Antiinflammatory Effects of Hydrogen Peroxide in Neutrophil Activation and Acute Lung Injury

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Rationale: Although reactive oxygen species (ROS) are generally considered to be proinflammatory and to contribute to cellular and organ dysfunction when present in excessive amounts, there is evidence that specific ROS, particularly hydrogen peroxide (H₂O₂), may have antiinflammatory properties.

Objectives: To address the role that increases in intracellular H₂O₂ may play in acute inflammatory processes, we examined the effects of catalase inhibition or the absence of catalase on LPS-induced inflammatory responses.

Methods: Neutrophils from control or acatalasemic mice, or control neutrophils incubated with the catalase inhibitor aminotriazole, were treated with LPS, and levels of reactive oxygen species, proteasomal activity, NF- κ B activation, and proinflammatory cytokine expression were measured. Acute lung injury (ALI) was produced by intratracheal injection of LPS into control, acatalasemic-, or aminotriazole-treated mice.

Measurements and Main Results: Intracellular levels of H₂O₂ were increased in acatalasemic neutrophils and in neutrophils exposed to aminotriazole. Compared with LPS-stimulated neutrophils from control mice, neutrophils from acatalasemic mice or neutrophils treated with aminotriazole demonstrated reduced 20S and 26S proteasomal activity, κ B- α degradation, NF- κ B nuclear accumulation, and production of the proinflammatory cytokines TNF- α and macrophage inhibitory protein (MIP)-2. The severity of LPS-induced ALI was less in acatalasemic mice and in mice treated with aminotriazole as compared with that found in control mice.

Conclusions: These results indicate that H₂O₂ has antiinflammatory effects on neutrophil activation and inflammatory processes, such as ALI, in which activated neutrophils play a major role.

Keywords: reactive oxygen species; neutrophils catalase; LPS; NF- κ B; acute lung injury

Reactive oxygen species (ROS) are generated during normal and disease-related metabolic processes and play an important role in modulating cell activation and function. Increased production of ROS is believed to be deleterious, contributing to accelerated aging and atherosclerosis and to the pathogenesis of cancer, diabetes mellitus, and neurodegenerative diseases (1–5). Although there are multiple different ROS, including superoxide, hydrogen peroxide (H₂O₂), and hydroxyl radical, because of rapid transition from one reactive oxygen intermediate to an-

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Reactive oxygen species are generally thought to have proinflammatory effects. However, recent studies suggest that different reactive oxygen intermediates may have distinct effects on cellular activation.

What This Study Adds to the Field

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other, most studies have assumed that increased generation of any ROS will result in cellular and organ dysfunction. However, recent data suggest that different ROS may have distinct effects on cellular activation. For example, whereas exposure to superoxide appears to increase the release of proinflammatory mediators, such as cytokines, by neutrophils and other cellular populations, elevated intracellular concentrations of H₂O₂ diminish LPS-induced neutrophil activation (6–9).

Antioxidants are involved in regulating the intracellular concentrations of ROS and are generally considered to have a beneficial role in diminishing oxidant stress and related cellular and tissue injury. Superoxide dismutase and catalase occupy linked functions in ROS elimination, with superoxide dismutase catalyzing the dismutation of superoxide into oxygen and H₂O₂ and catalase facilitating the decomposition of H₂O₂ to water and oxygen. A number of studies have shown a protective role for catalase in oxidant-induced cellular damage. For example, catalase overexpression prevents tissue injury in animals subjected to pathophysiologic situations associated with increased ROS generation, such as hypoxia-reoxygenation or doxorubicin-induced cardiotoxicity (10, 11). However, despite the apparent importance of catalase in modulating oxidant-induced cellular stress, mice or humans lacking active catalase are phenotypically normal (12), and some recent studies have shown that reduction in catalase activity is not necessarily associated with worse outcome after oxidant-induced stress. For example, acatalasemic mice are not more susceptible to hyperoxia-induced acute lung injury (ALI), and catalase overexpression failed to prevent allergic airways disease (13, 14).

Neutrophils benefit the host by participating in antimicrobial responses, but an excessive release of proinflammatory mediators by these cells can contribute to harmful inflammatory processes and organ dysfunction in settings, such as ALI, sepsis, or severe hemorrhage, in which activated neutrophils play a major role (15–17). Although activated neutrophils produce large amounts of ROS and are exposed to a pro-oxidant environment during acute inflammatory responses, there is only

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limited information concerning how ROS modulate neutrophil activity and neutrophil-dependent proinflammatory processes. Recently, we have shown that although increased levels of extracellular or intracellular superoxide have proinflammatory effects in neutrophils, resulting in enhanced nuclear translocation of nuclear factor (NF)- κ B and increased generation of NF- κ B-dependent cytokines, such as tumor necrosis factor (TNF)- α and macrophage inhibitory protein (MIP)-2, H₂O₂ appears to have antiinflammatory properties (6, 8, 9). In particular, incubation of neutrophils with H₂O₂ or H₂O₂-generating combinations of glucose and glucose oxidase resulted in diminished LPS-mediated NF- κ B activation and proinflammatory cytokine production through mechanisms that involve inhibition of proteasomal function and stabilization of cytoplasmic levels of the inhibitory protein I κ B- α (9). Such results suggest that increasing intracellular concentrations of H₂O₂ in neutrophils may be beneficial in pathophysiologic states associated with excessive neutrophil activation and accumulation, such as ALI.

To examine the hypothesis that enhanced intracellular concentrations of H₂O₂ have antiinflammatory properties *in vitro* and *in vivo*, we used acatalasemic mice as well as treatment of neutrophils and mice with the catalase inhibitor aminotriazole. The present studies demonstrate that the absence or blockade of catalase activity decreases LPS-induced neutrophil activation and the severity of ALI.

METHODS

Mice

Male C57BL/6, acatalasemic C3Ga.Cg-Cat b/J and control C3HeB/FeJ mice, 8 to 12 weeks of age, were purchased from Jackson Laboratory (Bar Harbor, ME). The mice were kept on a 12 hour/12 hour light-dark cycle with free access to food and water. All experiments were conducted in accordance with protocols approved by the institutional review board (Institutional Animal Care and Use Committee, UAB).

Reagents and Antibodies

Escherichia coli 0111:B4 endotoxin (LPS), H₂O₂, or aminotriazole (ATZ) were obtained from Sigma (St. Louis, MO). 2',7'-dichlorodihydrofluorescein (DCFH-DA) was purchased from Invitrogen (Carlsbad, CA). Cytokine ELISA kits were obtained from R&D Systems (Minneapolis, MN). MG132 and clasto-lactacystein β -lactone were purchased from Calbiochem (San Diego, CA). The antibodies to I κ B- α and phospho-Ser32/36 I κ B- α were purchased from Cell Signaling (Beverly, MA). Antirabbit horseradish peroxidase and anti-IKK γ were from Santa Cruz Biotechnology (Santa Cruz, CA), and antiactin antibody and pegylated catalase (PEG-CAT) were from Sigma. The Chromogenic LAL kit was obtained from Lonza (Walkersville, MD).

Neutrophil Isolation

Bone marrow neutrophils were isolated from the femurs and tibias of 8- to 12-week-old mice and purified by negative selection using primary antibodies specific for the cell surface markers F4/80, CD4, CD45R, CD5, and TER119 (StemCell Technologies, Vancouver, BC, Canada) as described previously (9). Neutrophil purity, as determined by Wright-Giemsa-stained cytospin preparations, was consistently greater than 97%. Neutrophils were cultured in RPMI 1640 medium containing FBS (0.5%) and treated as described in the figure legends. The percentage of cells that were viable and not apoptotic or necrotic, as determined by flow cytometry after staining with annexin V FITC and propidium iodide, was consistently greater than 95%.

Measurement of Catalase-like Activity

Catalase-like activity was determined by measuring the time-dependent decomposition of H₂O₂ by neutrophils treated with or without the catalase inhibitor aminotriazole (ATZ). Neutrophils (3.5×10^6) were incubated with ATZ (0 or 25 mM) in culture media (RPMI 1640, 0.5% FBS) for 90 minutes and treated with H₂O₂ (150 μ M) for 0, 5, 10, 20, and

30 minutes at 37°C, and the H₂O₂ concentration in the culture medium was determined using the xylenol orange assay (18). The rates of H₂O₂ decomposition by control or ATZ-treated neutrophils (3.5×10^6 /ml) were ~ 4.4 μ mol/min and 2.6 μ mol/min, respectively. Enzymatic activity of catalase was also measured in lung or liver homogenates as described previously (13, 19). Briefly, catalase-like activity was determined by incubation of lung or liver protein extracts in the presence of H₂O₂ (30 mM) in phosphate buffered saline (PBS) buffer for 5 minutes, and the time-dependent decrease in H₂O₂ concentration was monitored at 240 nm. The differences between initial rates of H₂O₂ decomposition were expressed as fold change as compared with control.

Imaging of DCF Fluorescence

Intracellular levels of ROS were determined using the redox sensitive probes DCFH-DA in conjunction with fluorescent microscopy (20, 21). Briefly, neutrophils (1.5×10^6 /well) in a four-well chambered cover glass (Nalge, Naperville, IL) were treated as indicated in the figure legends followed by incubation with DCFH-DA (20 μ M) for an additional 30 minutes, and images were acquired using a Leica DMIRBE inverted epifluorescence/Nomarski microscope outfitted with Leica TCS NT laser confocal optics (Leica Inc., Exton, PA) fluorescent confocal microscopy. The levels of fluorescence were averaged using SimplePCI software (Compix, Cranberry Township, PA). Images were processed using IPLab Spectrum and Adobe Photoshop (Adobe Systems, San Jose, CA) software.

Amplex Red Assay

The extracellular level of H₂O₂ in neutrophil cultures was determined using the Amplex Red/horseradish peroxidase method (22, 23). Briefly, bone marrow neutrophils (10^6 cells/well) were cultured with ATZ (0 or 25 mM) for 30 minutes in Ringer's lactate buffer followed by Amplex Red (100 μ M) and horseradish peroxidase (0.2 U/ml) inclusion for an additional 30 minutes at 37°C. Supernatants (50 μ L from each well) were transferred to 96-well plates, and fluorescence was measured spectrophotometrically (absorption: 544 nm/emission: 590 nm).

Purification of Nuclear Proteins and Western Blot Analysis

Nuclear proteins were purified from 7×10^6 neutrophils, whereas 3.5×10^6 cells were used to obtain whole cell lysates as previously described (9). The protein concentration of the supernatants was determined using Bradford reagent (BioRad, Hercules, CA) with bovine serum albumin as a standard. For Western blots, samples were mixed with Laemmli sample buffer and boiled for 5 minutes. Equal amounts of proteins were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride membrane (Immobilion P; Millipore, Billerica, MA). The membranes were probed with specific antibodies to I κ B- α , phospho-Ser 32/36 I κ B- α (Cell Signaling), or actin (Sigma), followed by detection with horseradish peroxidase-conjugated goat antirabbit IgG. Bands were visualized by enhanced chemiluminescence (SuperSignal; Pierce Biotechnology, Rockford, IL) and quantified by AlphaEaseFC Software (Alpha Innotech, San Leandro, CA). Each experiment was performed two or more times using cell populations obtained from separate groups of mice.

Measurement of Proteasome Activity

Cell lysates (100 μ L/sample) were obtained from neutrophil cultures (7.5×10^6 /well) using buffer containing 10 mM Tris (pH 7.5), 1 mM EDTA, 20% glycerol, 0.5% Triton X-100, and protease inhibitors (50 μ M PMSF, 50 μ M TPCK, 2 μ g/ml aprotinin, and 2 μ g/leupeptin). Cell extracts (15 μ g/100 μ L) were then incubated with ATP (1 mM) or SDS (0.6%) and the fluorogenic peptide substrates Suc-Leu-Leu-Val-Tyr-AMC (100 μ M) or Boc-Leu-Arg-Arg-AMC (100 μ M) to determine 26S or 20S proteasomal chymotrypsin-like or trypsin-like activity, respectively (24–27). Fluorescence was measured in a microtiter plate fluorometer (Biorad) at 2-minutes intervals over a 60-minute period at 37°C with an excitation filter of 380 nm and an emission filter of 460 nm. Proteasomal-independent activity was determined by performing the assay in the presence of the proteasome inhibitor MG132 (10 μ M). Proteasomal activity was determined using rate (fluorescence U/min) and standard AMC concentration curves, with calculated

Figure 1. Catalase inhibition by aminotriazole (ATZ) increases intracellular levels of hydrogen peroxide in bone marrow neutrophils. (A) Catalase-like activity was determined by measurement of neutrophil-time dependent H_2O_2 decomposition in the presence or absence of ATZ. Data are expressed as fold rate of H_2O_2 decomposition compared with control. (B) Neutrophils were incubated with or without ATZ (25 mM) for 30 minutes, and then 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (20 μ M) was included in the cultures for an additional 30 minutes. Representative confocal images are shown. Two additional experiments provided similar results. (C) DCF fluorescence was determined from randomly chosen fields from neutrophils treated as in B. (D) Amplex red fluorescence. All results are from three independent experiments. Means $\pm$ SEM are shown. $^{**}P < 0.01$.

values expressed as fold rates of control. The rates of chymotrypsin-like and trypsin-like activity (fluorescence U/min) were derived by subtracting the rates obtained in the presence of the proteasome inhibitors (MG132 or lactacystin) from the values obtained in their absence. Assays were performed at least in triplicate, and results are presented from three or more independent experiments using neutrophils purified from separate groups of mice ($n = 3-4$ in each group).

Electrophoretic Mobility Shift Assay

Nuclear extracts were obtained from bone marrow neutrophils, and electrophoretic mobility shift assays were performed as reported previously (28–30). In brief, the κ B DNA sequence of the Ig gene was used. Synthetic double-stranded sequences (with enhancer motifs underlined) were filled in and labeled with [32 P]dATP (GE Healthcare, Piscataway, NJ) using Sequenase DNA polymerase: B sequence 5'-GCCATG GGGGATCCCGAAGTCC-3' (Geneka Biotechnology).

Cytokine ELISA

ELISA were used to measure cytokines in bronchoalveolar lavage fluid (BALF) or in culture media from LPS-stimulated neutrophils. Levels of TNF- α , MIP-2, IL-6, or KC were determined using commercially available ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions and as previously described (8, 31).

Figure 2. Catalase inactivation by aminotriazole (ATZ) decreases LPS-induced nuclear factor (NF)- κ B nuclear accumulation and cytokine production by neutrophils. Neutrophils were treated with ATZ (0 or 25 mM) for 2.5 hours followed by stimulation with LPS (0 or 100 ng/ml) for 1 hour. Open bars represent -LPS; solid bars represent +LPS. (A) Representative EMSA showing effects of ATZ on LPS-induced nuclear accumulation of NF- κ B. (B) TNF- α mRNA expression in neutrophils incubated with ATZ (25 mM) or vehicle for 2.5 hours and then stimulated with LPS for 1 hour. (C and D) Neutrophils were exposed to ATZ (25 mM) for 2.5 hours followed by treatment with LPS (100 ng/ml) for 5 hours and then tumor necrosis factor (TNF)- α (C), and macrophage inhibitory protein (MIP)-2 (D) protein concentrations in the culture media were determined using ELISA. $n = 3$ independent experiments. Means $\pm$ SD are shown. $^{**}P < 0.01$ or $^{***}P < 0.001$ compared with cells treated with LPS alone.

Measurement of IKK β Kinase Activity

IKK β activity in neutrophils was determined using a direct kinase assay as previously described (32). Briefly, IKK β was immunoprecipitated from cellular extracts (300 mg protein per sample) using anti-IKK γ antibodies followed by determination of *in vitro* phosphorylation of recombinant GST-I κ B- α . The reaction was performed in standard kinase buffer (Cell Signaling) containing ATP (10 μ M), GST-I κ B- α (200 ng/sample), and [32 P]ATP (5 μ Ci) (Amersham, Piscataway, NJ) for 30 minutes at 30°C. The samples were then subjected to SDS-PAGE, and gels were exposed to radiographic film for 24 hours.

In Vivo ALI Model

To induce ALI, *E. coli* 0111:B4 LPS (1 mg/kg) in PBS was administered into the oropharynx (33). Briefly, mice were anesthetized with isoflurane, the tongue was gently extended, and LPS in PBS (50 μ l) was instilled into the distal part of the oropharynx. ATZ (500 mg/kg) in saline or saline alone was given intraperitoneally 2 hours before the

Figure 3. Effects of catalase inactivation by ATZ on LPS-induced IκB-α degradation. Neutrophils were incubated with ATZ (0 or 25 mM) for 2.5 hours and then stimulated with LPS (100 ng/ml) for the indicated time periods. Open bars represent -LPS; solid bars represent +LPS. (A) Effects of ATZ on LPS-induced IκB-α degradation. Cell extracts were subjected to SDS-PAGE and Western blot analysis using IκB-α or actin-specific antibodies. A representative Western blot and densitometry data from three independent experiments are shown. Means ± SD are shown. **P* < 0.05 or ***P* < 0.01. (B) 26S proteasomal chymotrypsin-like and trypsin-like activity in cell extracts obtained from neutrophils treated or not treated with ATZ (25 mM) for 2.5 hours. Results are shown from three independent experiments. Means ± SD are shown. **P* < 0.01 compared with untreated cells.

administration of LPS (1 mg/ml) to C57BL/6 mice. This dose of ATZ was previously used in murine models examining the efficacy or toxicity of ATZ (34, 35). In additional experiments, control C3HeB/FeJ or acatalasemic C3Ga.Cg-Cat b/J mice were given LPS (1 mg/kg

intratracheally). There were no deaths associated with ATZ or LPS administration.

Harvest of Lungs and BAL

Lungs were harvested 24 hours after LPS administration. BAL was obtained by cannulating the trachea with a blunt 20-gauge needle and then lavaging the lungs three times with 1 ml of iced PBS.

Wet-to-Dry Lung Weight Ratios

The wet-to-dry ratio was determined as reported previously (28, 36). All mice used for lung wet-to-dry weight ratios were of identical ages. Lungs were excised, rinsed briefly in PBS, blotted, and weighed to obtain the “wet” weight. Lungs were then dried in an oven at 80°C for 7 days to obtain the “dry” weight.

Myeloperoxidase Assay

Myeloperoxidase (MPO) activity was assayed as reported previously with minor modifications (28, 37). In brief, lung tissue was homogenized in 1 ml of potassium phosphate buffer (50 mM; pH 6.0) containing a reducing agent (N-ethylmaleimide; 10 mM) for 30 seconds on ice. The homogenate was centrifuged (12,000 × *g*) for 30 minutes at 4°C and washed twice in ice-cold buffer. The pellet was resuspended and sonicated on ice for 90 seconds in 10× volume of hexadecyltrimethylammonium bromide (HTAB) buffer (HTAB 0.5%) and potassium phosphate (50 mM; pH 6.0). Samples were incubated in a water bath for 2 hours at 56°C and centrifuged (12,000 × *g*) for 10 minutes. The supernatant was collected for assay of MPO activity as determined by measuring the H₂O₂-dependent oxidation of 3,3'-dimethoxybenzidine dihydrochloride (λ = 460 nm).

Statistical Analyses

For each experiment, neutrophils were isolated and pooled from groups of mice (*n* = 3–6), and all conditions were studied at the same time. One way ANOVA, the Tukey-Kramer Multiple Comparisons test (for multiple groups), or Student's *t* test (for comparisons between two groups) were used. A *P* value of < 0.05 was considered to be statistically significant.

RESULTS

Catalase Inhibition Increases Intracellular Levels of H₂O₂ in Neutrophils

Previous studies have shown that LPS-mediated activation of neutrophils is diminished after exposure to exogenous H₂O₂ or the H₂O₂-generating combination of glucose and glucose oxidase (8, 9). However, the role of intracellular enzymes involved in H₂O₂ decomposition and, in particular, of catalase in the regulation of neutrophil responses to proinflammatory stimuli has not been described. To address this question, we determined whether catalase inhibition with aminotriazole (3-amino-1,2,4-triazole, ATZ) alters LPS-induced neutrophil activation.

Culture of bone marrow neutrophils with ATZ decreased the rate of H₂O₂ decomposition by approximately 50% (Figure 1A). These results are consistent with catalase being a major—but not the sole—enzyme involved in the removal of H₂O₂ (38).

Figure 4. Increased levels of H₂O₂ in neutrophils isolated from acatalasemic mice. (A) Catalase-like activity in neutrophils isolated from control C3HeB/FeJ or acatalasemic C3Ga.Cg-Cat b/J mice; *n* = 3 independent groups of neutrophils. Means ± SEM are shown. ***P* < 0.01 comparing control with acatalasemic mice. (B) Representative confocal images of DCF fluorescence and (C) mean DCF fluorescence in neutrophils isolated from control or acatalasemic mice. All results are from three independent experiments. Means ± SD are shown. ***P* < 0.01.

Figure 5. LPS-induced cytokine production in acatalasemic and control neutrophils. Open bars represent $-LPS$; solid bars represent $+LPS$. (A) Neutrophils were preincubated with pegylated catalase (PEG-CAT; 0 or 125 U/ml) for 1.5 hours and then stimulated with LPS (0 or 100 ng/ml) for an additional hour. Tumor necrosis factor (TNF)- α mRNA levels were determined using reverse transcriptase polymerase chain reaction; $n = 4$ independent groups of neutrophils. Means $\pm$ SEM are shown. *** $P < 0.001$ comparing untreated control neutrophils with LPS-treated neutrophils. * $P < 0.05$ comparing LPS-treated control neutrophils with LPS-treated acatalasemic neutrophils and comparing PEG-CAT/LPS with LPS-treated acatalasemic neutrophils. (B) TNF- α mRNA levels in LPS-stimulated acatalasemic neutrophils preincubated with ATZ (0 or 25 mM) for 2.5 hours; $n = 3$ independent groups of neutrophils. Means $\pm$ SEM are shown. NS = not significant. (C and D) Neutrophils were stimulated with LPS (0 or 100 ng/ml) for 5 hours, and concentrations of TNF- α and macrophage inhibitory protein (MIP)-2 in the culture media were measured using ELISA. PEG-CAT (0 or 125 U/ml) was included in the cultures for 1.5 hours before the addition of LPS; $n = 4$ independent groups of neutrophils; values are means $\pm$ SD. * $P < 0.05$ comparing LPS-treated control neutrophils to LPS-treated acatalasemic neutrophils or LPS-treated acatalasemic neutrophils to LPS- and PEG-CAT-treated acatalasemic neutrophils.

Because catalase facilitates the decomposition of H_2O_2 to H_2O and O_2 , catalase inactivation should result in increased intracellular H_2O_2 concentrations. DCF fluorescence increased by approximately twofold in neutrophils treated with ATZ (Figures 1B and 1C). The DCFH-DA probe is not a specific indicator of H_2O_2 because it is oxidized by reactive nitrogen species and hydroxyl radical (39, 40). Because ATZ inhibits catalase but not other enzymes involved in H_2O_2 removal, the observed increase in DCF fluorescence in ATZ-treated neutrophils is likely to reflect elevated intracellular H_2O_2 concentrations. However, to confirm that ATZ does indeed increase H_2O_2 levels in neutrophils, we used the more specific Amplex Red/horseradish peroxidase assay (22, 23). ATZ resulted in significantly increased activity with this assay (Figure 1D). ATZ did not induce neutrophil death, either apoptotic or necrotic, as cell viability was

consistently greater than 95% and similar in control and ATZ-treated cultures as determined by annexin V FITC and propidium iodide staining (see Figure E1 in the online supplement). Culturing neutrophils with LPS did not alter DCF or Amplex Red fluorescence (data not shown).

Catalase Inactivation Decreases LPS-induced I κ B- α Degradation, NF- κ B Nuclear Accumulation, and Cytokine Production by Neutrophils

We recently found that exposure of neutrophils to H_2O_2 decreased LPS-induced nuclear translocation of NF- κ B, degradation of I κ B- α , and 26S proteasomal activity (9). To determine if the alterations in intracellular H_2O_2 concentrations achieved by inhibition of catalase could produce similar effects, we examined such NF- κ B-related signaling events in ATZ-treated neutrophils.

Inclusion of ATZ in neutrophil cultures inhibited LPS-induced nuclear translocation of NF- κ B as well as mRNA and protein expression of the NF- κ B-dependent cytokines TNF- α and MIP-2 (Figure 2). The time-dependent decrease in I κ B- α levels normally found after neutrophil stimulation with LPS was also inhibited in cells treated with ATZ (Figure 3A). There was a significant decrease in chymotrypsin-like, but not in trypsin-like, 26S proteasomal activity in ATZ-treated as compared with control neutrophils (Figure 3B).

NF- κ B Activation Is Diminished in LPS-stimulated Acatalasemic Neutrophils

Although ATZ blocks H_2O_2 decomposition due to the inactivation of catalase, ATZ can also affect heme synthesis and heme-containing proteins (41, 42), events that may influence intracellular oxidant balance. Therefore, we used neutrophils from acatalasemic mice to examine more specifically the role of catalase in modulating cellular activation. The loss of catalase activity due to a single-point mutation in its gene has previously been shown to decrease H_2O_2 decomposition in several tissues (43), but such effects on intracellular H_2O_2 concentrations have not been previously reported in acatalasemic neutrophils.

As expected, catalase-like activity was significantly decreased in neutrophils obtained from acatalasemic mice as compared with control mice (Figure 4A). Similar to results in ATZ-treated neutrophils, total ROS levels were increased in acatalasemic as compared with control neutrophils (Figures 4B and 4C). LPS-induced expression of TNF- α and MIP-2 was significantly decreased in acatalasemic neutrophils (Figures 5A, 5C, and 5D).

PEG-CAT, unlike native catalase, has been shown to accumulate in many types of cells and effectively diminishes the effects mediated by increased levels of intracellular H_2O_2 (44, 45). Pretreatment of acatalasemic neutrophils with PEG-CAT restored the levels of LPS-induced TNF- α mRNA expression (Figure 5A) and the production of TNF- α and MIP-2 (Figures 5C and 5D). Because the concentrations of endotoxin in the PEG-CAT were less than 0.1 EU/100 U, the ability of PEG-CAT to enhance cytokine expression is not due to further addition of LPS to the neutrophil cultures. Such results indicate that the absence of intracellular catalase is responsible for the alterations in LPS-induced responses found in acatalasemic neutrophils. Pretreatment with ATZ did not alter TNF- α expression in LPS-stimulated acatalasemic neutrophils (Figure 5B), showing that the ATZ-dependent inhibition of neutrophil activation (see Figures 2 and 3) was specifically due to catalase inactivation and concomitant increases in intracellular H_2O_2 levels rather than to other effects of ATZ.

Consistent with the diminished expression of TNF- α and MIP-2 in LPS-stimulated acatalasemic neutrophils, nuclear

Figure 6. Nuclear factor (NF)-κB activation, IκB-α degradation, and proteasomal activation is diminished in LPS-stimulated acatalasemic neutrophils. (A) Representative electrophoretic mobility shift assay obtained using nuclear fractions from LPS-stimulated control or acatalasemic neutrophils. Two additional experiments provided similar results. (B) Control or acatalasemic neutrophils were stimulated with LPS (100 ng/ml) for 60 minutes, and levels of IκB-α were determined using Western blot analysis. Representative Western blots are shown. A second experiment provided similar results. (C and D) Effects of pegylated catalase (PEG-CAT) on LPS-induced IκB-α degradation in acatalasemic neutrophils. Control or acatalasemic neutrophils were cultured with LPS (0 or 100 ng/ml) for 1 hour. PEG-CAT was included in the cultures of acatalasemic neutrophils for 1.5 hours before the addition of LPS. A representative Western blot and densitometry data from three independent experiments are shown. Means ± SD are shown. **P* < 0.05 comparing control with acatalasemic neutrophils. (E and F) 26S and 20S proteasomal chymotrypsin-like and trypsin-like activities in LPS-stimulated control or acatalasemic neutrophils; *n* = 4 independent experiments. Means ± SD are shown. ****P* < 0.001 or **P* < 0.05 comparing control with acatalasemic neutrophils.

translocation of NF-κB after LPS stimulation was also decreased as compared with control neutrophils (Figure 6A). LPS-induced degradation of IκB-α was less pronounced in acatalasemic neutrophils (Figure 6B), similar to results found after ATZ treatment (*see* Figure 3A), and was partially restored by the inclusion of PEG-CAT in the cultures (Figures 6C and 6D). Decreased 26S and 20S proteasomal chymotrypsin-like, but not trypsin-like activity, was present in LPS-stimulated neutrophils from acatalasemic mice as compared with that present in control neutrophils (Figures 6E and 6F). Acatalasemic and control neutrophils had similar IKKβ activity after LPS exposure (Figure E2A). LPS-induced phosphorylation of IκB-α was similar or even slightly increased at early time points in acatalasemic as compared with control neutrophils (Figure E2B).

Catalase Inhibition Diminishes LPS-induced ALI

Activation of NF-κB and enhanced production of NF-κB-dependent proinflammatory mediators contribute to the development of ALI, a pathophysiologic process in which neutrophils play a major role (17, 46–48). Because catalase inhibition or the absence of active catalase diminished LPS-induced nuclear translocation of NF-κB and expression of TNF-α and MIP-2 in neutrophils, we hypothesized that such therapy could reduce the severity of lung injury.

A significant decrease (~50%) in catalase-like activity was detected in lung and liver homogenates obtained from mice treated with ATZ (Figure 7A). ATZ treatment resulted in reduced severity of LPS-induced lung injury, as determined by

wet-to-dry ratios, MPO activity in lung homogenates, and total cell and neutrophil numbers in BALF (Figures 7B and 7C). The levels of TNF-α, MIP-2, IL-6, and KC in BALF from LPS-treated mice given ATZ were significantly decreased compared with those present in control animals that received LPS alone (Figure 7D). ATZ alone had no significant effects on pulmonary neutrophil accumulation or lung cytokine levels as compared with the values present in saline treated mice (Figures 7C and 7D).

Acatalasemic Mice Are Resistant to LPS-induced ALI

A significant decrease (~60%) in catalase-like activity was detected in lung homogenates obtained from acatalasemic mice (Figure 8A). Similarly, decreased catalase-like activity was present in liver homogenates from acatalasemic as compared with control mice (Figure 8A).

The severity of LPS-induced lung injury, including the development of interstitial edema (as measured by wet-to-dry ratio) and neutrophil accumulation into the pulmonary interstitium and airspaces, was significantly reduced in acatalasemic as compared with control mice (Figure 8B and 8C). Levels of proinflammatory cytokines and chemokines, specifically TNF-α, KC, MIP-2, and IL-6, were also significantly decreased in the lungs of LPS-treated acatalasemic mice (Figure 8D).

To examine the role that enhanced intracellular catalase activity might have on LPS-mediated ALI, mice were pretreated with PEG-CAT in saline or saline before being exposed to LPS. PEG-CAT alone had no effects on measures of lung injury or pulmonary cytokine levels. In contrast, administration

Figure 7. Effects of catalase inhibition by aminotriazole (ATZ) on LPS-induced acute lung injury. *Open bars* represent control mice; *solid bars* represent acatalasemic mice. (A) Catalase-like activity in liver and lung homogenates obtained from mice treated with saline or ATZ (500 mg/kg intraperitoneally) 4 hours before organ collection; $n =$ three mice in each group. Means $\pm$ SEM are shown. *** $P < 0.01$ compared with saline-treated mice. (B and C) Mice were given ATZ (500 mg/kg) in saline or saline alone intraperitoneally then 2 hours later received LPS 1 mg/kg intratracheally. Lungs were harvested, and bronchoalveolar lavage fluid (BALF) was obtained 24 hours after LPS administration. The fold increase in lung wet-to-dry ratios was compared with that in mice given saline alone. Lung homogenate myeloperoxidase (MPO) concentrations and total cell as well as neutrophil numbers in BALF are shown. (D) Cytokine concentrations in BALF; $n = 6$ mice for each condition. Means $\pm$ SEM are shown. * $P < 0.05$ or ** $P < 0.01$ comparing LPS-treated mice given saline alone with mice given ATZ.

of PEG-CAT before LPS resulted in increased wet to dry ratios, accumulation of neutrophils into the pulmonary parenchyma (MPO activity) and BALF, as well as increased pulmonary TNF- α and MIP-2 levels as compared with values found in control, LPS-exposed mice (Figure 9).

DISCUSSION

ROS have generally been considered to be proinflammatory and to contribute to organ dysfunction. A putative mechanism for such effects is ROS-induced activation of transcriptional factors, particularly NF- κ B, with resultant enhanced production of cytokines and other proinflammatory mediators. However, despite studies showing that oxidants can enhance NF- κ B activation in cell lines and primary cell populations, this has not been a universally observed effect (50–52). For example, although H₂O₂ can enhance nuclear translocation of NF- κ B in some cell populations, particularly those that are macrophage derived (53, 54), in other cell types, H₂O₂ has been found to inhibit inflammatory responses (55–57). In particular, exposure of neutrophils to extracellular H₂O₂ diminished LPS-induced

activation of NF- κ B and expression of NF- κ B-dependent proinflammatory cytokines (8, 9). Such findings suggested that interventions that increased intracellular concentrations of H₂O₂ might be beneficial in neutrophil-driven proinflammatory conditions, such as ALI.

The present experiments are consistent with an antiinflammatory role for increased intracellular concentrations of H₂O₂. In these studies, inactivation or absence of catalase was associated with increased intracellular H₂O₂ and resulted in inhibition of activation of the 20S and 26S proteasome, degradation of I κ B- α , nuclear translocation of NF- κ B, and expression of NF- κ B-dependent cytokines in LPS-stimulated neutrophils. In addition, the severity of LPS-induced ALI was decreased in mice that were acatalasemic or in which catalase had been inhibited with aminotriazole and was increased in mice treated with PEG-CAT.

There are a number of intracellular antioxidant enzymes involved in facilitating the decomposition of H₂O₂ to H₂O and O₂, with catalase appearing to occupy a major role in this process among most cell populations that have been examined (12, 13, 58). However, there has been only limited information concerning the role of catalase in modulating intracellular levels of H₂O₂ in neutrophils. In the present studies, increased H₂O₂

Figure 8. Acatalasemic mice are less susceptible to LPS-induced acute lung injury. (A) Catalase-like activity as determined in lung or liver homogenates from control C3HeB/FeJ and acatalasemic C3Ga.Cg-Cat b/J mice; $n = 3$ mice in each group. Means $\pm$ SEM are shown. *** $P < 0.001$. (B) Change in lung wet-to-dry ratios as well as (C) myeloperoxidase (MPO) content in lung homogenates and total cell and neutrophil numbers in bronchoalveolar lavage fluid (BALF) 24 hours after intratracheal LPS administration; $n = 6$ mice in each experimental group. Means $\pm$ SEM are shown. * $P < 0.05$ comparing LPS-treated control mice with LPS-treated acatalasemic mice. (D) Tumor necrosis factor (TNF)- α , macrophage inhibitory protein (MIP)-2, IL-6, and keratinocyte-derived chemokine (KC) concentrations in BALF 24 hours after LPS administration; $n = 6$ mice in each experimental group. Means $\pm$ SEM are shown. * $P < 0.05$ compared with LPS-treated control mice.

levels were found in LPS stimulated acatalasemic neutrophils and in neutrophils in which catalase was inhibited with aminotriazole. These results indicate that catalase plays a significant role in the removal of endogenously produced H₂O₂ in neutrophils. However, other antioxidant enzymes, in addition to catalase, can modulate intracellular levels of H₂O₂ and potentially alter neutrophil activation through H₂O₂-dependent mechanisms. For example, the inhibitory effects of H₂O₂ on LPS-stimulated NF- κ B activation was recently demonstrated in primary tracheal epithelial cells isolated from glutaredoxin 1 (Grx1)-deficient mice (55).

In the present experiments and in previous studies (9), increases in intracellular H₂O₂ were associated with decreased 26S and 20S proteasomal activity. Because degradation of I κ B- α after TLR4 induced cellular activation occurs through mechanisms involving the 26S proteasome, such inhibitory effects of H₂O₂ on proteasomal function would be expected to result in increased cytoplasmic levels of I κ B- α that would inhibit nuclear translocation of NF- κ B through continued association between the two molecules. Previous studies have shown that treatment of resting or LPS-stimulated neutrophils with H₂O₂ or the H₂O₂ generating combination of glucose and glucose oxidase is associated with elevations in cytoplasmic concentrations of I κ B- α (9). The present experiments also found that the inactivation or absence of catalase resulted in decreased LPS-induced degrada-

tion of I κ B- α , despite no change in IKK β activity, consistent with the antiinflammatory actions of increased intracellular H₂O₂ being due to effects on 26S proteasomal activity. Nevertheless, it is possible that elevated concentrations of H₂O₂ may inhibit NF- κ B activation through other mechanisms, such as by reducing ubiquitination of I κ B- α , an event that precedes proteasomal degradation of I κ B- α (59), or through modulating NF- κ B phosphorylation or association with transcriptional coactivators, such as CBP or p300.

These studies used acatalasemic mice and treatment with aminotriazole as two different approaches for exploring the role of catalase in modulating intracellular H₂O₂ levels and neutrophil-associated inflammatory responses. Both approaches showed similar benefits of catalase inhibition or absence in diminishing LPS-induced neutrophil activation and the severity of ALI. In addition, enhanced intracellular catalase activity, achieved by administration of PEG-CAT, resulted in greater severity of ALI, consistent with decreased intracellular H₂O₂ being a proinflammatory event.

Although acatalasemic mice lack active catalase, the chronic nature of this cellular alteration likely results in the development of compensatory mechanisms that may be protective from oxidant-induced stress. To address this issue by transiently blocking catalase activity, we used treatment with aminotriazole, a potent and irreversible inhibitor of catalase that has been

Figure 9. Effects of pegylated catalase (PEG-CAT) administration on LPS-induced lung injury. PEG-CAT (0 or 15,000 U/kg) in saline was administered intraperitoneally to mice 4 hours before LPS (1 mg/kg) in phosphate buffered saline (PBS) or PBS alone intratracheally. Lung homogenates and bronchoalveolar lavage fluid (BALF) were collected 24 hours after LPS injection. (A) Wet-to-dry ratios and myeloperoxidase (MPO) activity in lung homogenates. (B) Number of total white cells and neutrophils in BALF. (C and D) levels of tumor necrosis factor (TNF)- α and macrophage inhibitory protein (MIP)-2 in whole lung homogenates or BALF; $n = 4$ to 6 mice in each group. Means $\pm$ SEM are shown. * $P < 0.05$ compared with LPS and LPS/PEG-CAT-treated mice.

shown to increase intracellular H_2O_2 levels *in vitro* and *in vivo* (34). Although the effects of aminotriazole on H_2O_2 decomposition appear to be selective for catalase and not other antioxidant enzymes, such as peroxyreductases, aminotriazole can affect heme synthesis and heme-containing proteins and may indirectly affect intracellular H_2O_2 levels through catalase-independent mechanisms (41, 42). However, because the effects of aminotriazole on intracellular H_2O_2 levels, proteasome activity, degradation of $I\kappa B$ - α , activation of NF- κB , and production of proinflammatory cytokines in LPS-treated neutrophils were similar to those found in LPS-stimulated acatalasemic neutrophils, it appears likely that such actions of aminotriazole in neutrophils are due to its inhibitory effects on catalase rather than interactions with other intracellular enzymes. In addition, the protective effects of aminotriazole in reducing the severity of LPS-induced pulmonary inflammation suggest that inhibition of catalase may be useful as a therapeutic approach for ALI and other inflammatory processes in which neutrophils play a major role.

Although preclinical studies have shown that antioxidants can diminish the severity of ALI, such findings were primarily obtained using transgenic mice overexpressing antioxidants or as a pretreatment (49, 60–62). Clinical trials with antioxidants for acute illnesses, such as ALI or sepsis, have been disappointing, and no agent has been shown to be effective in critically ill patients (63–68).

The present results provide a potential mechanism for the lack of efficacy of nonspecific antioxidant therapy in clinical trials enrolling patients with ALI, sepsis-induced organ dysfunction, and other acute inflammatory conditions in which neutrophils play a major role. Because H_2O_2 appears to be antiinflammatory in neutrophils, the administration of nonspecific antioxidants, even though they may reduce concentrations of some proinflammatory ROS, such as superoxide, may also induce enhanced neutrophil activation and neutrophil-induced tissue damage by decreasing intracellular concentrations of H_2O_2 . More targeted antioxidants that increase intracellular concentrations of H_2O_2 , either by facilitating the dismutation of superoxide to H_2O_2 or diminishing the decomposition of H_2O_2 to H_2O and O_2 , would be

expected to be more beneficial than nonspecific agents. The present results, showing that inactivation or absence of catalase is antiinflammatory in neutrophils and reduces the severity of ALI, support this hypothesis. Similarly, the efficacy of EC-SOD and EC-SOD mimetics, which facilitate the transition of superoxide to H_2O_2 , in reducing the severity of ALI in experimental models (69, 70), also is consistent with benefit being associated with antioxidant approaches that specifically result in increases in intracellular H_2O_2 concentrations.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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