

# Antioxidant Treatment Ameliorates Respiratory Syncytial Virus–induced Disease and Lung Inflammation

Shawn Monique Castro, Antonieta Guerrero-Plata, Giovanni Suarez-Real, Patrick A. Adegboyega, Giuseppe N. Colasurdo, Amir M. Khan, Roberto P. Garofalo, and Antonella Casola

Departments of Pediatrics, Pharmacology and Toxicology, Pathology, Microbiology and Immunology, and Sealy Center for Vaccine Development, University of Texas Medical Branch, Galveston; and Department of Pediatrics, University of Texas Health Science Center, Houston, Texas

**Rationale:** Respiratory syncytial virus (RSV) is a major cause of lower respiratory tract infection in children. No treatment has been shown to significantly improve the clinical outcome of patients with this infection. Recent evidence suggests that oxidative stress could play an important role in the pathogenesis of acute and chronic lung inflammatory diseases. We do not know whether RSV induces pulmonary oxidative stress and whether antioxidant treatment can modulate RSV-induced lung disease.

**Objectives:** To investigate the effect of antioxidant administration on RSV-induced lung inflammation, clinical disease, and airway hyperreactivity (AHR).

**Methods:** BALB/c mice were infected with  $10^7$  plaque-forming units of RSV, in the presence or absence of orally administered butylated hydroxyanisole (BHA), an antioxidant. Malondialdehyde and 4-hydroxynonenal were measured in bronchoalveolar lavage (BAL) by colorimetric assay. Cytokines and chemokines were measured in BAL by Bio-Plex and leukotrienes were measured by enzyme-linked immunosorbent assay. AHR to methacholine challenge was measured by whole-body plethysmography.

**Results:** BHA treatment significantly attenuated RSV-induced lung oxidative stress, as indicated by the decrease of malondialdehyde and 4-hydroxynonenal content in BAL of RSV-infected mice. RSV-induced clinical illness and body weight loss were also reduced by BHA treatment, which inhibited neutrophil recruitment to the lung and significantly reduced pulmonary cytokine and chemokine production after RSV infection. Similarly, antioxidant treatment attenuated RSV-induced AHR.

**Conclusion:** Modulation of oxidative stress represents a potential novel pharmacologic approach to ameliorate RSV-induced acute lung inflammation and potentially prevent long-term consequences associated with RSV infection, such as bronchial asthma.

**Keywords:** antioxidant; chemokines; lung inflammation; oxidative stress; respiratory syncytial virus

Respiratory syncytial virus (RSV) is a major cause of respiratory tract infection in children worldwide and is the leading cause of virally induced bronchiolitis (1). Each year, approximately 100,000 children are hospitalized with RSV disease, with an estimated annual cost close to \$300 million in the United States alone (2, 3). There is no safe and efficacious vaccine for RSV, and immunity is incomplete, resulting in repeat attacks of acute

## AT A GLANCE COMMENTARY

### Scientific Knowledge on the Subject

Oxidative stress has been shown to play an important role in the pathogenesis of acute and chronic lung inflammatory diseases, such as asthma, lung fibrosis, and chronic obstructive pulmonary disease.

### What This Study Adds to the Field

Respiratory syncytial virus induces oxidative stress *in vivo*. Antioxidant administration significantly reduces lung pulmonary inflammation and ameliorates clinical disease due to respiratory syncytial virus.

illness throughout adulthood. The long-term consequences of RSV infection, which include increased airway resistance and recurrent wheezing (1), are associated risk factors for the development of asthma (4). Although the mechanisms of RSV-induced airway disease and the associated long-term consequences have yet to be clearly defined, the local inflammatory response is believed to play a fundamental role. Airway epithelial cells are the target of RSV infection, and they respond to the infection by producing a variety of mediators involved in lung immune/inflammatory responses, like cytokines, chemokines, and interferons, and by up-regulating adhesion molecules and major histocompatibility complex antigens on the cell surface (reviewed in Reference 5).

Reactive oxygen species (ROS) are highly reactive molecules implicated in cellular damage. In the past few years, there has been increased recognition of their role as redox regulators of cellular signaling (reviewed in References 6 and 7). We have previously shown that RSV-infected airway epithelial cells generate ROS and that antioxidant treatment with butylated hydroxyanisole (BHA), as well as a panel of chemically unrelated antioxidants, blocks RSV-induced signal transduction cascades, leading to chemokine expression *in vitro* through inhibition of transcription factors belonging to interferon regulatory factor (IRF) and signal transducers and activators of transcription (STAT) families (8, 9). Recent studies have indicated, directly or indirectly, an important role of ROS produced by epithelial and inflammatory cells in the pathogenesis of acute and chronic lung inflammatory diseases, such as acute respiratory distress, asthma, and chronic obstructive pulmonary disease (reviewed in References 10–12). Surprisingly, little is known regarding the oxidative stress response in patients with virally induced lung inflammation. In an animal model of influenza infection, inhibition of oxygen radicals through administration of antioxidants or increased lung superoxide dismutase levels significantly reduced lung injury and improved the survival rate of infected animals,

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Correspondence and requests for reprints should be addressed to Antonella Casola, M.D., Department of Pediatrics, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555-0366. E-mail: ancasola@utmb.edu

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suggesting that oxidative stress can play a significant role in the pathogenesis of virally induced pneumonia (13–17). We have previously shown that RSV induces reactive nitrogen species and nitric oxide synthase (NOS) in the lungs of infected mice, and that inhibition of NOS expression significantly reduces RSV-induced lung inflammation (18). However, it is not known whether RSV infection induces significant lung oxidative stress damage and whether inhibiting ROS production by antioxidants modifies RSV-induced lung disease. Therefore, the aim of this study was to investigate the effect of antioxidant administration on RSV-induced lung inflammation, clinical disease, and airway hyperreactivity (AHR).

## METHODS

### RSV Preparation

The RSV A2 strain was grown in Hep-2 cells and purified by centrifugation on discontinuous sucrose gradients as described elsewhere (19). The virus titer of the purified RSV pools was  $8$  to  $9 \log_{10}$  plaque-forming units (PFU) per milliliter using a methylcellulose plaque assay. No contaminating cytokines were found in these sucrose-purified viral preparations (20). LPS, assayed using the limulus hemocyanin agglutination assay, was not detected. Virus pools were aliquoted, quick-frozen on dry ice/alcohol, and stored at  $-70^{\circ}\text{C}$  until used. Sucrose-purified extracts from uninfected Hep-2 cells were also generated under the same conditions.

### Inoculation Procedure and Mice

Female BALB/c mice were purchased from Harlan (Houston, TX) and were housed in pathogen-free conditions in the animal research facility of the University Texas Medical Branch (UTMB), Galveston, Texas, in accordance with the National Institutes of Health and UTMB institutional guidelines for animal care. Under light anesthesia, mice were inoculated intranasally with either sucrose-purified Hep-2 extracts (sham-infected) or sucrose-purified RSV at  $1 \times 10^7$  PFU, diluted in sterile phosphate-buffered saline (PBS) for a total inoculation volume of  $50 \mu\text{l}$ , as previously described (21). BHA was prepared by dissolving the right amount of compound in a  $100\text{-}\mu\text{l}$  volume of corn oil, which was administered by gavage, once a day, without anesthesia. A total of four experimental groups consisting of two treatment groups, vehicle (corn oil) and BHA, for each infection group, sham and RSV, were used for all experiments. There was no effect of the vehicle alone; therefore, the groups sham + vehicle and RSV + vehicle are usually identified in the figures as sham and RSV. In AHR experiments, mice were infected with RSV at  $1 \times 10^5$  PFU.

### Clinical Illness

We used a well-established clinical illness grading scale for mice to establish the severity of infection (0 = healthy; 1 = barely ruffled fur; 2 = ruffled fur but active; 3 = ruffled fur and inactive; 4 = ruffled fur, inactive, and hunched; 5 = dead) (21). In addition, daily determination of body weight was used to monitor the progression of disease over the experimental period. These parameters have been shown to closely correlate with lung pathology in experimental paramyxovirus infection of BALB/c mice (22). Visual differences were observed and captured by digital photography.

### AHR

The effect of BHA on RSV-induced AHR was measured in mice using a whole-body plethysmograph (Buxco, Troy, NY) as previously described (18, 23). Measurement of airway responses was performed on individual, unrestrained, and nonanesthetized mice within a two-chamber plethysmograph. AHR was expressed as an enhanced pause (Penh). Penh, a dimensionless parameter used to measure pulmonary resistance, is calculated by changes in chamber pressure induced by methacholine challenge during inspiration and expiration. After a brief acclimatization to the chamber, the mice received an initial baseline challenge of saline followed by increasing doses of nebulized methacholine (3, 6, 12, 24, and 50 mg/ml). Recordings were taken for 3 min after each nebulization. The respiratory rate in breaths per minute was extrapolated

from readings of every 10 breaths. The box pressure waveforms generated from the respiratory cycle were used to calculate peak expiratory pressure (PEP), peak inspiratory pressure (PIP), and the time of expiration. Penh was then calculated using the following formula:  $\text{Penh} = \text{Pause} \times \text{PEP}/\text{PIP}$ . Penh values were averaged and reported as a percentage of baseline saline values.

### Pulmonary Inflammation

Bronchoalveolar lavage (BAL) was obtained from the lungs of mice at various time points postinfection. In brief, anesthetized and tracheotomized mice were cannulated with a 1-ml syringe, and the lungs flushed three times with 1 ml of sterile, cold PBS. Total cellular influx and differential cell counts were measured in the BAL of all experimental groups. Total cell counts were determined by staining  $50 \mu\text{l}$  of BAL with trypan blue and counting viable cells using a hemocytometer. For differentials,  $100 \mu\text{l}$  of BAL was used to generate cytopsin preparations. Slides were dried, fixed, and stained with Protocol Hema3 (Fisher Diagnostics, Middletown, VA). A total of 300 cells were counted per sample using light microscopy.

### Lung Pathology

Selected mice in each group were killed at various time points, and the entire lung was perfused, removed, and fixed in 10% buffered formalin and embedded in paraffin. Multiple  $4\text{-}\mu\text{m}$  longitudinal cross-sections were stained with hematoxylin and eosin. The slides were analyzed and scored for cellular inflammation under light microscopy by a board-certified pathologist, as previously described (21). The pathology score reported includes the number of abnormal perivascular and peribronchial regions divided by the total number of perivascular and peribronchial regions and are reported as the percentage of inflamed tissue.

### Effect of Antioxidants on Viral Replication

Lungs were excised on Days 3, 5, and 7 postinfection, snap-frozen in liquid nitrogen, and stored at  $-70^{\circ}\text{C}$ . Viral titers were determined by plaque assay using confluent Hep-2 cells grown in 24-well plates, as previously described (24).

### Lipid Peroxidation in BAL

BAL was centrifuged at 15,000 rpm for 1 min at  $4^{\circ}\text{C}$ . BHT was added to the supernatant to prevent further oxidation, and the samples were immediately frozen in liquid nitrogen. Measurement of lipid peroxidation markers malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) was carried out using a lipid peroxidation kit from Calbiochem (EMD Biosciences, Inc., San Diego, CA). To detect MDA and 4-HNE,  $200 \mu\text{l}$  of BAL was added to  $650 \mu\text{l}$  of a 3:1 solution of N-methyl-2-phenylindole in acetonitrile:ferric ions in methanol and quickly vortexed. A total of  $150 \mu\text{l}$  of methanesulfonic acid were added and incubated at  $45^{\circ}\text{C}$  for 1 h. The samples were cooled and measured at 586 nm using a spectrophotometer.

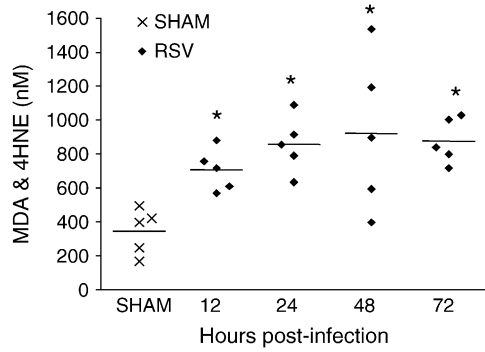
Measurements of 8-isoprostane were performed using a competitive enzyme immunoassay from Cayman Chemical (Ann Arbor, MI), according to the manufacturer's instructions.

### Chemokine and Cytokine Protein Profile

To assess the production of chemokines and cytokines, BAL was obtained at 12, 24, 48, and 72 h postinfection. Samples were tested for multiple cytokines using the Bio-Plex Cytokine Mouse 18-Plex panel (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer's instructions, as previously described (25). The panel includes the following cytokines: interleukin (IL)-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12 (p40), IL-12 (p70), IL-17, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, IFN- $\gamma$ , KC, MIP-1 $\alpha$ , MCP-1, RANTES, and tumor necrosis factor (TNF)- $\alpha$ . The broad assay range was from 0.2 to 5,000 pg/ml.

### Cysteinyl Leukotriene Determination

BAL was used to measure leukotriene C<sub>4</sub> (LTC<sub>4</sub>) by a competitive ACE enzyme immunoassay (Cayman Chemical). The sensitivity of the assay was 7.8 pg/ml. The specificity of the assay for traditionally classified cysteinyl leukotrienes (cys-LTs) LTC<sub>4</sub> and LTC<sub>5</sub> was 100%. The cross-reactivities



**Figure 1.** Lipid peroxidation products in the bronchoalveolar lavage (BAL). malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) were detected by a colorimetric assay in BAL of respiratory syncytial virus (RSV)- and sham-infected mice at various times postinfection. The bar in the scatter plot represents the mean of five different animals. The figure is representative of two different experiments. \**p* < 0.01 relative to sham-infected mice.

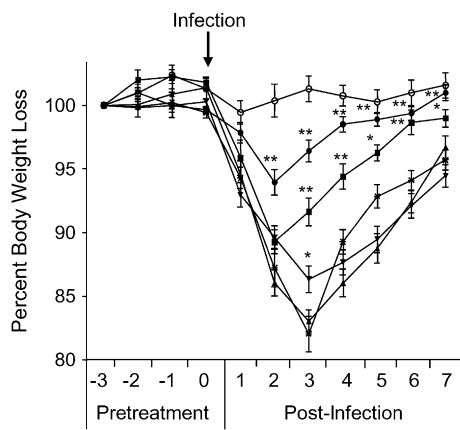
of the assay with LTC<sub>4</sub> metabolites are 48% with LTD<sub>5</sub>, 46% with LTD<sub>4</sub>, 28% with LTE<sub>4</sub>, 7% with LTE<sub>5</sub>, and 2% with LTE<sub>4</sub>.

**Statistical Analysis**

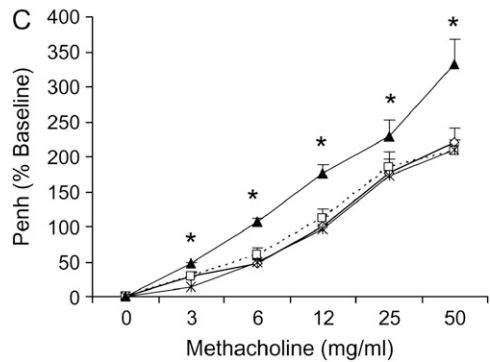
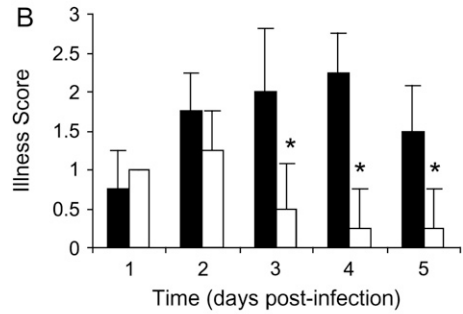
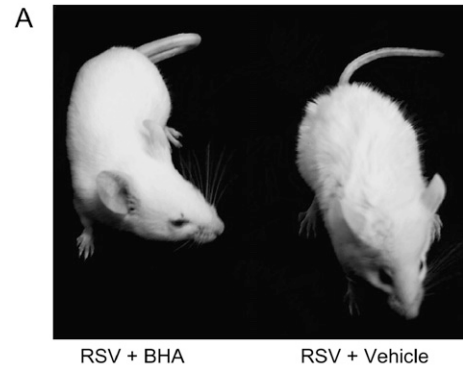
When two groups are compared, the values are analyzed using an unpaired, two-tailed, Student’s *t* test (GraphPad Prism; GraphPad Software, Inc., San Diego, CA). Results are expressed as mean ± SD unless otherwise stated. One-way analysis of variance with Dunnett’s multiple comparison tests were performed to analyze body weight loss in RSV versus RSV + BHA treatment groups.

**RESULTS**

RSV infection induces oxidative stress in the lung. Lipid peroxidation products, such as MDA, 4-HNE, and isoprostanes, are useful markers of pulmonary oxidative stress and by themselves



**Figure 2.** Effect of butylated hydroxyanisole (BHA) administration on RSV-induced weight loss. Mice were treated with increasing concentrations of BHA for 3 d before RSV infection and during the first 7 d of infection. The following is a representative diagram of three different experiments. All data points represent the mean of at least four animals. Open circles, sham; cross marks, RSV; triangles, RSV + BHA 50 mg/kg; inverted triangles, RSV + BHA 100 mg/kg; solid squares, RSV + BHA 150 mg/kg; solid circles, RSV + BHA 250 mg/kg. \**p* < 0.05 relative to sham-infected mice; \*\**p* < 0.01 relative to sham-infected mice.



**Figure 3.** Effect of BHA on clinical illness and airway hyperreactivity (AHR). Mice were infected with RSV and treated with BHA 250 mg/kg for the following 7 d. (A) Differences in the appearance of fur in RSV + BHA- versus RSV + vehicle-treated mice at Day 3 postinfection. (B) Clinical illness scores of RSV + BHA (open squares) and RSV + vehicle (solid squares) were measured from Day 1 to Day 5 postinfection. Sham-infected mice treated with either vehicle or BHA received a healthy illness score = 0 throughout the course of the experiment (data not shown). Data are expressed as mean ± SD of five different animals and are representative of three different experiments. \**p* < 0.01 relative to RSV-infected mice. (C) The effect of BHA treatment on RSV-induced AHR during methacholine challenge was measured at Day 4 postinfection by whole-body plethysmography. All treatment groups were given an initial dose of saline and subsequently challenged with increasing concentrations of methacholine (mg/ml). Penh is reported as a percentage increase from baseline saline challenge. Data are expressed as mean ± SD of eight different animals. Open squares, sham; RSV, solid triangles; cross marks, RSV + BHA. \**p* < 0.01 relative to RSV-infected mice.

have been shown to mimic all the pathophysiologic features of asthma (bronchoconstriction, AHR, mucus hypersecretion, enhanced arachidonic acid cascade, increased synthesis of chemoattractants, epithelial damage, and microvascular leakage; reviewed in Reference 26). To determine whether RSV infection induced oxidative damage of the lung, MDA and 4-HNE were measured

in the BAL of RSV-infected mice at different times postinfection. As shown in Figure 1, RSV infection was associated with a significant increase of MDA and 4-HNE levels in BAL samples at all time points tested, when compared with sham mice, indicating that lung oxidative stress damage indeed occurs in RSV infection. MDA and 4-HNE values from the sham group did not change over time (data not shown).

### BHA Attenuates RSV-induced Clinical Illness

To determine whether antioxidant administration was capable of altering RSV-induced disease, we initially assessed the effect of three different BHA treatment protocols on body weight loss. In protocol 1, animals were pretreated with various doses of BHA, ranging from 50 to 250 mg/kg, 3 d before and during the first 5 d of infection. As shown in Figure 2, mice inoculated with RSV alone progressively lost weight during the first 3 d of infection, with a peak of 15 to 20% loss at Day 3 postinfection. The 250-mg/kg dose of BHA consistently attenuated RSV-induced body weight loss, as the mice experienced a weight loss of 6% and regained their original body weight earlier. A statistically significant difference in body weight loss was also observed with the 150-mg/kg dose, whereas the 100-mg/kg had some effect only at Day 3 postinfection, which represents the peak of body weight loss. We then determined whether the 250-mg/kg dose was still effective if treatment was started the same day of RSV infection (protocol 2) or at 1 d postinfection (protocol 3). Protocol 3, or post-treatment with BHA, failed to attenuate RSV-induced body weight loss and was not further investigated (data not shown). There was no statistically significant difference in body weight loss between protocols 1 and 2 (data not shown); therefore, all

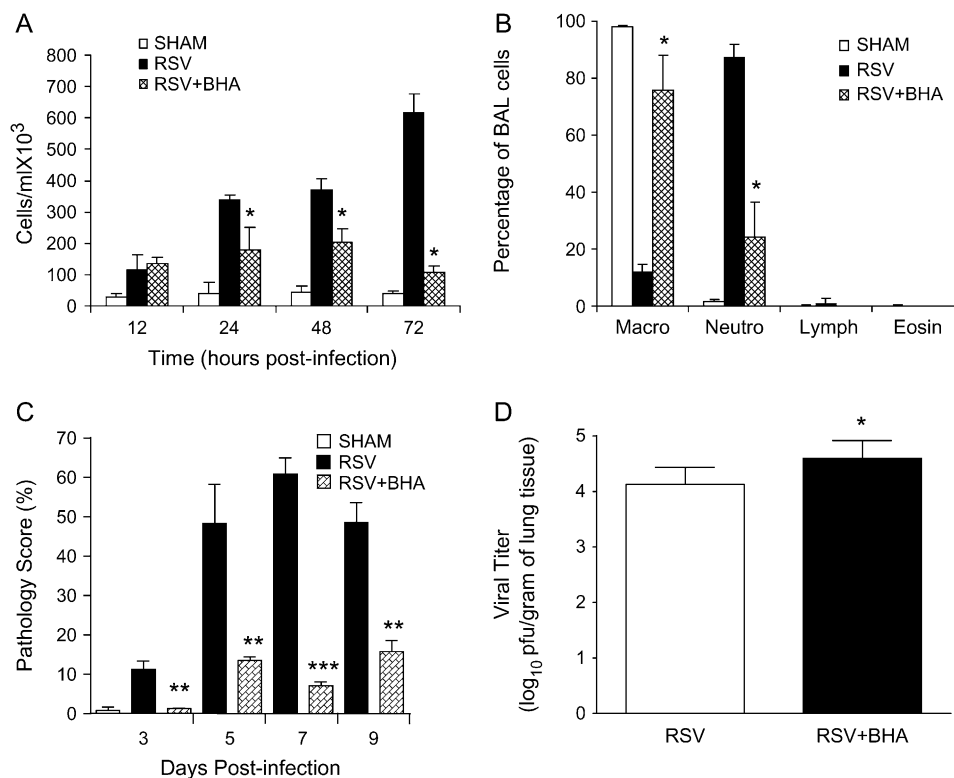
subsequent experiments were performed using the dose of BHA 250 mg/kg given during the first 5 d of RSV infection.

A positive effect of antioxidant treatment was also observed on other clinical parameters of RSV infection that constitute the viral-induced illness score (*see METHODS* for details). Typically, the peak of illness severity coincides with the peak of RSV-induced body weight loss and occurs between Day 3 and 4 of infection (27). We observed a significant difference in appearance of BHA-treated versus untreated RSV-infected mice (Figure 3A), as well as in the total illness score, starting at Day 3 postinfection (Figure 3B), indicating that antioxidant treatment is effective in modulating RSV-induced clinical disease.

We and others have previously shown that RSV infection induces AHR in response to methacholine challenge (18, 23, 28). As shown in Figure 3C, we observed a significant difference between RSV- and RSV + BHA-treated animals, because administration of BHA strongly attenuated RSV-induced AHR at all methacholine doses. BHA treatment did not alter baseline Penh values or airway response to methacholine in sham-infected animals.

### Effect of BHA on RSV-induced Pulmonary Inflammation

To determine whether BHA altered RSV-induced lung inflammation, total and differential cell counts in the BAL were measured. We observed a significant attenuation of cellular influx by BHA treatment in RSV-infected mice, starting at Day 1 postinfection and continuing throughout the infection, with the most significant difference observed at Day 3 postinfection (Figure 4A), which, in our mouse model, corresponds to the peak of inflammatory cell recruitment in the BAL after RSV infection.



**Figure 4.** Effect of BHA on airway inflammation and viral replication. (A) Total number of cells was measured in BAL of sham and RSV-infected mice, either untreated or treated with BHA, at various times postinfection. Data are expressed as mean  $\pm$  SD of five different animals and representative of three different experiments. \* $p < 0.01$  relative to RSV-infected mice. (B) Differential cell count was measured in BAL of sham and RSV-infected mice, either untreated or treated with BHA, at Day 3 postinfection. \* $p < 0.01$  relative to RSV-infected mice. (C) Mice were infected with RSV or sham-infected and treated with either vehicle or BHA. At various days postinfection, mice were killed and lungs were excised, fixed in 10% buffered formalin, and embedded in paraffin. Lung sections were stained with hematoxylin and eosin, and peribronchial, perivascular inflammation was scored. Data are expressed as mean  $\pm$  SD of four animals/group. Data shown are representative of three independent experiments. \*\* $p < 0.01$  relative to RSV-infected mice; \*\*\* $p < 0.001$  relative to sham-infected mice. (D) Mice were infected with RSV and either treated with vehicle or BHA. At Day 5 postinfection, lungs were excised and viral load was determined by plaque assay. Data shown are representative of three independent experiments ( $n = 10$ , mean  $\pm$  SD). \* $p < 0.05$  relative to RSV-infected mice.

Macrophages are the normal resident cell type found within the airways in uninfected mice. RSV infection induces a significant lung recruitment of neutrophils, which become the predominant inflammatory cell observed in the BAL in the first few days of infection (29). As shown in Figure 4B, BHA administration significantly blocked neutrophil recruitment into the airways, with little difference observed among the different experimental groups in lymphocyte and eosinophil populations.

To further examine the effect of BHA treatment on RSV-induced inflammation, lungs of infected mice were analyzed for histopathology. The perivascular and peribronchial mononuclear cell recruitment in the lung was examined at Days 3, 5, 7, and 9 postinfection. A dramatic decrease in lung inflammation between untreated and BHA-treated infected mice was observed as early as Day 3 postinfection, bringing the histopathology score of the treated RSV-infected mice to levels similar to that of uninfected control animals, with a similar, although less striking, effect at Days 5, 7, and 9 postinfection, as shown in Figure 4C.

Because inflammatory cells, especially neutrophils, can play an important role against viral replication, at the early stage of infection (30, 30), we examined whether BHA altered RSV replication. In our mouse model of RSV infection, increased viral titer can be detected as early as Day 3 postinfection, with peak viral titer occurring at Day 5 postinfection, and viral clearance by Day 7 postinfection (27). We found a consistent increase of approximately a half log in viral titer in BHA-treated animals at Days 4 and 5 postinfection, as shown in Figure 4D, where antioxidant administration increased peak viral load from  $4.12 \pm 0.31$  to  $4.59 \pm 0.32 \log_{10}$ . RSV replication was usually no longer detectable at Day 7 postinfection in both untreated and BHA-

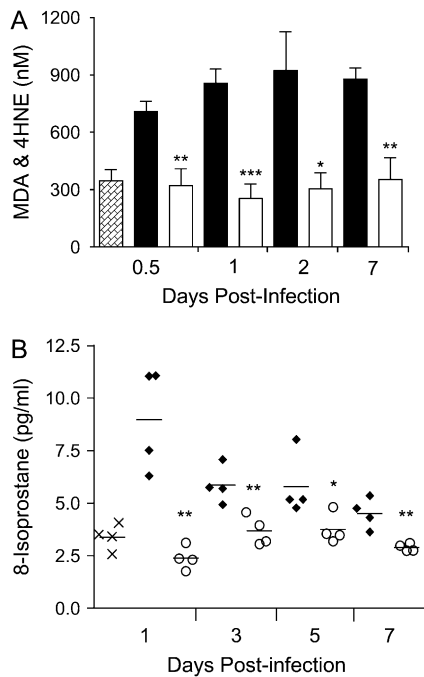
treated infected mice, although occasionally we could recover virus at a very low titer ( $10^1$ ) in the treated animals.

### BHA Attenuates RSV-induced Oxidative Stress in the Lung

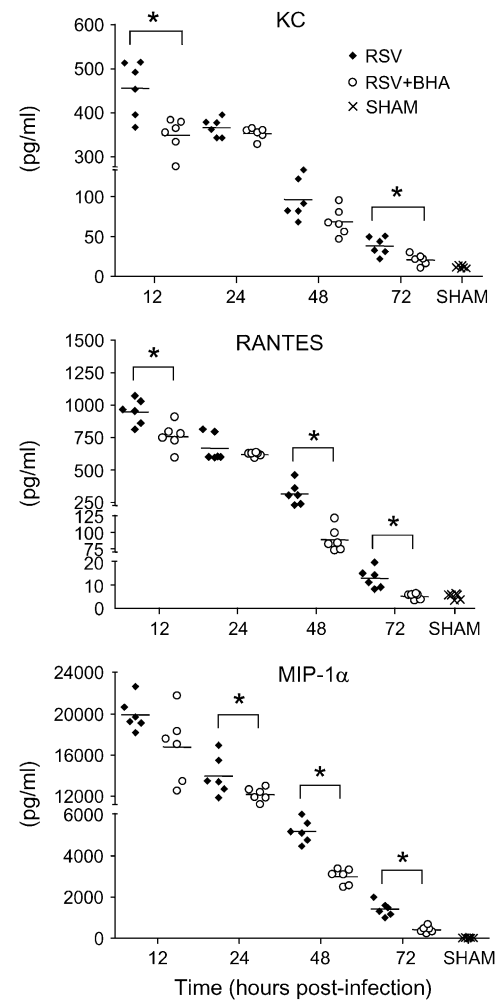
To determine whether antioxidant treatment affected RSV-induced oxidative damage of the lung, MDA and 4-HNE, as well as 8-isoprostanes, were measured in the BAL of RSV-infected mice, with or without BHA treatment. As shown in Figure 5, the average levels of MDA and 4-HNE were between 2- and 2.5-fold higher in RSV-infected BAL samples in comparison to control mice at all time points of RSV infection. BHA administration clearly lowered RSV-induced lipid peroxidation to values equivalent to control at all time points. Similarly, RSV-induced 8-isoprostane production was significantly reduced at all time points tested.

### Effect of BHA on Chemokine and Cytokine Induction

RSV infection is a potent inducer of chemokine production, and increased chemokine release has been shown to play an important role in RSV-induced lung inflammation and to correlate with disease severity (reviewed in Reference 5). In our



**Figure 5.** Effect of BHA on lipid peroxidation. (A) MDA and 4-HNE or (B) 8-isoprostane were detected by either colorimetric assay or ELISA in BAL of mice infected with RSV and treated with either vehicle (solid bars) or BHA (open bars) at various times postinfection. Hatched bars, sham-treated mice. The bar in the scatter plot represents the mean of five different animals. ×, sham; solid diamonds, RSV; open circles, RSV + BHA. Data shown are representative of two independent experiments. \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001 relative to RSV-infected mice.



**Figure 6.** Effect of BHA on chemokine release in the BAL. Chemokine production was measured by Bio-Plex bead suspension array in the BAL of mice infected with RSV and treated with either vehicle or BHA at various times postinfection. The bars in the scatter plot represent the mean of six different animals. Data shown are representative of three independent experiments. \*p < 0.01 relative to RSV-infected mice.

model, the peak of chemokine production occurs during the first 3 d of infection (25), after which chemokines are no longer measurable. To determine whether BHA treatment was able to modulate RSV-induced chemokine secretion, we analyzed chemokine levels in BAL samples collected during the first 3 d of infection. As shown in Figure 6, RANTES, KC, and MIP-1 $\alpha$  were strongly up-regulated in the lungs of infected animals, starting as early as 12 h postinfection. BHA treatment consistently reduced all three chemokine protein levels, especially at 48 and 72 h postinfection.

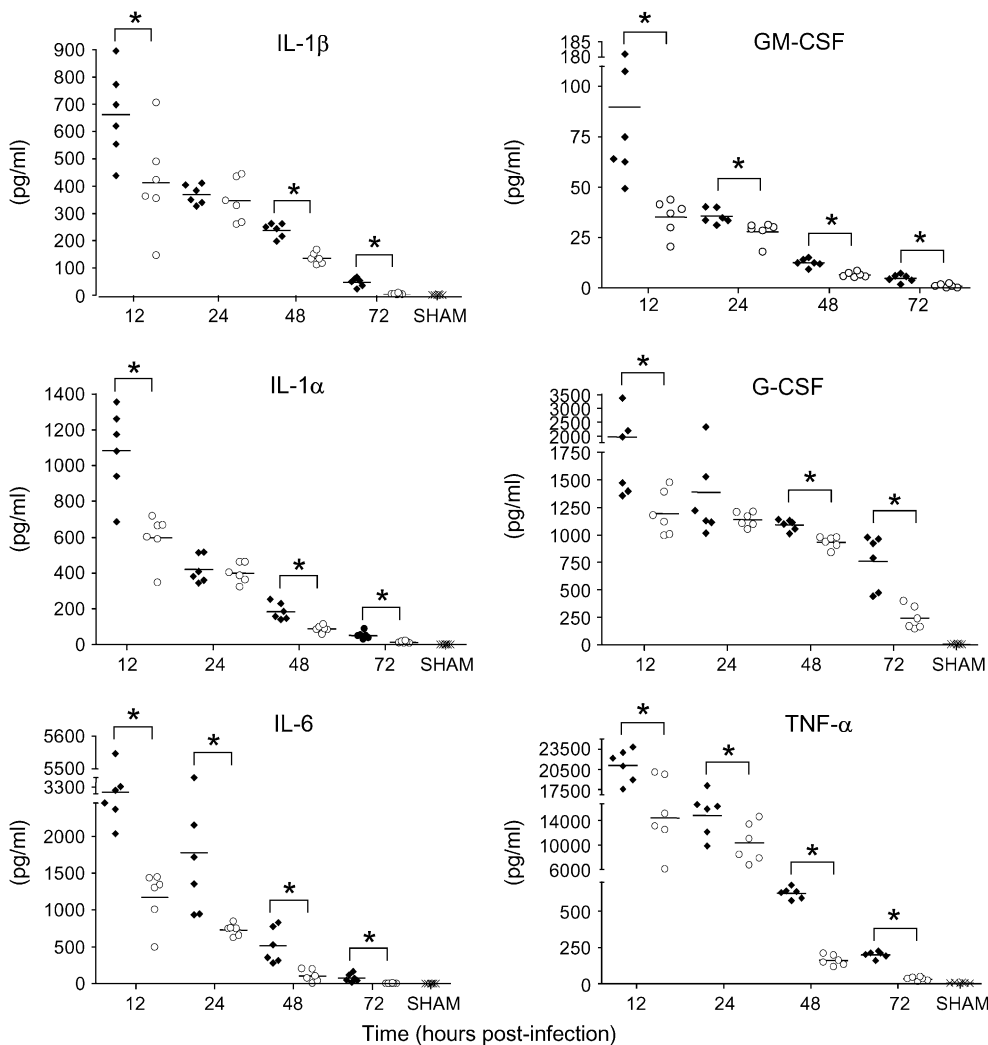
Proinflammatory cytokines, such as IL-1, IL-6, and TNF, as well as immunomodulatory cytokines, such as IL-10, IL-12, and IFN- $\gamma$ , have been implicated in the pathogenesis of RSV-induced lung disease (reviewed in References 5 and 31). As with chemokine secretion, significant cytokine production after RSV infection can be measured mainly during the first 3 d of infection, with the exception of IL-12 and IFN- $\gamma$  (25). Similar to what we observed for chemokine production, BHA treatment effectively reduced the secretion of all tested RSV-induced cytokines at Days 1, 2, and 3 postinfection (Figures 7 and 8). IFN- $\gamma$  secretion was also significantly reduced at later time points (Day 5: RSV,  $128.7 \pm 63.3$ , vs. RSV + BHA,  $22.1 \pm 5.3$  pg/ml; Day 7: RSV,  $294.8 \pm 69$ , vs. RSV + BHA,  $5.8 \pm 1.7$ ), indicating that antioxidant treatment exerts a general antiinflammatory activity in the context of RSV infection of the lung.

### Effect of BHA on RSV-induced LT Production

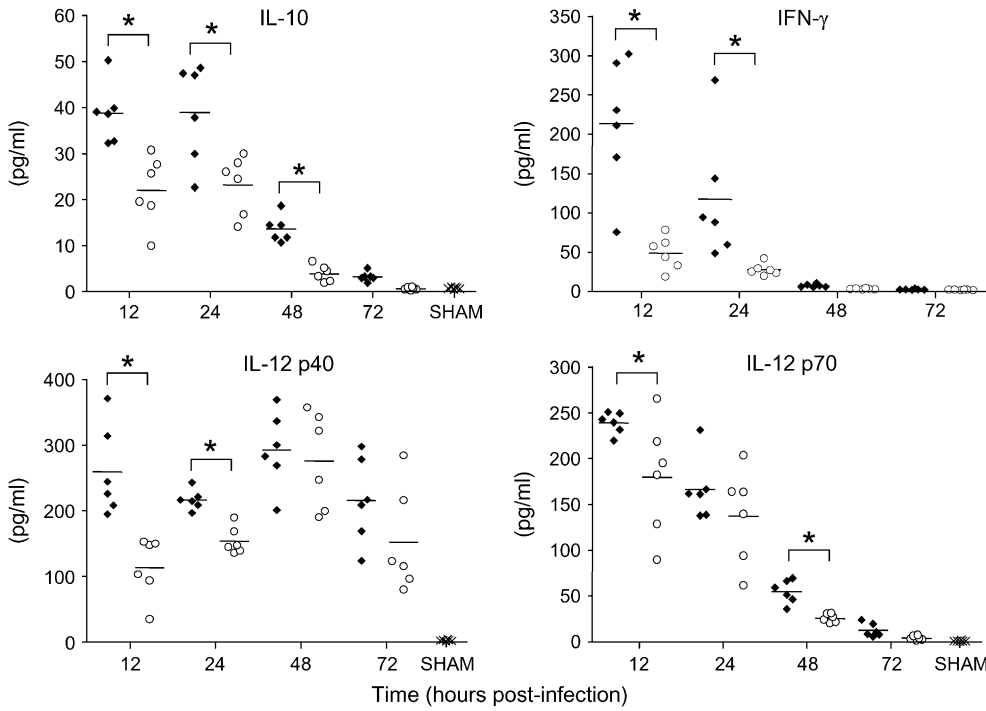
Cys-LTs, which include LTC<sub>4</sub>, LTD<sub>4</sub>, and LTE<sub>4</sub>, are important mediators of airway inflammation and bronchoconstriction (reviewed in Reference 32). They have been shown to increase in the respiratory secretion of children infected with RSV (33) and are associated with RSV-induced wheezing (34). We and others have shown that inhibition of LT synthesis (35) or treatment with LT receptor antagonist (36) inhibits lung inflammation and AHR in a mouse model of RSV infection. To determine whether the antioxidant BHA had an effect on RSV-induced cys-LT release, LTC<sub>4</sub> was measured in BAL at various times postinfection. As shown in Figure 9, BHA-treated mice had significantly lower levels of LTC<sub>4</sub>, which was particularly evident at Days 3 and 5 postinfection.

### DISCUSSION

Oxidative stress has been implicated in the pathogenesis of several acute and chronic airway diseases, such as asthma and chronic obstructive pulmonary disease (reviewed in Reference 37). ROS generation, due to the respiratory burst of activated phagocytic cells recruited to the airways during viral infections, is an important antiviral defense, necessary for viral clearance. However, a robust production of ROS can lead to depletion of antioxidants and cause oxidative stress. Surprisingly, little is



**Figure 7.** Effect of BHA on proinflammatory cytokines release in BAL. Cytokine production was measured by Bio-Plex bead suspension array in the BAL of mice infected with RSV and treated with either vehicle or BHA at various times postinfection. The bars in the scatter plot represent the mean of six different animals. Data shown are representative of three independent experiments.  $\times$ , sham; solid diamonds, RSV; open circles, RSV + BHA. \* $p < 0.01$  relative to RSV-infected mice.

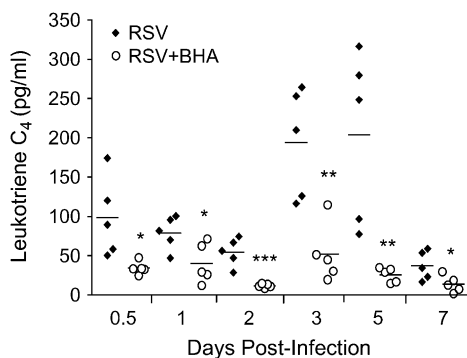


**Figure 8.** Effect of BHA on immunomodulatory cytokines in BAL. Cytokine production was measured by Bio-Plex bead suspension array in the BAL of mice infected with RSV and treated with either vehicle or control at various times postinfection. The bars in the scatter plot represent the mean of six different animals. Data shown are representative of three independent experiments. ×, sham; solid diamonds, RSV; open circles, RSV + BHA. \*p < 0.01 relative to RSV-infected mice.

known regarding the role of ROS in respiratory virus-induced lung diseases, with the exception of influenza. It was not known whether modulation of ROS production with antioxidants would be of clinical benefit in the context of RSV infection, for which no effective therapeutic treatment is currently available. In this study, we took advantage of an *in vivo* model of RSV infection to test the effect of antioxidant treatment on RSV-induced lung disease. We show for the first time that RSV infection induces lung oxidative stress, as evidenced by increase in the lipid peroxidation products. This is an important finding, because this information was available only for a mouse model of influenza infection (15). Remarkably, there are no studies investigating lipid peroxidation products in children or adults infected with respiratory viruses. These biomarkers of oxidative stress have been shown to be elevated in plasma and breath condensate of pa-

tients, both children and adults, with various acute and chronic inflammatory lung diseases, and to correlate with severity of illness in certain disease conditions (reviewed in Reference 38). In our study, antioxidant treatment with BHA significantly decreased the content of MDA and 4-HNE in BAL of RSV-infected mice and ameliorated both body weight loss and clinical illness, indicating that modulation of oxidative stress in the context of RSV infection can lead to improvement in lung disease. It is important to note that these findings were reproduced by the use of another antioxidant agent, dimethyl sulfoxide (DMSO). Oral administration of DMSO at the time of infection significantly reduced RSV-induced body weight loss and clinical disease (data not shown). However, given the systemic toxicity of DMSO, we did not use it for further experiments.

Although it is difficult to determine the precise mechanisms by which antioxidants provided protection in the context of RSV infection, attenuation of inflammation is likely a key one. We and others have shown that RSV-induced chemokine and cytokine release and subsequent pulmonary inflammation play a fundamental role in the pathogenesis of RSV-induced disease (reviewed in References 5, 39, and 40). Lack of production of specific chemokines, such as MIP-1α (21), or modulation of chemokine secretion, for example by perflubron treatment (24), has been shown to significantly reduce RSV-induced pulmonary inflammation. BHA treatment significantly reduced pulmonary cytokine and chemokine production after RSV infection, resulting in inhibition of lung recruitment of inflammatory cells, especially neutrophils, which are the major cell type responsible for oxidative burst in response to infectious stimuli. BHA treatment was effective if given before or at the moment of RSV infection, but did not result in significant improvement of clinical disease administered 1 d after infection, when clinical disease and lung inflammation are already present. This finding suggests that early therapeutic intervention may be necessary to improve clinical outcome in RSV infection. In fact, treatment of children with RSV-induced lower respiratory tract infection with anti-inflammatory drugs, such as steroids, has been shown to be mostly



**Figure 9.** Effect of BHA on RSV-induced leukotriene release. Leukotriene C<sub>4</sub> production was measured by ELISA in BAL of RSV-infected mice treated with either vehicle or BHA at various time points after infection. The bars in the scatter plot represent the mean of five different animals. Data shown are representative of three independent experiments. \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001 relative to RSV-infected mice.

ineffective in improving clinical disease, as these drugs are usually administered when children are hospitalized, days after manifestation of initial symptoms (reviewed in Reference 41).

Although BHA administration slightly increased RSV replication in the lung, there is no documented correlation between RSV viral titer and severity of illness. Antiviral treatment with ribavirin alone or immunotherapy with specific immunoglobulin does not provide significant clinical improvement, indicating that RSV-induced lung disease is most likely the result of the inflammatory response more than a direct viral cytopathic effect (reviewed in Reference 42). On the other hand, inhibition of NO production, which is antiviral against RSV *in vitro* and *in vivo* (18, 43), significantly reduces pulmonary inflammation and airway hyperresponsiveness, although it increases viral replication (18), suggesting again that modulation of inflammation is fundamental to controlling RSV disease.

RSV infection early in life has been associated with AHR and recurrent wheezing, and possibly long-term pulmonary abnormalities (4, 44, 45). In the mouse model of RSV infection, whole-body plethysmography has been used to measure airway resistance, and we have found that AHR to inhaled methacholine correlates with pulmonary resistance when measured with more invasive techniques (18, 36, 46). In our study, we found that antioxidant treatment attenuated RSV-induced AHR, possibly due to a reduction in cys-LT production. Cys-LTs are potent bronchoconstrictors, as well as proinflammatory mediators, and their role in the pathogenesis of airway inflammation and obstruction has been recently recognized as a new target for therapeutic intervention (reviewed in Reference 32). They have been shown to increase in the respiratory secretion of children infected with RSV (33, 47) and are associated with RSV-induced wheezing (34). In a mouse model of RSV infection, increased levels of cys-LTs correlate with increased airway responsiveness (35), and either inhibition of LT production (35) or treatment with LT receptor antagonist (36) inhibits AHR. In children, treatment with a cys-LT receptor antagonist decreased exacerbations of reactive airway disease after RSV bronchiolitis (48). In our study, BHA administration significantly decreased LTC<sub>4</sub> secretion, with parallel reduction of AHR after methacholine challenge, suggesting a causal relationship of the two phenomena, although we cannot exclude that inhibition of other important mediators could be responsible for AHR reduction after BHA treatment.

Although little information is available regarding respiratory infections, there is a body of literature showing that antioxidants can afford protection against virally induced morbidity and mortality and are important in host immune responses (reviewed in References 49 and 50). For example, butylated hydroxytoluene, an analog of BHA, provided increased survival of chickens infected with Newcastle disease virus (51). In conclusion, the ability of antioxidants to attenuate symptoms and pathology in RSV infection warrants further investigation of these agents as a novel therapeutic approach to virally induced pulmonary disease.

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