beads; *blue*, nuclei) are shown. Scale bars: 10 μm (A). For quantification of phagocytic activity, at least 200 cells per well were counted and the numbers of beads per cell were represented as a percentage of the 21% O<sub>2</sub> (0 μM) control group (B). Each value represents the mean ± SEM of three independent experiments for each group. \**P* ≤ 0.05 compared with the 95% O<sub>2</sub> (0 μM) control group; #*P* ≤ 0.05 compared with 21% O<sub>2</sub> (0 μM) control group. O<sub>2</sub>, 95% oxygen; RA, 21% oxygen.

immunity against bacterial infections by improving macrophage phagocytic activity under hyperoxic conditions.

### AA Significantly Decreases Oxidative Stress in Hyperoxia-Exposed Macrophages

To determine the mechanisms underlying AA-attenuated hyperoxia-compromised

macrophage phagocytic function (Figure 2), ORP, an indicator of oxidative stress, was measured in RAW 264.7 cells exposed to hyperoxia in the presence of a series of AA concentrations (0–1,000 μM). Macrophages exposed to hyperoxia had significantly higher ORP compared with those exposed to room air (21% O<sub>2</sub>; 227.7 versus 205.1 mV;  $F_{(5,32)} = 11.19$ ,

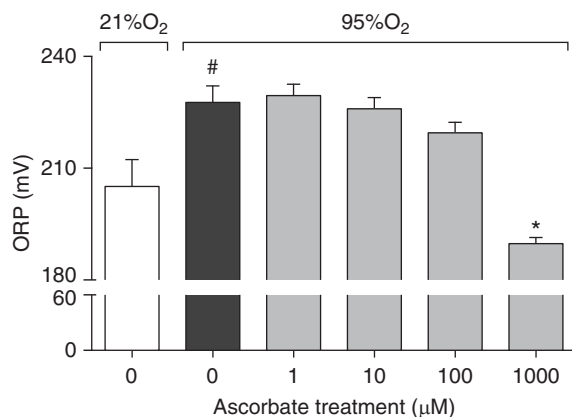
$P < 0.01$ ; Figure 3). Cells incubated with AA showed a concentration-dependent decrease in ORP, which was significantly less at 1,000 μM, compared with macrophages exposed to hyperoxia in the absence of AA (189.8 versus 227.7 mV;  $F_{(5,32)} = 11.19$ ,  $P < 0.0001$ ; Figure 3). These results suggest that AA enhances macrophage functions by blunting hyperoxia-induced oxidative stress.

### Exposure to Hyperoxia Reduces Intracellular AA Levels in Macrophages

Immune cells, especially macrophages, maintain very high concentrations of AA compared with plasma (40). To determine if exposure to hyperoxia alters AA levels in macrophages, the intracellular concentrations of AA were measured. Because AA was undetectable in cultured RAW 264.7 cells (data not shown), RAW cells were incubated with 1,000 μM AA for 1 hour before hyperoxia exposure. Exposure to hyperoxia significantly reduced the intracellular AA levels compared with control RAW cells exposed to room air ( $0.47 \pm 0.07$  versus  $1.81 \pm 0.24$  arbitrary unit (AU);  $t = 5.66$ ,  $df = 11$ ,  $P < 0.001$ ; Figure 4A). These data indicate that intracellular AA levels in macrophages are rapidly exhausted upon hyperoxia exposure.

We further investigated whether continuous AA (10–1,000 μM) supplementation for 24 hours with simultaneous exposure of RAW cells to hyperoxia can maintain intracellular AA levels. Data presented in Figure 4B show that RAW cells with supplementation of 1,000 μM AA had similar intracellular AA levels upon exposure to hyperoxia as that of room air-exposed cells (101.5 versus 100%;  $t = 0.08$ ,  $df = 24$ ; Figure 4B). However, intracellular AA levels were significantly lower in hyperoxia-exposed cells when RAW cells were supplemented with 10 or 100 μM AA as compared with the room air control groups (35.94 and 48.81 versus 100%;  $t = 3.72$  and 2.97, respectively,  $df = 24$ ,  $P < 0.05$ ; Figure 4B). These results suggest that a continuous supply with appropriate concentrations of AA mitigates hyperoxia-induced decrease in intracellular AA levels and maintains the intracellular antioxidant capacity.



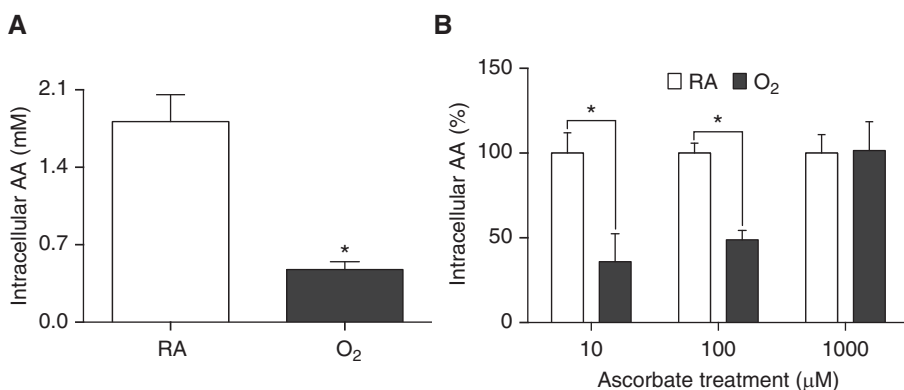


**Figure 3.** AA decreases oxidative stress in hyperoxia-exposed macrophages. RAW 264.7 cells either remained at RA (21% O<sub>2</sub>) or were exposed to 95% O<sub>2</sub> for 24 hours in the presence of AA (0–1,000 μM). Cell lysates were prepared and used to measure oxidation–reduction potential (ORP). Values represent the mean ± SEM of three independent experiments. \* $P \leq 0.05$  compared with 95% O<sub>2</sub> (0 μM) control group; # $P \leq 0.05$  compared with 21% O<sub>2</sub> (0 μM) control group.

### AA Significantly Reduces the Extracellular Accumulation of HMGB1 in Hyperoxia by Inhibiting Its Translocation

Previous studies in our laboratory have shown that elevated levels of airway HMGB1 contribute to hyperoxia-compromised macrophage functions (7, 17). To determine whether or not AA enhances the functions of hyperoxic macrophages by reducing the levels of extracellular HMGB1, we determined the levels of HMGB1 in the airways of mice exposed to hyperoxia, as described in the MATERIALS AND METHODS section. Figure 5A

illustrates that mice receiving AA supplementation had significantly lower HMGB1 levels in BALF compared with the unsupplemented controls ( $18 \pm 6.7$  versus  $100 \pm 10.1\%$ ;  $t = 7.26$ ,  $df = 4$ ,  $P < 0.0001$ ; Figure 5A). To further delineate the AA-induced increase in macrophage function, the levels of extracellular HMGB1 were assessed in the culture media of RAW 264.7 cells that were exposed to hyperoxia in the presence of AA (0–1,000 μM). Macrophages exposed to hyperoxia had a significant increase in the levels of extracellular HMGB1 accumulation as compared with the 21% O<sub>2</sub> control



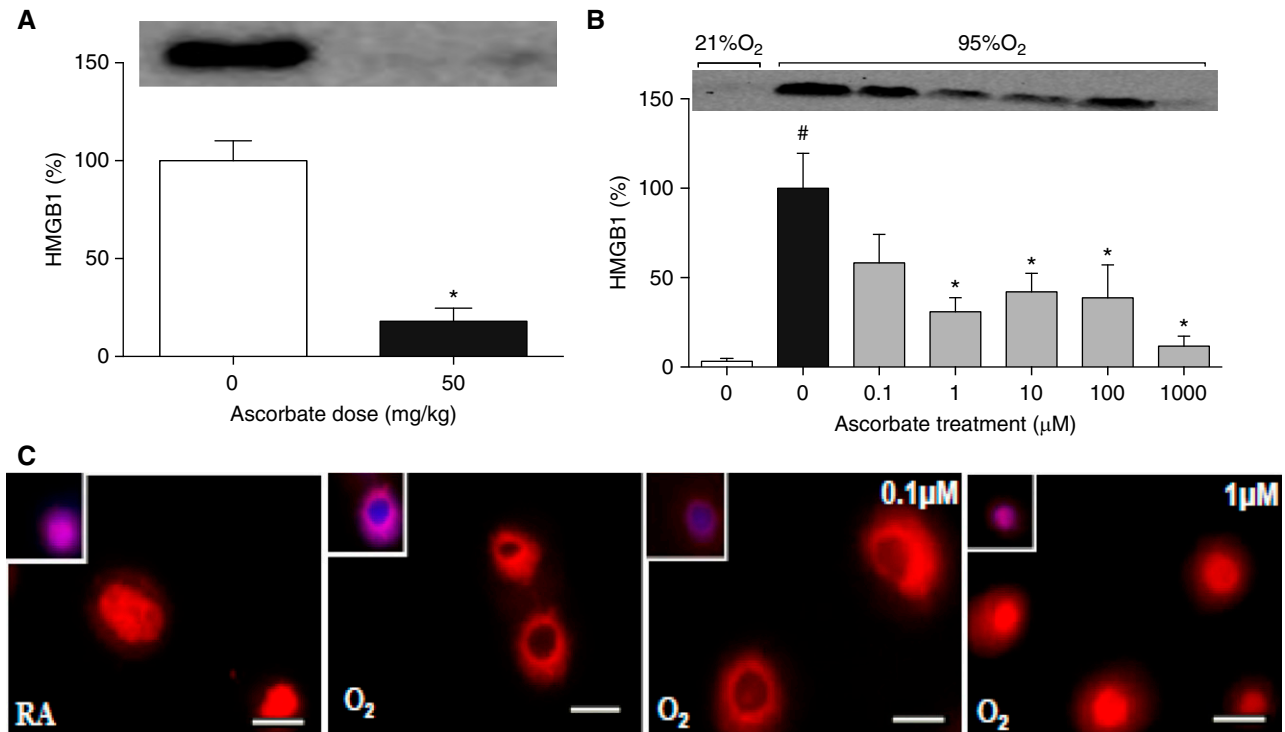
**Figure 4.** Exposure to hyperoxia reduces intracellular AA levels in macrophages. RAW 264.7 cells were pretreated with AA (1,000 μM) for 1 hour, followed by replacing media with fresh media, and cells were exposed to either RA (21% O<sub>2</sub>) or 95% O<sub>2</sub> for 24 hours (A). In a different set of experiments, RAW 264.7 cells either remained at RA or were exposed to 95% O<sub>2</sub> for 24 hours in the presence of AA (0–1,000 μM) (B). Intracellular AA levels were determined using HPLC–ultraviolet spectrometry. Values represent the mean ± SEM of three independent experiments. \* $P \leq 0.05$  compared with RA control group.

(100 versus 3.2%;  $F_{(6,14)} = 11.92$ ,  $P < 0.0001$ ; Figure 5B). AA at 1, 10, 100, and 1,000 μM significantly reduced the levels of extracellular HMGB1 as compared with cells exposed to hyperoxia in the absence of AA (30.9, 42.0, 38.7, and 11.7 versus 100%, respectively;  $F_{(6,14)} = 11.92$ ,  $P < 0.01$ ; Figure 5B).

The translocation of HMGB1 from the nucleus to the cytoplasm is a critical step in the extracellular secretion of HMGB1 (42, 43). Therefore, we determined the effect of AA on the nucleocytoplasmic translocation of HMGB1 in hyperoxic macrophages. Macrophages exposed to hyperoxia exhibited intense staining of HMGB1 (red) in the cytoplasm as compared with control cells that remained in room air (21% O<sub>2</sub>), where the predominant stain was in the nucleus (Figure 5C). The macrophages incubated with 1–1,000 μM of AA displayed distinct staining of HMGB1 in the nucleus compared with the negligible nuclear staining in macrophages incubated with either hyperoxia alone or in the presence of 0.1 μM AA (Figure 5C; images for 10, 100, and 1,000 μM not shown). These results indicate that AA significantly reduces the hyperoxia-elevated HMGB1 accumulation in the extracellular milieu by inhibiting its translocation from the nucleus to the cytoplasm.

### AA Inhibits Nuclear Translocation of NF-κB in Hyperoxic Macrophages

NF-κB activation plays a critical role in mediating the release of HMGB1 from cultured macrophages under stress stimuli, including exposure to hyperoxia (17, 38, 44). NF-κB activation was assessed by immunostaining the p65 subunit of NF-κB, as described in the published studies. In nonactivated cells, NF-κB is primarily localized in the cytoplasm, as indicated by a prominent cytoplasmic stain and a distinct hollow nucleus in cells that remained at 21% O<sub>2</sub> (Figure 6A). Upon hyperoxic exposure, NF-κB translocates into the nucleus, as indicated by a profound nuclear staining (Figure 6A; images for 100 and 1,000 μM not shown). However, macrophages exposed to hyperoxia and incubated with AA at 1, 100, and 1,000 μM had significantly reduced nuclear staining compared with cells exposed to hyperoxia alone (0.82, 0.83, and 0.84 versus 0.90, respectively;  $F_{(5,2374)} = 49.58$ ,  $P < 0.0001$ ; Figures 6A and 6B). These results suggest



**Figure 5.** AA decreases the hyperoxia-induced release of high-mobility group box protein B1 (HMGB1) by inhibiting its translocation from the nucleus to the cytoplasm. Male C57BL/6 mice, exposed to 98% O<sub>2</sub> or higher for 48 hours, were inoculated with PA ( $1 \times 10^7$  CFU) intratracheally. Mice were randomized to receive either AA (50 mg/kg) or saline intraperitoneally. HMGB1 levels in the BALF were analyzed by Western blot analysis. \* $P \leq 0.05$  compared with hyperoxia control group (A). RAW 264.7 cells either remained at RA (21% O<sub>2</sub>) or were exposed to 95% O<sub>2</sub> for 24 hours with AA (0–1,000 μM). HMGB1 levels in cell culture media were analyzed by Western blot analysis. The data obtained are represented in percentage of the 95% O<sub>2</sub> control group. \* $P \leq 0.05$  compared with 95% O<sub>2</sub> (0 μM) control group; # $P \leq 0.05$  compared with 21% O<sub>2</sub> (0 μM) control group (B). The translocation of HMGB1 was assessed by immunostaining the cells with anti-HMGB1 antibody (red). DAPI stain was used to visualize the nuclei (blue). Scale bars: 10 μm (C). Each value represents the mean  $\pm$  SEM of three independent experiments.

that AA inhibits HMGB1 release, at least in part, by preventing hyperoxia-induced NF- $\kappa$ B activation.

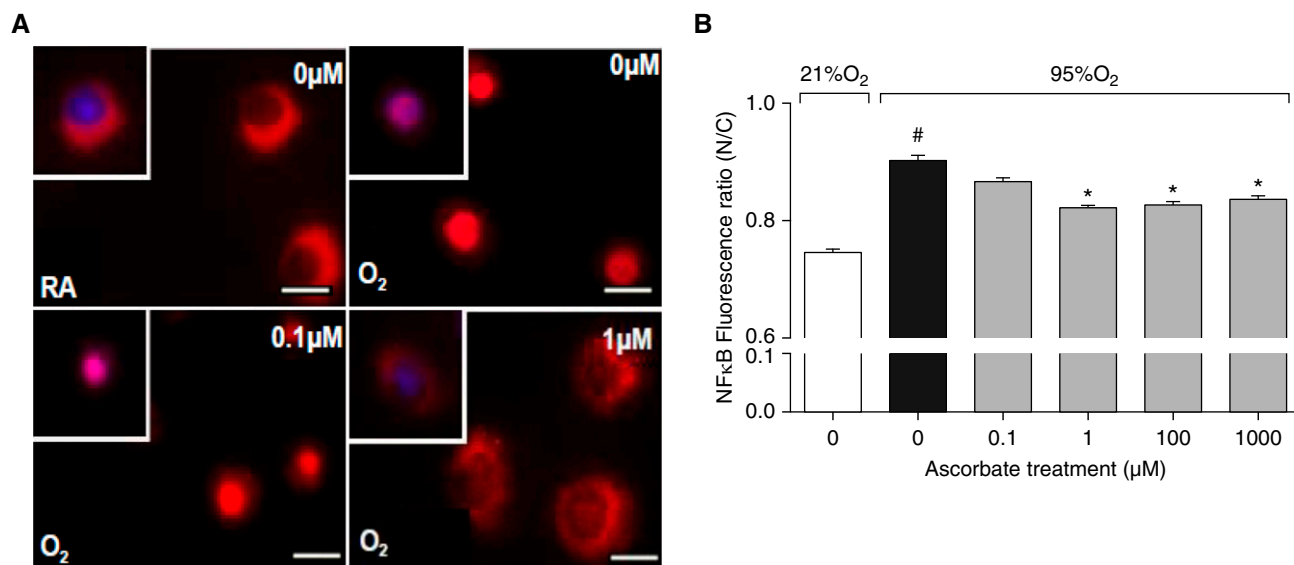
## Discussion

The results of this study indicate that AA protects against hyperoxia-induced lung injury and enhances innate immunity against pulmonary PA infection in a mouse model of VAP. In addition, AA can effectively restore hyperoxia-compromised phagocytic function of cultured macrophages. AA-increased macrophage phagocytic function was associated with a decrease in hyperoxia-induced: (1) elevation in intracellular oxidative stress; and (2) accumulation of extracellular HMGB1. The AA-mediated decrease in the extracellular accumulation of HMGB1 was associated with inhibition of the translocation of nuclear HMGB1 to the cytoplasm, which

was associated with attenuated NF- $\kappa$ B activation.

Prolonged exposure to hyperoxia increases the susceptibility of subjects to lung microbial infections (7, 13, 45). The results in Figure 1 show that AA significantly increases bacterial clearance in the airways and lungs of mice exposed to hyperoxia (Figures 1A and 1B). Hyperoxia-induced lung permeability, a marker of tissue injury, can also be reduced by AA (Figure 1C). This increase in host immunity was associated with an improved survival of these animals (Figure 1D). Interestingly, AA did not significantly increase bacterial clearance and lung injury under normoxic (healthy) conditions. Similarly, AA (100 mg/kg/12 h) does not significantly increase the clearance of *Streptococcus pneumoniae* in normoxic, healthy mice (46). These results may provide further explanations, other than the route of administration of AA, for the difference in

the incidence of infections observed in clinical trials of AA administration. Glazebrook and Thomson (47) reported that AA administration did not affect the incidence of viral infections in healthy youth, whereas Heyland and colleagues (48) found that patients on mechanical ventilation receiving supplementation of AA, in combination with other antioxidants, had reduced occurrence of intensive care unit-acquired pneumonia (71 [~42.3%] versus 95 patients [~52.5%] in the control group without antioxidant supplementation,  $P = 0.06$ ). A similar protective effect of AA (50 or 200 mg/kg/24 h) against organ failure and tissue injury has also been observed in patients with severe sepsis (49). These findings suggest that the protective effect of AA is specific to pathological conditions with AA deficiency. Oxidative stress induced by prolonged exposure to hyperoxia or by pronounced inflammatory responses may cause a



**Figure 6.** AA inhibits the nuclear translocation of NF- $\kappa$ B in hyperoxic macrophages. RAW 264.7 cells either remained at RA (21%  $O_2$ ) or were exposed to 95%  $O_2$  for 24 hours in the presence of AA (0–1,000  $\mu$ M). The localization of NF- $\kappa$ B was assessed by immunostaining the cells with anti-p65 antibody (red). DAPI stain was used to visualize nuclei (blue). Scale bars: 10  $\mu$ m (A). Fluorescence was quantified using ImageJ software (National Institutes of Health, Bethesda, MD) and represented as the fluorescence ratio of nucleus to cytoplasm (N/C) (B). \* $P \leq 0.05$  compared with 95%  $O_2$  (0  $\mu$ M) control group; # $P \leq 0.05$  compared with 21%  $O_2$  (0  $\mu$ M) control group.

critical reduction or even depletion of the AA reservoir in the system. AA supplementation brings about repletion to attenuate the adverse effects of oxidative stress. This hypothesis is supported by the results presented in Figures 4A and 4B, which show that exposure to hyperoxia depletes intracellular AA levels in cultured macrophages, and continuous supplementation with 1,000  $\mu$ M AA can replenish and maintain the levels of intracellular AA.

Prolonged exposure to hyperoxia has been shown to result in excessive production of mitochondrial ROS in lung cells (20). Our results indicate that hyperoxia increases intracellular oxidative stress in cultured macrophages (Figure 3). Supplementation with AA can reduce hyperoxia-increased levels of ORP to similar levels observed under normoxic conditions (Figure 3). In hyperoxic macrophages without AA supplementation, oxidative stress can cause actin oxidation and subsequently increase stress fiber formation (16, 18), and oxidation of actin's regulatory proteins, thereby causing dysfunction in normal actin polymerization (24, 50), leading to the compromised macrophage phagocytic function. AA has also been shown to decrease the levels of the hydrogen peroxide-induced ROS (51). Interestingly, plasma AA provides

protection against aqueous peroxyl radicals in healthy human subjects (29). Macrophages normally accumulate and maintain very high intracellular levels of AA compared with plasma via specific sodium-dependent transporters, thus providing an effective mechanism to neutralize excessive ROS in these cells (40). However, levels of intracellular AA can be significantly decreased after activation of phagocytosis (52). Thus, AA supplementation under oxidative stress may help to maintain the levels of intracellular AA, and subsequently the antioxidant capacity of the alveolar macrophages during phagocytosis, leading to the improvement in phagocytic activity, as in the results shown in Figure 2. Similar to these observations in macrophages, AA has also been shown to improve phagocytic functions of monocytes and neutrophils in humans (53, 54) and murine peritoneal macrophages (55, 56). These results demonstrate that AA can effectively maintain the phagocytic functions of macrophages under oxidative stress.

HMGB1, a potent proinflammatory cytokine, can impair macrophage phagocytosis when it is present in the extracellular milieu (7, 37). High levels of airway HMGB1 are observed in animals exposed to hyperoxia and/or mechanical ventilation, as well as in patients with VAP

(7, 11, 57–59). The results of this study indicate that AA can inhibit hyperoxia-induced HMGB1 accumulation in the extracellular milieu (Figure 5). This effect was associated with an increase in macrophage phagocytosis function (Figure 2), and a decrease in the cytoplasmic translocation of HMGB1 from nuclei in hyperoxic macrophages, which is the first step in the release of HMGB1 into the extracellular milieu (Figure 5). Consistent with these results, it has been reported that the administration of AA significantly inhibited LPS-stimulated HMGB1 secretion from cultured macrophages (60, 61). These results suggest that AA significantly increases macrophage phagocytosis not only by reducing the intracellular oxidative stress, but also by inhibiting the translocation and the release of nuclear HMGB1 from these cells.

NF- $\kappa$ B is a redox-sensitive transcription factor (62–65) that is activated under oxidative stress conditions, including hyperoxia (66–68). Activation of NF- $\kappa$ B has been shown to play a critical role in the release of HMGB1 from macrophages exposed to hyperoxia or LPS (17, 44, 69). Our results indicate that AA can significantly inhibit NF- $\kappa$ B activation by attenuating the hyperoxia-induced nuclear translocation of NF- $\kappa$ B in cultured macrophages (Figure 6). This effect is





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