The Compromise of Macrophage Functions by Hyperoxia Is Attenuated by Ethacrynic Acid via Inhibition of NF- κ B–Mediated Release of High-Mobility Group Box-1

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Abstract

The prolonged exposure to hyperoxia can compromise macrophage functions and contribute to the development of ventilator-associated pneumonia. High levels of extracellular high-mobility group box-1 (HMGB1) in the airways of mice exposed to hyperoxia can directly cause macrophage dysfunction. Hence, inhibition of the release of nuclear HMGB1 into the extracellular milieu may help to maintain macrophage functions under hyperoxic conditions. The present study investigates whether ethacrynic acid (EA) affects hyperoxia-induced HMGB1 release from macrophages and improves their functions. Macrophage-like RAW 264.7 cells and bone marrow-derived macrophages were exposed to different concentrations of EA for 24 hours in the presence of 95% O₂. EA significantly decreased the accumulation of extracellular HMGB1 in cultured media. Importantly, the phagocytic activity and migration capability of macrophages were significantly enhanced in EA-treated cells. Interestingly, hyperoxia-induced NF- κ B activation was also inhibited in these cells. To determine whether NF- κ B plays a role in hyperoxia-induced HMGB1 release, BAY 11-7082, an inhibitor of NF- κ B activation, was used. Similar to EA, BAY 11-7082 significantly inhibited the accumulation of extracellular HMGB1 and improved hyperoxia-compromised macrophage migration and phagocytic activity. Furthermore, 24-hour hyperoxic exposure of

macrophages caused hyperacetylation of HMGB1 and its subsequent cytoplasmic translocation and release, which were inhibited by EA and BAY 11-7082. Together, these results suggest that EA enhances hyperoxia-compromised macrophage functions by inhibiting HMGB1 hyperacetylation and its release from macrophages, possibly through attenuation of the NF- κ B activation. Therefore, the activation of NF- κ B could be one of the underlying mechanisms that mediate hyperoxia-compromised macrophage functions.

Keywords: hyperoxia; macrophage; high-mobility group box-1; phagocytosis; NF- κ B

Clinical Relevance

We demonstrate that modulating extracellular levels of high-mobility group box-1 by inhibitors to NF- κ B activation could be used as a potential approach to improve hyperoxia-compromised macrophage functions. If these results can be translated to humans, it is possible that ethacrynic acid could be used as part of the treatment for patients with ventilator-associated pneumonia.

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Oxygen therapy, using mechanical ventilation with supraphysiological concentrations of oxygen (hyperoxia), is routinely used to treat patients in respiratory distress (1). However, a significant number of patients on ventilators have an enhanced susceptibility to infection, and can develop ventilator-associated pneumonia (VAP) (2, 3). VAP is the most frequent nosocomial infection, and is responsible for high mortality rates in patients receiving oxygen therapy (4, 5). Alveolar macrophages (AMs) are the primary phagocytes of the innate immune system in the lung (6, 7). AMs play a critical role in the cellular defense against respiratory infections (8, 9). However, prolonged exposure to hyperoxia can compromise the ability of AMs to phagocytose bacteria (10–12), which may play a critical role in the pathogenesis of VAP. In addition, cultured macrophages exposed to hyperoxia and AMs isolated from hyperoxia-exposed animals exhibit impairment in chemotaxis, adherence, and pathogen killing (13, 14). Thus, identifying factors that contribute to hyperoxia-compromised macrophage functions may provide insights into the development of novel therapeutic strategies that improve host defense against bacterial infection for patients with VAP.

Recently, we reported that high-mobility group box-1 (HMGB1) protein plays a critical role in mediating hyperoxia-compromised innate immunity (15, 16). HMGB1 is a member of the nonhistone chromosomal proteins known as high-mobility group proteins, and is present in almost all eukaryotic cells (17, 18). Typically, HMGB1 remains primarily in the nucleus, and is involved in stabilizing nucleosome structure and regulating gene transcription and DNA repair (19, 20). However, nuclear HMGB1 can be released into the extracellular milieu from either dead cells or viable cells in response to exogenous bacterial endotoxins, proinflammatory cytokines, and other stimuli, such as IFN- γ , hydrogen peroxide, or CpG (cytosine and guanine separated by only one phosphate) DNA (21–25). Once released into the extracellular milieu, HMGB1 can act as a proinflammatory cytokine (26, 27). We have recently identified a novel role for extracellular HMGB1 in compromising host defense against bacterial infection in the lungs (15, 28). We demonstrated that the presence of high levels of airway HMGB1 can impair the phagocytic activity of AMs and

compromise the host's ability to clear invading micro-organisms (15). Thus, inhibition of airway HMGB1 or prevention of HMGB1 release into the airways is critical for maintaining normal functions of AMs in mediating host defense against pulmonary bacterial infection. Numerous compounds have been shown to inhibit LPS-stimulated HMGB1 release from macrophages (26, 29, 30), although the underlying mechanisms remain to be elucidated. Ethacrynic acid (EA), a phase 2 enzyme inducer and loop diuretic (31, 32), is used to treat edema in patients with severe allergic reactions to sulfas or other loop diuretics (32, 33). Interestingly, EA can inhibit LPS-induced HMGB1 release from cultured macrophages in a concentration-dependent manner (34).

The aim of this study was to determine whether EA can modulate HMGB1 translocation from the nucleus to the cytoplasm and, subsequently, its release into the extracellular milieu, and affect macrophage functions under hyperoxic conditions. Here, we show that EA significantly inhibited HMGB1 release from cultured murine macrophages that were exposed to hyperoxia, by targeting HMGB1 hyperacetylation that was associated with hyperoxia-induced NF- κ B activation. Importantly, EA can restore the ability of hyperoxic macrophages to phagocytose and migrate.

Materials and Methods

Cell Culture and Reagents

Murine macrophage-like RAW 264.7 cells (TIB-71; American Type Culture Collection, Manassas, VA) were cultured in RPMI 1,640 medium (Gibco BRL, Grand Island, NY) supplemented with 10% FBS (Atlanta Biologicals, Lawrenceville, GA), 1% penicillin, and 1% streptomycin (Life Technologies, Grand Island, NY). Cells were maintained at 37°C in normoxia (5% CO₂/21% O₂) for 24 hours, allowed to grow to 70–80% confluency, and subcultured every 3 days. Hyperoxic exposure was performed in sealed, humidified chambers (Billups-Rothenberg Inc., Del Mar, CA) flushed with 95% O₂/5% CO₂ at 37°C, as previously described (12). An oxygen analyzer (MSA Medical Products, Pittsburgh, PA) was used to monitor the O₂ levels. EA and BAY 11-7082 were purchased from Sigma-Aldrich (St. Louis, MO).

Isolation and Culture of Bone Marrow-Derived Macrophages

Bone marrow cells were harvested from 8- to 12-week-old female C57BL/6 mice and cultured as described previously (35, 36). Briefly, femurs were removed, cleaned, and placed into tubes containing sterile PBS. The bones were placed in 70% ethanol for

Figure 1. Effect of ethacrynic acid (EA) on hyperoxia-induced high-mobility group box-1 (HMGB1) release from RAW 264.7 cells and bone marrow-derived macrophages (BMDMs). RAW 264.7 cells and BMDMs were exposed to 95% O₂ in the presence of different concentrations of EA for 24 hours. The levels of extracellular HMGB1 in the culture media of (A) RAW 264.7 cells and (B) BMDMs were determined by Western blot analysis. The levels of HMGB1 were quantified by measuring the integrated density value (IDV) of the immunoreactive bands on Western blots and expressed as % HMGB1, considering 95% O₂ as 100%. Each value represents the mean $\pm$ SEM of three and two independent experiments for each group in RAW 264.7 cells and BMDMs, respectively. * P < 0.05; ** P < 0.01 compared with cells exposed to 95% O₂ in DMSO alone.

1 minute and washed with PBS under sterile conditions. The bones were gently flushed with a syringe filled with PBS to extract bone marrow, and the cell suspension thereafter was centrifuged. The cell pellet was resuspended and cultured in a 75-cm² flask containing RPMI 1,640 supplemented with L-glutamine (Cellgro, Manassas, VA), 10% heat-inactivated FBS, 1% of 1 M HEPES solution and 15% L929 cell-conditioned medium (as a source of granulocyte/macrophage colony-stimulating factor) (37). The media were changed every 36 hours and, after approximately 6 days in culture, macrophages were detached by gentle scraping. Cells were counted and cell viability was assessed using Trypan blue exclusion. The cells were seeded and cultured in six-well tissue culture plates for further experiments.

Evaluation of Cell Viability

To measure the effect of EA and BAY 11-7082 on cell viability, RAW 264.7 cells were seeded in 48-well plates and allowed to adhere overnight at 37°C. RAW 264.7 cells were exposed to either 95% O₂ alone or 95% O₂ in the presence of different concentrations of EA and BAY 11-7082 for 24 hours. After exposure to oxygen or test compounds, RAW 264.7 cells were incubated with 0.5% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 2 hours at 37°C. The MTT assay is a rapid colorimetric assay that measures the activity of the mitochondrial enzyme, succinate dehydrogenase (38). The mitochondrial dehydrogenase of viable cells cleaves the tetrazolium ring of MTT, yielding purple formazan derivative crystals. At the end of the 2-hour incubation, removing MTT stopped the reaction and isopropanol was added to the wells to solubilize the formazan crystals for 15 minutes. The amount of MTT formazan product was determined by measuring the absorbance of the resulting purple solution at 570 nm using a microplate reader (Multiscan, Thermo Electron Corp., Milford, MA).

Western Blot Analysis

RAW 264.7 cells were cultured in serum-free Opti-MEM I medium (Gibco BRL) in 12-well plates and then exposed to hyperoxia, and treatments were performed as previously described. After hyperoxic exposure, the levels of HMGB1 in the culture media of treated cells were measured

by Western blot analysis. The samples were loaded onto 10% SDS-polyacrylamide gels (Bio-Rad, Hercules, CA) and then transferred to Immobilon-P membranes (Millipore, Bedford, MA). Nonspecific binding sites on the membrane were blocked by incubating the membrane with 5% nonfat dry milk (Bio-Rad) in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST) for 1 hour at room temperature. Subsequently, membranes were washed three times with TBST and incubated overnight at 4°C with rabbit anti-HMGB1 polyclonal primary antibody (1:500, H9664; Sigma-Aldrich, St. Louis, MO) diluted in 5% nonfat dry milk. After three washes with TBST, the membranes were incubated with anti-rabbit horseradish peroxidase-coupled secondary antibody (1:5,000; Bio-Rad) for 1 hour at room temperature. Subsequently, membranes were washed three times again with TBST, and the immunoreactive proteins were visualized using the enhanced chemiluminescence reagent kit (GE Healthcare, Piscataway, NJ) per the

manufacturer's instructions. The images were developed using the FluorChem system (Alpha Innotech, Santa Clara, CA).

Immunofluorescence Analysis

Immunofluorescence analysis was used to assess the intracellular localization of HMGB1 and NF-κB using a fluorescent microscope (Nikon, Melville, NY). RAW 264.7 cells were seeded in 12-well plates and allowed to adhere overnight at 37°C. Cells were exposed to either 95% O₂ alone or in the presence of different concentrations of EA and BAY 11-7082 for 24 hours. After incubation with the test compounds for 24 hours, cells were fixed with 2% phosphate-buffered formaldehyde (pH 7.4) for 15 minutes and washed three times with PBS. Cells were then permeabilized with 0.2% Triton X-100 (Sigma-Aldrich, St. Louis, MO) and nonspecific binding sites were blocked with 10% normal goat serum (Chemicon, Temecula, CA) for 20 minutes. Cells were then washed with 1% BSA in PBS and incubated with anti-HMGB1 or

Figure 2. Effects of EA on HMGB1 localization in hyperoxic RAW 264.7 cells. RAW 264.7 cells, grown on coverslips, were exposed to room air (21% O₂) or 95% O₂ in the presence of different concentrations of EA for 24 hours. HMGB1 localization was visualized by immunofluorescence microscopy (A), and quantification of fluorescence intensity (B) was performed using ImageJ software (National Institutes of Health, Bethesda, MD). Counterstaining with 4',6-diamidino-2-phenylindole (DAPI) was used to visualize nuclei (insets). Each value represents the mean ± SEM of three independent experiments for each group. **P* < 0.05, compared with cells exposed to 95% O₂ in DMSO alone.

anti-NF- κ B p65 primary antibodies (1:200, sc-372; Santa Cruz Biotechnology, Dallas, TX) overnight at 4°C. The cells were incubated with secondary antibody using goat anti-rabbit IgG conjugated with Alexa fluor 594 (Molecular Probes, Eugene, OR) for 1 hour. Normal blocking serum without primary antibody was used as a negative control. The nuclei of the cells were visualized by counterstaining with 4',6-diamidino-2-phenylindole. HMGB1 and NF- κ B translocation was observed under a fluorescent microscope.

Phagocytosis Assay

The phagocytosis assay was performed as previously described, with minor modifications (12). Briefly, RAW 264.7 cells were seeded in 48-well plates and allowed to adhere overnight at 37°C. RAW 264.7 cells were exposed to either 95% O₂ alone or 95% O₂ in the presence of a series of concentrations of EA (1, 2, 4, and 8 μ M) and BAY 11-7082 (0.5, 1, and 2 μ M) for 24 hours. RAW 264.7 cells were incubated at 37°C for 60 minutes with opsonized FITC-labeled latex beads (Polysciences, Warrington, PA) at a ratio of 100:1 (beads: cell). Macrophages were incubated with 0.04% Trypan blue in PBS for 10 minutes to quench beads that were not internalized by macrophages. To visualize the uptake of FITC-labeled latex beads, macrophages were fixed with 4% paraformaldehyde for 10 minutes, washed with PBS, and stained with 4',6-diamidino-2-phenylindole. To visualize cytoskeleton, cells were stained with Texas Red X-phalloidin (Molecular Probes) in 1% BSA overnight. The phagocytosis or uptake of the beads was assessed visually using a fluorescent microscope and then counting 250 individual macrophages per well in duplicates from three independent experiments for each treatment group.

Macrophage Migration or Wound Healing Assay

RAW 264.7 cells were seeded in 24-well plates and allowed to grow to saturation density. A scratch was made vertically along the length of the well using a pipette tip, and the scratched area was washed with PBS. The cell debris removed created a wound region in the plate, which was photographed (0 h, before treatment). The wound regions were allowed to heal by placing the plates in room air, hyperoxia alone, or hyperoxia with different concentrations of EA and

BAY 11-7082 for 24 hours. Pictures of the scratched area were taken again (24 h, after treatment), and the number of cells that migrated to the scratched area was counted.

Analysis of HMGB1 by Liquid Chromatography Tandem Mass Spectrometry

All chemicals and solvents were of the highest available grade (Sigma-Aldrich, Poole, UK). Samples were precleared with 50 μ l protein G-Sepharose beads for 1 hour at 4°C. Supernatant HMGB1 was immunoprecipitated with 5 μ g rabbit anti-HMGB1 (Abcam, Cambridge, UK) for 16 hours at 4°C, as previously described (39). Samples were subjected to *Staphylococcus aureus* Protease V8 (GluC; New England Biolabs, Herts, UK) digestion, according to the manufacturer's instructions, and desalted using ZipTip C18 pipette tips (Millipore, Consett, UK). Characterization of acetylated lysine residues within HMGB1 was determined as described previously (40, 41) using an AB Sciex TripleTOF 5,600 (Sciex Inc., Warrington, UK).

Statistical Analysis

The data are presented as mean ($\pm$ SEM) of at least three independent experiments, and were analyzed for statistical significance using the Student's *t* test with Excel software (Microsoft Corp., Seattle, WA). A *P* value less than 0.05 was considered statistically significant.

Results

EA Inhibits Hyperoxia-Induced HMGB1 Release

Previously, we have shown that prolonged exposure to hyperoxia induces the release of nuclear HMGB1 from cultured macrophages into the extracellular milieu, leading to compromised macrophage phagocytosis (15). To test whether EA inhibits the hyperoxia-induced release of HMGB1 from macrophages, RAW 264.7 cells and primary bone marrow-derived macrophages (BMDMs) were incubated with EA in the presence of 95% O₂. Figure 1 shows that EA at 1, 2, and 4 μ M

Figure 3. EA restores the phagocytic ability of macrophages exposed to hyperoxia. RAW 264.7 cells were exposed to room air (21% O₂) or 95% O₂ in the presence of different concentrations of EA for 24 hours. Cells were subsequently incubated with FITC-labeled latex minibeads for 1 hour and stained with phalloidin to visualize the cells. (A) shows representative immunofluorescence images of RAW 264.7 cells phagocytosing minibeads [bright dots]. At least 250 individual macrophages per well were counted to quantify the extent of phagocytic activity (B). Each value represents the mean $\pm$ SEM of three independent experiments for each group. **P* < 0.05, compared with cells exposed to 95% O₂ in DMSO alone.

significantly inhibited the accumulation of HMGB1 in the culture media of hyperoxic RAW 264.7 cells (Figure 1A) (9.31 ± 0.50 arbitrary units [AU], 2.42 ± 0.61 AU, and 7.89 ± 0.61 AU, respectively, versus 16.39 ± 1.08 AU of the control cells) and BMDMs (Figure 1B), with the maximum effect occurring at $2 \mu\text{M}$. The reduced levels of extracellular HMGB1 from EA-treated cells were not due to a decrease in the number of viable cells, as the number of viable cells in groups incubated with EA was not significantly altered compared with the control cells incubated with the vehicle alone (see Figure E1 in the online supplement). However, $8 \mu\text{M}$ of EA decreased the number of viable cells compared with the vehicle controls (0.232 ± 0.018 AU compared with 0.272 ± 0.005 AU of cells incubated with vehicle control), suggesting that EA might be somewhat cytotoxic to RAW 264.7 cells under hyperoxic conditions. This cytotoxic effects likely accounts for the accumulation of extracellular HMGB1 in RAW 264.7 cells incubated with $8 \mu\text{M}$ EA (Figure 1).

The Inhibition of Hyperoxia-Induced HMGB1 Release by EA Results from Inhibition of the Cytoplasmic Translocation of Nuclear HMGB1

HMGB1 release involves the process of translocation from the nucleus to the cytoplasm and subsequently to the extracellular milieu (42). To determine if the decrease in HMGB1 release under hyperoxic conditions was the result of EA affecting the translocation of nuclear HMGB1, cellular localization of HMGB1 was determined in RAW 264.7 cells incubated with EA. HMGB1 was primarily localized in the nucleus of RAW 264.7 cells that were maintained in room air (Figure 2, $21\% \text{ O}_2$). On the other hand, HMGB1 was found in the cytoplasm, with hollow nuclear staining in some (arrows) of the many RAW 264.7 cells exposed to hyperoxia for 24 hours (Figure 2, $95\% \text{ O}_2$). Incubation of macrophages with 0.05% DMSO (vehicle control) did not significantly alter HMGB1's cellular localization (Figure 2, $0 \mu\text{M}$). However, EA, at 1 , 2 , or $4 \mu\text{M}$, significantly inhibited the evident staining of HMGB1 in the cytoplasm in hyperoxic RAW 264.7 cells (Figures 2A and 2B). These data suggest that the reduction in HMGB1 release by EA is due to its inhibition of HMGB1 translocation from the nucleus to the

cytoplasm. Consistent with the accumulation of extracellular HMGB1 in cells incubated with $8 \mu\text{M}$ EA, a marked reduction in total HMGB1 staining was observed in these cells, further confirming that EA at $8 \mu\text{M}$ is cytotoxic to hyperoxic macrophages (Figure 2, $8 \mu\text{M}$).

EA Restores Macrophage Functions under Hyperoxic Conditions

The exposure of macrophages to hyperoxia significantly impairs their ability to phagocytose micro-organisms (12). To determine whether EA can restore the phagocytic ability of hyperoxic macrophages, phagocytic activity was determined in RAW 264.7 cells incubated with EA. Figure 3 shows that RAW 264.7 cells phagocytosed significantly fewer beads after exposure to hyperoxia for 24 hours compared with those that remained in room air ($33.46 \pm 7.02\%$ of hyperoxia compared with $100 \pm 17.54\%$ of room air

[$21\% \text{ O}_2$]; $P < 0.05$). The vehicle control, 0.05% DMSO (Figure 3, $0 \mu\text{M}$), had no significant effect on the hyperoxia-compromised phagocytic ability of RAW 264.7 cells (Figure 3). In contrast, increased numbers of beads were phagocytosed by RAW 264.7 cells incubated with 1 and $2 \mu\text{M}$ of EA (Figure 3), with a significant improvement at $2 \mu\text{M}$ ($76.75 \pm 5.20\%$ of $2 \mu\text{M}$ compared with $33.46 \pm 7.02\%$ of hyperoxia; $P < 0.01$). These results suggest that EA restores the hyperoxia-induced suppression of phagocytic ability. However, 4 or $8 \mu\text{M}$ EA did not significantly protect macrophages under hyperoxic conditions.

The ability to migrate to the site of infection is a vital step for AMs to clear invading pathogens. Figure 4 shows that exposure of RAW 264.7 cells to hyperoxia significantly suppressed macrophage migration ($18.01 \pm 10.04\%$ of hyperoxia versus $100 \pm 9.75\%$ of room air; $P < 0.01$), as indicated by a decrease in the number of

Figure 4. Effects of EA on hyperoxia-suppressed macrophage migration. (A) RAW 264.7 cells were seeded and incubated overnight in 24-well plates. Cells were scratched and photographed immediately (0 h) as the 0 hour. Cells were then allowed to migrate in room air ($21\% \text{ O}_2$) or hyperoxia ($95\% \text{ O}_2$) in the presence of different concentrations of EA for 24 hours. The scratched regions were then photographed again at 24 hours to visualize the migration of cells (after treatment). (B) The graph shows the percentage of cells migrating to the wound regions. Each value represents the mean $\pm$ SEM of three independent experiments. * $P < 0.05$ compared with cells exposed to $95\% \text{ O}_2$ in vehicle DMSO alone; # $P < 0.05$ compared with cells remained in room air.

cells that traveled to the scratched area (Figure 4A). The incubation of RAW 264.7 cells with 2 μ M EA significantly attenuated the hyperoxia-induced suppression of the migration ($105.45 \pm 20.78\%$ of versus $18.01 \pm 10.04\%$; $P < 0.05$). The incubation of cells with 8 μ M EA did not restore hyperoxia-compromised macrophage migration (Figure 4B).

EA Suppresses NF- κ B Activation

NF- κ B has been shown to play a critical role in HMGB1 release from RAW 264.7 cells in response to endotoxin (26, 29, 30). To determine the mechanism underlying EA's restorative effect on hyperoxia-compromised macrophage functions, the translocation of the p65 subunit of NF- κ B, a marker for NF- κ B activation, was assessed in the RAW 264.7 cells. Under normoxic conditions, the p65 subunit of NF- κ B was primarily localized in the cytoplasm (Figure 5A, 21% O₂). Exposure of the macrophages to 95% O₂ for 24 hours induced p65 translocation into the nucleus (Figure 5). Translocation of p65 was also observed in control cells incubated with the vehicle, 0.05% DMSO alone (Figure 5A, 0 μ M). In contrast, RAW 264.7 cells incubated with EA at 1, 2, or 4 μ M markedly reduced p65 translocation into the nucleus, as indicated by the predominant cytoplasmic stain (Figure 5A). Phosphorylation of p65 on serine 536 plays an important role in LPS-induced NF- κ B activation (43). To further ascertain that EA inhibits NF- κ B activation under hyperoxic conditions, levels of phosphorylation of p65 were determined in macrophages exposed to 95% O₂ with different concentrations of EA for 2 hours. Results shown in Figure 5B indicate that EA inhibited hyperoxia-induced levels of phosphorylation of the p65 subunit, with a significant effect at 2 μ M. These data indicate that EA inhibits hyperoxia-induced NF- κ B activation, suggesting that the effect of EA on HMGB1 release under hyperoxic conditions occurred by inhibiting hyperoxia-induced NF- κ B activation.

The NF- κ B Inhibitor, BAY 11-7082, Decreases HMGB1 Release and Restores Macrophage Functions in Hyperoxia

To confirm the above observation that EA rescues hyperoxia-compromised

Figure 5. EA inhibits nuclear translocation of NF- κ B p65 subunit and phosphorylation of p65 (p-p65) on serine 536 in hyperoxic RAW 264.7 cells. (A) RAW 264.7 cells, grown on coverslips, were exposed to room air (21% O₂) or to 95% O₂ in the presence of different concentrations of EA for 24 hours and analyzed for the localization of NF- κ B p65 subunit by an immunofluorescence assay. After oxygen exposure, cells were washed with PBS, fixed, permeabilized, and stained to localize the NF- κ B p65 subunit. The nuclear translocation of p65 was visualized using immunofluorescence microscopy. Counterstaining with DAPI was used to visualize nuclei (insets). (B) RAW 264.7 cells were exposed to room air (21% O₂) or to 95% O₂ in the presence of different concentrations of EA for 2 hours, and phosphorylation of p65 on serine 536 was assessed by Western blot analysis. Representative image of immunoreactive bands on Western blot is shown. Bar graph shows the integrated density value of p-p65 bands. The data are expressed as mean $\pm$ SEM of two independent experiments. * $P < 0.05$, compared with cells exposed to 95% O₂ in DMSO alone.

macrophage functions via inhibiting HMGB1 release by attenuating NF- κ B activation, BAY 11-7082, a well known NF- κ B inhibitor (44), was used in the following experiment. Whereas the p65 subunit of NF- κ B was primarily observed in the nuclei of RAW 264.7 cells exposed to 95% O₂ in the absence of BAY 11-7082 (Figure 6A), it was mainly localized in the cytoplasm of RAW 264.7 cells incubated with 1 μ M of BAY 11-7082 (Figure 6A). Corresponding to the attenuated NF- κ B activation in the presence of 1 μ M BAY 11-7082, HMGB1 was mainly localized in the nucleus of hyperoxic RAW 264.7 cells incubated with BAY 11-7082 (Figure 6B). To determine whether BAY 11-7082 inhibited hyperoxia-induced HMGB1 release, RAW 264.7 cells were exposed to 95% O₂ in the presence of 0, 0.5, 1, or 2 μ M

BAY 11-7082. Figure 6C shows that BAY 11-7082 significantly inhibited HMGB1 release, with 2 μ M BAY 11-7082 producing the greatest inhibitory effect (Figure 6C). DMSO (0.05%), the vehicle control, in contrast, did not significantly alter hyperoxia-induced HMGB1 release (Figure 6C). These results suggest that inhibition of hyperoxia-induced NF- κ B activation can attenuate HMGB1 release under hyperoxic conditions. Similar to EA, the results presented in Figure 6D show that BAY 11-7082 restored hyperoxia-induced suppression of phagocytic ability of macrophages. In addition, hyperoxia-induced inhibition of macrophage migration was significantly attenuated by BAY 11-7082 at 0.5 and 1 μ M (Figure 6E), although the rescuing effect was blunted by BAY 11-7082 at 2 μ M, perhaps due to its cytotoxicity.

Figure 6. BAY 11-7082 inhibits the hyperoxia-induced activation of NF- κ B and HMGB1 release, and restores hyperoxia-suppressed macrophage functions. RAW 264.7 cells, grown on coverslips, were exposed to hyperoxia in the presence of different concentrations of BAY 11-7082 for 24 hours. (A and B) After exposure, cells were washed with PBS, fixed, permeabilized, and stained to localize (A) NF- κ B p65 subunit or (B) HMGB1. The localization of the NF- κ B p65 subunit or HMGB1 was visualized using immunofluorescence microscopy. Representative images are shown. (C) Levels of HMGB1 in the cell supernatants were determined by Western blot analysis. The levels of HMGB1 were quantified by measuring an integrated density value (IDV) in arbitrary units (AU) of the immunoreactive bands on Western blots. Each value represents the mean $\pm$ SEM of three independent experiments for each group. * $P < 0.05$, ** $P < 0.01$ compared with cells exposed to 95% O₂ in DMSO alone. (D) RAW 264.7 cells, grown on coverslips, were exposed to room air (21% O₂) or 95% O₂ alone in the presence of different concentrations of BAY 11-7082 for 24 hours. Cells were subsequently incubated with FITC-labeled latex minibeads for 1 hour and stained with phalloidin and DAPI. At least 50 cells/slide were counted to quantify the extent of phagocytosis. Each value represents the mean $\pm$ SEM of three independent experiments for each group. * $P < 0.05$, ** $P < 0.01$ compared with cells exposed to 95% O₂ in DMSO alone. (E) RAW 264.7 cells were seeded and incubated overnight in 24-well plates. Cells were scratched and photographed immediately (0 h). The cells remained in room air (21% O₂) or were exposed to 95% O₂ in the presence of different concentrations of BAY 11-7082 for 24 hours. The scratched regions were photographed again to visualize migration of cells (after treatment). The graph shows the percentage of migrating cells to the wound region. Each value represents the mean $\pm$ SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$ compared with cells exposed to 95% O₂ in DMSO alone.

EA and BAY 11-7082 Reduce Hyperacetylation of HMGB1 in Hyperoxic Macrophages

The acetylation status of key lysine residues on the two nuclear localization signal sites of HMGB1 is a critical regulatory mechanism for intracellular shuttling of HMGB1 between the nucleus and the cytoplasm (45). Hyperacetylation of HMGB1 leads to active release of HMGB1 from activated macrophages (45). To examine the acetylation state of HMGB1

under hyperoxic conditions, liquid chromatography tandem mass spectrometry was used as described previously (16, 46). Under normoxic conditions, no detectable HMGB1 was found in cell supernatants, whereas hypoacetylated HMGB1 was found in the cell lysates (Figures 7A and 7B). These data confirm that HMGB1 exists primarily in the nonacetylated form in macrophages under normoxic conditions. After exposure to hyperoxia for 24 hours, hyperacetylated

HMGB1 was found in both the cell supernatants and lysates (Figures 7A and 7B), suggesting that hyperoxia-induced HMGB1 release is mediated via hyperacetylation of the nuclear location signal sites, thereby blocking its shuttling back to the nuclei. The incubation of macrophages with EA or BAY 11-7082 significantly attenuated hyperoxia-induced HMGB1 hyperacetylation (Figures 7A and 7B), suggesting that EA and BAY 11-7082 attenuate HMGB1 translocation from the nucleus to the cytoplasm and, subsequently, its release by inhibiting its hyperacetylation.

Discussion

Hyperoxia-compromised macrophage functions are implicated in the pathogenesis of VAP. We have previously shown that the accumulation of high levels of extracellular HMGB1 in the airways during prolonged exposure to hyperoxia impairs macrophage phagocytic function, a host response essential for defense against bacterial infection (15). In this study, we show that the incubation of cultured macrophages with EA significantly reduced the hyperoxia-induced accumulation of extracellular HMGB1 by inhibiting its translocation from the nucleus to the cytoplasm. The EA-induced inhibition of the release of nuclear HMGB1 occurred in parallel with a restoration of macrophage phagocytic and migration activities. Attenuation of hyperoxia-activated NF- κ B might be important in maintaining hypoacetylation of HMGB1, which is associated with inhibited HMGB1 release and restored macrophage functions under hyperoxic conditions.

EA Rescues Hyperoxia-Compromised Macrophage Functions

AMs are one of the initial and pivotal mediators of lung innate immunity (8, 47). The ability of AMs to migrate and to phagocytose micro-organisms is essential for the effective clearance of invading pathogens from the lungs (48). Using RAW 264.7 cells, a model for AMs, to study hyperoxia-compromised macrophage functions (10, 12, 15, 49) and AMs isolated from hyperoxic mice, we and others have shown that prolonged exposure to hyperoxia can impair their phagocytic activity and ability to migrate (10, 12, 50).

Figure 7. EA and BAY 11-7082 inhibit hyperoxia-induced acetylation of HMGB1. Representative spectra of the liquid chromatography mass spectrometric (LC-MS) characterization of peptides produced from HMGB1 derived from macrophage cell lysates (A) and supernatants (B) enzymatically cleaved with endopeptidase *Staphylococcus aureus* protease V8 (Gluc). Macrophages were exposed to 21 or 95% O₂ in the presence or absence of either EA or BAY 11-7082. The presence of the peptides with molecular weights 1,624 and 1,133 Da indicate the hypoacetylation of lysine residues within nuclear localization signal (NLS) 1 and 2, respectively ($n = 1$). The presence of the peptides with molecular weights 1,750 and 1,343 Da indicate the hyperacetylation of lysine residues within NLS 1 and 2, respectively. The experiment was performed once. (C) Proposed schematic overview of the mechanism of hyperoxia-induced HMGB1 acetylation and its inhibition by EA or BAY 11-7082.

The hyperoxia-induced dysfunction of AMs can impair the ability of the host defense to clear invading bacteria, thereby contributing to the development of VAP (10, 12, 51, 52). Furthermore, the bacteria that elicit VAP are typically resistant to antibiotics used in the treatment of VAP (53, 54). Therefore, novel treatments that can effectively improve macrophage functions under hyperoxic conditions may be beneficial for the treatment of patients with VAP. In this study, we found that EA significantly restored the ability of hyperoxia-compromised macrophages to phagocytose micro-organisms and to migrate (Figures 3 and 4) (55). In contrast to our observations, others have reported that EA has adverse effects on immune cell functions. For example, EA can inhibit the phagocytosis of either zymosan particles by polymorphonuclear leukocytes that were isolated from the peritoneal cavity of rabbits, or *Staphylococcus epidermidis* by AMs

isolated from rabbit lungs (56, 57). The discrepancies between our results and those from other laboratories may be due to differences in the concentrations of EA. In our study, EA was used at low micromolar concentrations, ranging from 1 to 8 μM, whereas, in other studies, EA was used at much higher concentrations (100–1,000 μM) (56, 57). Interestingly, we also found that EA, at 8 μM, failed to improve hyperoxia-compromised macrophage phagocytosis and migration. Together, these data suggest that EA, at low micromolar concentrations, attenuates impaired macrophage functions under hyperoxic conditions.

EA Inhibits HMGB1 Release from Hyperoxia-Exposed Macrophages

Our results indicate that EA significantly inhibits nuclear HMGB1 release from RAW 264.7 cells and also primary macrophages (BMDMs) exposed to hyperoxia (Figure 1). A critical step in the release of HMGB1 into

the extracellular milieu is its relocation from the nucleus into the cytoplasmic endolysosomes (23, 42, 58). Although HMGB1 was localized in the nucleus under normoxic conditions, it was evidently localized in the cytoplasm of many macrophages in the hyperoxic environment (Figure 2). The translocation of HMGB1 from the nucleus to the cytoplasm was significantly less pronounced in macrophages exposed to hyperoxia in the presence of EA (Figure 2).

Thus, EA can reduce HMGB1 release from hyperoxic macrophages, at least partly by affecting this cytoplasmic translocation. This finding is congruent with the inhibitory effects of EA on the release of HMGB1 in macrophages exposed to endotoxin (34). Therefore, EA can effectively inhibit the release of nuclear HMGB1 from cultured macrophages in response to both gram-negative bacterial infection and oxidative stress.

Figure 8. A hypothesized pathway indicating how NF- κ B may modulate macrophage functions via its effect on hyperoxia-induced HMGB1 release. Under normoxic conditions, macrophages can efficiently phagocytose invading pathogens. HMGB1 regularly shuttles between the nucleus and the cytoplasm, depending on its acetylation status. Whereas underacetylated HMGB1 is imported into the nucleus, hyperacetylation of HMGB1 promotes its export to the cytoplasm. Under normoxic conditions, HMGB1 is underacetylated. Thus, the nuclear import predominates the export, resulting in its prevalent localization in the nucleus. The prolonged exposure of macrophages to hyperoxia inhibits their phagocytic function and causes HMGB1 release into the extracellular milieu. Before its release, HMGB1 translocates from the nucleus to the cytoplasm as a result of its hyperacetylation upon prolonged exposure to hyperoxia. The exposure of macrophages to hyperoxia also leads to the translocation of NF- κ B into the nucleus, indicative of its activation. Nuclear NF- κ B has been shown to activate histone acetyltransferases (HATs) (72), which can hyperacetylate HMGB1, thereby favoring its translocation to the cytoplasm and, subsequently, its extracellular release. EA and BAY 11-7082 significantly inhibited the activation of NF- κ B, which further inhibited hyperoxia-induced release of acetylated HMGB1 and its translocation. It is likely that NF- κ B modulates macrophage functions by hyperacetylation of HMGB1, thereby inhibiting its release. *Solid arrows* represent known pathways; *dashed arrows* represent possible pathways.

EA and BAY 11-7082 Inhibit Hyperoxia-Induced HMGB1 Release by Blocking Hyperacetylation of HMGB1

HMGB1 regularly shuttles between the nucleus and the cytoplasm. Post-translational modifications, such as oxidation, methylation, acetylation, and phosphorylation, are reported to be important in this nucleocytoplasmic shuttling (59–61). Because the functions of HMGB1 rely on its cellular localization, these modifications can affect not only HMGB1 cellular localization, but also its functions (20). Recent findings suggest that acetylation of some lysine residues on HMGB1 regulates its active release from activated monocytes and macrophages (45, 62). In addition, oxidative stress can result in

the release of hyperacetylated HMGB1 from cells, including hepatocytes (63). Our results indicate that EA and BAY 11-7082 inhibited HMGB1 release under hyperoxic conditions by targeting the hyperacetylation of HMGB1 (Figures 7 and E2). Recently, it has been reported that the active release of HMGB1 by double-stranded RNA-dependent protein kinase and inflammasomes might involve pyroptosis, a programmed inflammatory cell death pathway (62). Therefore, hyperoxia-induced release of hyperacetylated HMGB1 might be via pyroptosis, because as no signs of death of these cells occurred under hyperoxic conditions, unless the cells were incubated with higher concentrations of EA or BAY 11-7082.

Hyperoxia-Induced HMGB1 Release Is Associated with NF- κ B Activation

NF- κ B, a redox-sensitive transcription factor, has been implicated in mediating the increased accumulation of proinflammatory cytokines in the airways under hyperoxic conditions (64). Results presented in this report indicate that NF- κ B activation in macrophages exposed to hyperoxia was accompanied by an increase in the levels of extracellular HMGB1 (Figures 1 and 5). NF- κ B activation has also been observed in the lungs of mice and pulmonary lymphocytes isolated from hyperoxia-exposed mice (64, 65). It has been reported that LPS-induced activation of NF- κ B plays a pivotal role in HMGB1 release from cultured macrophages (34). The inhibition of NF- κ B activation with an adenovirus-expressing I κ B α mutation can attenuate HMGB1 translocation in cultured cells, as well as in a cecal ligation and puncture-induced animal model of sepsis (66). In addition, inhibitors of IKK α and IKK β have been shown to inhibit HMGB1-induced chemotaxis (67). In this study, the association between the increased levels of extracellular HMGB1 and hyperoxia-induced NF- κ B activation suggests that NF- κ B plays a role in oxidative stress-induced HMGB1 release. This hypothesis is supported by results presented in Figures 1 and 5 showing that inhibition of hyperoxia-induced NF- κ B activation by EA was associated with an attenuation of HMGB1 release. The direct inhibitory effect of EA on NF- κ B activation may result from its inhibitory effects on multiple checkpoints in the NF- κ B activation pathway, which includes the degradation of I κ B α and I κ B β , phosphorylation of p65, NF- κ B translocation, DNA binding, and transcription (43, 68). Results shown in Figure 5 confirmed the involvement of NF- κ B translocation and phosphorylation of p65 in hyperoxia-induced NF- κ B activation. The correlation between hyperoxia-induced HMGB1 release and NF- κ B activation was further supported by the finding that BAY 11-7082, a well characterized inhibitor of NF- κ B, significantly inhibited HMGB1 release (Figure 6). The widespread activation of NF- κ B in different lung cells under hyperoxic conditions suggests that it may play a role in the pleiotropic regulation of HMGB1 release in the lungs (69–71). It has been reported that LPS-induced activation of NF- κ B plays

a pivotal role in HMGB1 release from cultured macrophages (34).

NF- κ B has been reported to activate histone acetyltransferases (72). Thus, hyperoxia-induced NF- κ B activation could lead to hyperacetylation of HMGB1, thereby promoting its translocation to the cytoplasm in a hyperacetylated form, and, subsequently, its extracellular release via pyroptosis (62). In cells incubated with LPS, NF- κ B activation is also associated with increased HMGB1 acetylation and pronounced localization of HMGB1 in the cytoplasm (45, 73). Thus, inhibition of NF- κ B by EA and BAY 11-7082 could effectively reduce HMGB1 hyperacetylation through preventing NF- κ B-induced histone acetyltransferase activation, subsequently resulting in reduced accumulation of extracellular HMGB1 in the airway and, eventually,

improved macrophage functions. Overall, our study demonstrates that EA and BAY 11-7082 significantly attenuate hyperoxia-induced HMGB1 release from macrophages, most likely by inhibiting its hyperacetylation by NF- κ B. The reduced levels of extracellular HMGB1 may play a critical role in improving macrophage functions that are compromised by hyperoxia. In addition, we show that the effects of EA and BAY 11-7082 on hyperoxia-induced HMGB1 release are associated with attenuated NF- κ B activation, suggesting that NF- κ B mediates HMGB1 release from macrophages under hyperoxic conditions. Figure 8 illustrates a hypothesized pathway indicating how NF- κ B may modulate macrophage functions via its effect on hyperoxia-induced HMGB1 release. Interestingly, a two-way,

randomized, cross-over study indicates that the average $C_{\max}$ of EA is approximately 6.1 μ g/ml after intravenous administration in human subjects over 15 minutes (74), which is approximately equivalent to 2 μ M of EA, the most effective concentration used in this study. Therefore, our results, provided that they can be translated to humans, suggest that EA could be used as part of the treatment regimen or a potential lead compound in treating patients with VAP. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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