ORIGINAL RESEARCH

Increased Concentration of Iodide in Airway Secretions Is Associated with Reduced Respiratory Syncytial Virus Disease Severity

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Abstract

Recent studies have revealed that the human and nonrodent mammalian airway mucosa contains an oxidative host defense system. This three-component system consists of the hydrogen peroxide (H_2O_2)-producing enzymes dual oxidase (Duox)1 and Duox2, thiocyanate (SCN^-), and secreted lactoperoxidase (LPO). The LPO-catalyzed reaction between H_2O_2 and SCN^- yields the bactericidal hypothiocyanite ($OSCN^-$) in airway surface liquid (ASL). Although SCN^- is the physiological substrate of LPO, the Duox/LPO/halide system can generate hypoiodous acid when the iodide (I^-) concentration is elevated in ASL. Because hypoiodous acid, but not $OSCN^-$, inactivates respiratory syncytial virus (RSV) in cell culture, we used a lamb model of RSV to test whether potassium iodide (KI) could enhance this system *in vivo*. Newborn lambs received KI by intragastric gavage or were left untreated before intratracheal inoculation of RSV. KI treatment led to a 10-fold increase in ASL I^- concentration, and this I^- concentration was approximately 30-fold higher than that measured in the serum. Also, expiratory effort, gross lung lesions, and pulmonary expression of an RSV antigen and IL-8 were reduced in the KI-treated lambs as compared with nontreated control lambs. Inhibition of LPO activity significantly increased lesions, RSV mRNA, and antigen. Similar experiments in 3-week-old lambs demonstrated that KI administration was associated with reduced gross lesions, decreased RSV titers in bronchoalveolar lavage fluid, and reduced RSV antigen expression. Overall, these data indicate that high-dose KI supplementation can be used *in vivo* to lessen the severity of RSV infections, potentially through the augmentation of mucosal oxidative defenses.

Keywords: mucosal immunity; oxidative defense system; respiratory syncytial virus; potassium iodide; ovine model

Clinical Relevance

This study demonstrates that enhancement of dual oxidase/lactoperoxidase oxidative responses by potassium iodide administration reduces respiratory syncytial virus disease severity in a lamb model that has much similarity to disease condition in human infants. The work is readily translational.

Respiratory syncytial virus (RSV) is a major cause of acute lower respiratory infection in infants and young children and is a leading cause of infantile bronchiolitis worldwide (1, 2). In industrialized countries, human RSV (hRSV) accounts for up to 70% of hospitalized bronchiolitis cases (1–3). Although the antiviral drugs palivizumab and ribavirin have virucidal activity against RSV *in vivo*, there are no fully satisfactory therapeutic regimens or vaccines (4–6). It is estimated that 3% of RSV cases in the United States and 10% of cases worldwide result in hospitalization; the reported number of new cases of lower respiratory infection due to RSV in children under 5 years of age in 2005 was 33.8 million (7, 8). Thus, there is a need for new therapies for RSV infection.

The host defense activity of the airway epithelium is critical for the continuous inactivation and removal of inhaled microbes from the respiratory tract. Although the role of mucociliary clearance and epithelial host defense proteins and peptidesin lung immunity is well established, the recognition that airway epithelial cells express an oxidative

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microbicidal system is recent (9–13; reviewed in Refs. 11 and 12). This three-component oxidative system consists of two H_2O_2–generating enzymes of airway epithelia—dual oxidase (Duox)1 and Duox2—together with a pseudohalide anion (thiocyanate [SCN^-]) and the enzyme lactoperoxidase (LPO). The Duox enzymes generate H_2O_2 into the apical extracellular space where H_2O_2 reacts with secreted SCN^- in a LPO-catalyzed reaction to form the antibacterial hypothiocyanite ($OSCN^-$) molecule ($H_2O_2 + SCN^- \rightarrow OSCN^-$) (10, 13–21). LPO and SCN^- are highly concentrated in the airway surface liquid (ASL). The Duox/LPO/SCN^- system generates sufficient $OSCN^-$ to eliminate bacteria *in vitro* and *in vivo* (13, 22, 23). *In vitro*, the substitution of iodide (I^-) for SCN^- in this system yields hypoiodous acid ($H_2O_2 + I^- \rightarrow$ HOI) instead of the physiological product $OSCN^-$ (24, 25). HOI has potent microbicidal activity against bacteria (23) and viruses (26), including activity against RSV, whereas $OSCN^-$ exhibits little antiviral activity (24). We previously reported that I^- is concentrated in the nasal ASL of human subjects after oral I^- supplementation using an FDA-approved formulation (24). However, it was not tested whether I^- supplementation led to high I^- concentration in the ASL of lower airways and whether a high I^- concentration in the ASL could affect the severity of respiratory viral infections.

The ovine respiratory tract functionally expresses the Duox/LPO/halide system (23), is susceptible to human strains of RSV, and develops microscopic lesions similar to those in infants (reviewed in References 27–30). Some other animal species used to model RSV infection (e.g., mice and rats) lack airway submucosal glands (31), which are the LPO-secreting structures in the airways of larger mammals. Without LPO production in the airways, the Duox/LPO/halide system is not fully functional. Therefore, lambs were used to test the hypothesis that I^- supplementation can protect the newborn lung against RSV infection. The life cycle of RSV is restricted to the epithelia of the respiratory tract. Because the Duox/LPO/halide system is dependent on mature epithelia and submucosal glands that can produce adequate levels of enzymes and halide transport proteins, studies were completed in newborn and 3-week-old lambs.

Materials and Methods

I^- Concentration Measurements in Airway Secretions and Serum

The concentrations of I^- and SCN^- in the nasal secretions and serum of lambs were measured after the administration of NaI (1.7 mg/kg body weight [$n = 5$]) or PBS (control [$n = 5$]) via the external jugular vein. Nasal secretions and blood samples were collected at 0 hours (just before the injection of NaI) and at 4, 12, and 36 hours. Blood (8 ml) was drawn from the external jugular vein, and serum was collected. Nasal secretions were collected with microsampling probes (BC-402C; Olympus, Center Valley, PA) as previously described (24, 32). Nasal fluid was recovered from the microsampling probes by microcentrifugation. Serum samples and nasal secretions were diluted in water and analyzed for anion composition using ion-exchange chromatography as described previously (24, 32).

The experimental design described above was slightly modified to determine the effect of intragastric delivery of potassium iodide (KI) on the concentrations of I^- and SCN^- in airway secretions. Briefly, lambs ($n = 5$) received KI-containing PBS (1.8 mg KI/kg body weight) via an 18 French Foley catheter followed by 6 ml of water flush to clear. Nasal secretions and serum samples were collected at 0 hours (just before the intragastric gavage) and at 4, 12, and 36 hours. At 4 and 12 hours, one animal per time point was killed after the collection of serum and nasal secretions to harvest tracheobronchial secretions as previously described (24, 32). The remaining three animals were killed at 36 hours for the collection of tracheobronchial secretions.

Lamb RSV Challenge Experiments

Lambs were randomly assigned to three groups: the first group was inoculated with RSV, the second group was inoculated with RSV and treated with KI, and the third group was neither inoculated with RSV nor treated with KI. Using this experimental design, a pilot experiment was performed in which the human pathogen RSV strain A2 was delivered through a fiberoptic bronchoscope (10^8 plaque-forming units of RSV in 10 ml cell culture medium) into the trachea of newborn lambs (2–3 d of age [$n = 5$]). In this pilot experiment, the KI-treated lambs ($n = 5$) received 1.8 mg KI/kg body weight in PBS by intragastric gavage 4 hours before the inoculation of RSV A2 and daily thereafter. Control lambs ($n = 3$) received I^-–free PBS via intragastric gavage and sterile cell culture medium (10 ml) via bronchoscope. In subsequent experiments involving 2- to 3-day-old lambs and 3-week-old lambs, a more pathogenic RSV strain (Memphis 37) (27) was delivered (3.5×10^7 plaque-forming units) to lambs in 6 ml of culture medium using a Sprint nebulizer (PARI, Midlothian, VA) and a cone face mask fitted with a rubber gasket that provided a seal around the mandible and nose. The dose of KI was increased to 10 mg KI/kg body weight/d. Control lambs received I^-–free PBS intragastrically (6 ml) via nebulization, and an additional group of lambs received dapsone, which inhibits LPO activity. Additional details are provided in the online supplement.

Results

ASL I^- Levels after Intravenous and Intragastric I^- Delivery

To evaluate the extent to which systemic I^- administration to lambs affects the ASL concentration of I^-, nasal secretions and serum samples were collected from lambs that received sodium iodide (NaI) in PBS intravenously (1.7 mg NaI/kg body weight) or PBS only (negative control). Ion-exchange chromatography analysis of these samples showed that the I^- concentration increased in the nasal secretions to approximately 340 μM at the 4-hour time point, whereas the serum I^- concentration only increased to approximately 14 μM (Figures 1A and 1B). By the 36-hour time point, the I^- concentration in the nasal secretions returned to near baseline levels. The SCN^- concentration decreased in the nasal secretions when the I^- concentration reached its peak in the serum (*see* Figure E2 in the online supplement), suggesting that SCN^- and I^- compete for the same transporters in the airway. I^- administration to humans also leads to a greatly increased [I^-] in the nasal ASL (24). Thus, our data indicate that the upper airway of lambs recapitulates the halide-transporting activity of the human nasal mucosa.

Next, we examined whether intragastric administration of I^- leads to elevated concentration of I^- in the secretions of the upper and lower airways. We used intragastric gavage to deliver KI

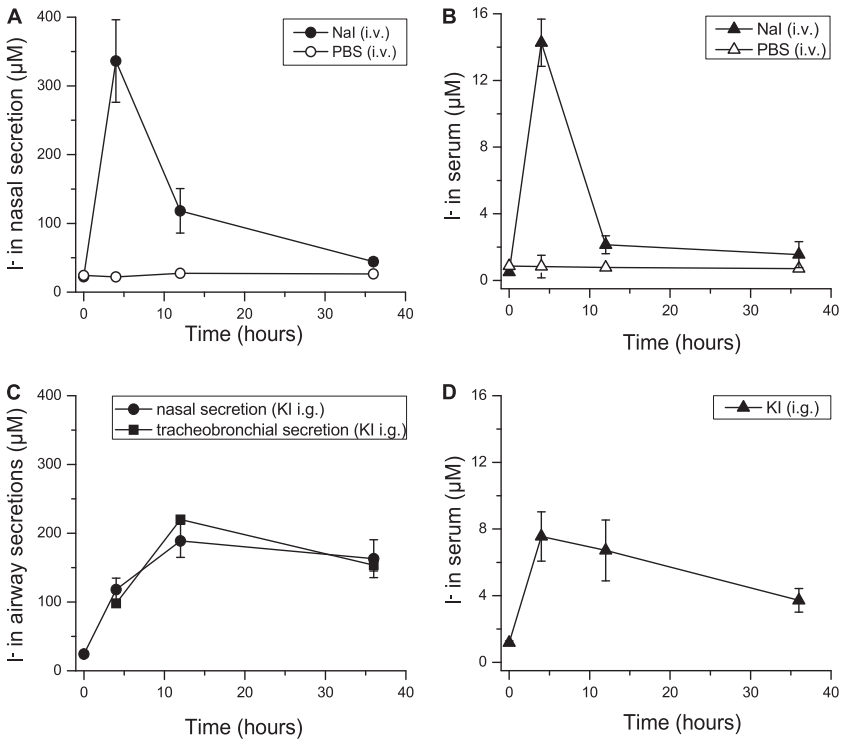

Figure 1. Iodide (I^-) concentrations in the airway secretions and serum of lambs after systemic administration of I^-. (*A*) I^- concentration in nasal secretions of lambs at the indicated time points after intravenous injection of NaI (*closed circles*; $n = 5$) or PBS (*open circles*; $n = 5$) at 0 hours. (*B*) I^- concentrations in the serum of lambs at the indicated time points after intravenous (i.v.) administration of NaI (*closed triangles*; $n = 5$) or PBS (*open triangles*; $n = 5$) at 0 hours. (*C*) I^- concentration in the nasal and tracheobronchial secretions of lambs (*closed circles* and *closed squares*, respectively) at the indicated time points after intragastric potassium iodide (KI) administration at 0 hours. The numbers of animals used for the collection of nasal secretions were 5 (0 and 4 h), 4 (12 h), and 3 (36 h). The numbers of animals used for the collection of tracheal secretions were 1 (4 h), 1 (12 h), and 3 (36 h). (*D*) I^- concentration in the serum of lambs ($n \geq 3$) at the indicated time points after intragastric delivery of KI. *Error bars* = SEM.

(1.8 mg KI/kg body weight) to lambs. Nasal secretions and serum samples were harvested at 0, 4, 12, and 36 h. Tracheobronchial secretions were collected immediately after killing the lambs at 4, 12, and 36 hours. Analysis of these samples showed that intragastric KI administration led to a more prolonged increase of I^- in the serum, nasal secretions, and tracheobronchial secretions than the intravenous delivery of NaI (Figure 1). Furthermore, the upper and lower airway secretions contained similar levels of I^- at each time point (Figure 1C). These data indicate that intragastric delivery of I^- to lambs leads to elevated I^- concentration in the ASL of the lower and upper airways.

Pilot Study: I^- Effects Human RSV Strain A2 *In Vivo*

A pilot study was completed to establish an initial proof-of-principle that I^- would have some effect on reducing RSV replication. Lambs received intragastric KI supplementation or control (i.e., I^-–free) solution 4 hours before to intratracheal inoculation of hRSV A2 and daily thereafter. The severity of hRSV A2 infection was evaluated 6 days later when the RSV-induced lung lesions are maximal in this model (25, 33–36). In the lungs of KI-treated lambs, we found reduced hRSV A2 RNA levels (Figure 2A) and a reduction in gross and histological lesions (consolidation score, 2.22 ± 0.9) compared with untreated lambs infected with hRSV A2 (consolidation score, 4.9 ± 0.8) ($P < 0.05$). After 6 days of I^- supplementation, treated lambs had significantly increased [I^-] in tracheal ASL compared with nonsupplemented control lambs (Figure 2B). These findings suggest that I^- administration is associated with reduced RSV disease severity.

I^- Effects on RSV M37 Infection in Newborn (2- to 3-day-old) Lambs

Because the pilot studies demonstrated reductions in lung lesions and in RSV RNA levels, additional studies were completed in 2- to 3-day-old lambs to further investigate the extent to which I^- reduces RSV severity in newborn lambs. The virus strain RSV Memphis 37 (M37) was used in these studies because M37 is less laboratory adapted than RSV A2, and the lung disease caused by M37 is very similar in lambs and human infants.

Effect of KI on clinical parameters, lesions, and bronchoalveolar lavage fluid. There was a significant reduction in the incidence and severity of enhanced expiratory effort (forced expiration) in lambs receiving KI treatment at Days 2, 3, 4, 5, and 6 after inoculation (Figure 3A) compared with RSV-inoculated lambs without KI prophylaxis. Control lambs showed no increased expiratory effort. Among virus-treated lambs, 2 of 12 KI-treated RSV-inoculated lambs exhibited mildly increased expiratory effort, whereas 9 of 12 lambs given RSV without KI treatment had increased expiratory effort that was moderate to marked. There were no significant differences between groups of lambs (RSV M37 without KI treatment; RSV M37 with KI treatment; control) in weight gain, temperature, or respiratory rate. Lung lesions were significantly reduced in KI-treated lambs compared with untreated RSV-inoculated lambs (Figure 3B). Gross lesions were present in 11 of 12 of lambs receiving RSV M37 without KI; these were marked in 7 of 12 and mild in 4 of 12 lambs. In contrast, 2 of 12 KI-treated lambs receiving RSV M37 had marked gross lesions, 9 of 12 had mild lesions, and 1 of 12 had no lesions. To evaluate the effect of KI treatment on the inflammatory cell populations within the lung, bronchoalveolar lavage fluid (BALF) differential cell counts were performed. In newborn lambs, no statistically significant differences were detected between groups. However, there was a trend for the RSV-infected lambs that received KI treatment to have an increased proportion of lymphocytes ($P = 0.096$; ANOVA). Consolidation scores determined by histopathology for lambs inoculated with RSV M37 lacking KI treatment (0.64 ± 0.32) were not significantly altered from lambs treated with KI (0.70 ± 0.29); controls (no RSV; no KI) lacked lesions

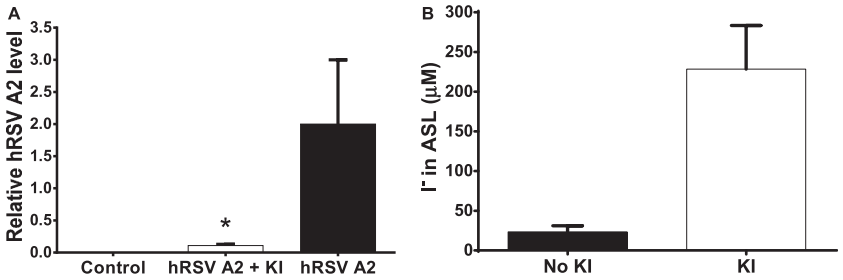

Figure 2. Effect of oral KI supplementation on human respiratory syncytial virus (hRSV) strain A2 mRNA levels and airway surface liquid (ASL) [I^-]. Respiratory syncytial virus (RSV) replication (*A*) and [I^-] in tracheal ASL (*B*) were evaluated 6 days after inoculating lambs with hRSV A2; the indicated groups of animals also received daily doses of KI (1.8 mg/kg BW). (*A*) RSV mRNA signal normalized to total RNA loaded per RT-qPCR: control, $n = 5$; RSV+KI, $n = 5$; RSV, $n = 5$. (*B*) No KI, $n = 5$; KI, $n = 5$. *Error bars* = SEM. $^*P < 0.05$.

(0.00 consolidation score). Lambs receiving dapsone, KI, and RSV had significantly increased levels of expiratory effort, gross and histologic lesion scores compared with control and KI-treated lambs (no dapsone), and increased gross and microscopic lesions compared with M37 alone (no KI or dapsone) (Figure 3A–3D). The findings demonstrate that KI treatment reduces some, but not all, clinical parameters of RSV infection and that inhibition of LPO (with dapsone) eliminates the anti-RSV effects of the Duox/LPO enhanced with KI. Dapsone itself did not affect RSV infectivity *in vitro* (Figure E5).

Effect of KI on RSV replication and cytokines. By immunohistochemistry, RSV viral antigen abundance was significantly reduced in epithelial cells lining alveoli and airways (bronchioles and bronchi) in KI-treated lambs compared with RSV-inoculated lambs with no KI treatment (Figures 3C–3E). In lambs receiving dapsone, KI, and RSV had significantly increased levels of RSV mRNA and antigen compared with control and KI-treated lambs (no dapsone) and also compared with M37 alone (no KI or dapsone) (Figures 3C and 3D). Levels of RSV N gene mRNA and of immune and inflammatory gene mRNAs were assessed by RT-qPCR (37–40). RSV mRNA levels trended lower in KI-treated lambs but not significantly (Figure E3). IL-8 mRNA levels were significantly reduced in KI-treated lambs compared with RSV-inoculated lambs lacking KI (Figure E3). Also, IFN-γ mRNA levels were significantly higher in KI-treated lambs compared with lambs receiving RSV M37 without KI treatment (Figure E3). Control lambs lacked RSV mRNA and increases or alterations in innate and adaptive immunity genes. The levels of IFN-β, monocyte chemotactic protein 1α, macrophage inflammatory protein 1α, RANTES, sheep β-defensin 1, surfactant protein A, TGF-β, and programmed cell death 1 ligand 1 mRNA in KI-treated lambs trended higher compared with lambs inoculated with RSV M37 without KI prophylaxis. In contrast, RNA levels of RSV and mRNA levels of Clara cell 10-kD protein, IL-6, IL-10, IFN-γ–induced protein 10, monocyte chemotactic protein 2, macrophage inflammatory protein 1β, surfactant protein D, and TNF-α trended higher for lambs receiving RSV M37 compared with RSV-inoculated lambs treated with KI. Further details on the RT-qPCR procedure are provided in the online supplement. These findings demonstrate that KI treatment is associated with reductions in some parameters of RSV infection.

I^- Effects on RSV M37 Infection in 3-Week-Old Lambs

It is possible that with increased lung maturation, the Duox or LPO enzymes or other components of the Duox/LPO/halide system undergo or exhibit increased expression and function compared with newborn lungs. Therefore, the extent to which KI treatment reduces RSV disease severity in 3-week-old lambs was determined.

Effect of KI on clinical parameters, lesions, and BALF. Lung lesions were less frequent in 3-week-old lambs with KI treatment (8.6 ± 1.9) compared with RSV-inoculated lambs lacking KI (4.9 ± 1.6), but this difference was not significant; control animals lacked lesions. Consolidation scores determined by histopathology for 3-week-old lambs inoculated with RSV M37 and lacking KI treatment (1.1 ± 0.6) trended higher than those from lambs treated with KI (0.70 ± 0.7) but lacked statistical significance; controls (no RSV) lacked lesions (0.00 consolidation score). Clinically, there were no significant differences in expiratory effort among the control or treatment groups in 3-week-old lambs. There were no significant differences between groups of 3-week-old lambs (RSV M37 without KI treatment; RSV M37 with KI treatment; control) in weight gain, temperature, heart and respiratory rates, and expiratory effort. To evaluate the effect of KI treatment on inflammatory cell populations within the lung, differential cell counts were performed on BALF. In 3-week-old animals, KI treatment significantly reduced the proportion of macrophages within BALF in RSV-infected lambs. No significant differences were detected in the populations of other cells, although there was a trend for the KI-treated lambs to have a higher proportion of lymphocytes ($P = 0.057$) compared with RSV-infected lambs that did not receive treatment (Figure 4A). Comparison between the two RSV-infected groups and uninfected controls were not possible in this age group because too few control lamb samples were present.

Effect of KI on RSV replication. RSV titers in BALF were significantly reduced in 3-week-old lambs treated with KI compared with lambs inoculated with RSV lacking KI (Figure 4B). Control lambs (receiving no RSV or KI) lacked viral titers. RSV viral antigen abundance determined by immunohistochemistry was significantly reduced in the epithelial cells lining the airways (bronchi and bronchioles) and the alveoli in 3-week-old lambs receiving KI compared with RSV-inoculated lambs lacking KI (Figure 4C). The levels of RSV nucleoprotein mRNA measured by RT-qPCR were significantly reduced in 3-week-old lambs receiving KI compared with RSV-inoculated lambs lacking KI (Figure 4D).

Ontologic Expression of Duox1, Duox2, and LPO Genes in Lamb Lung

Because the lung of newborns continues to develop and mature after birth and because expression of Duox1, Duox2, and LPO is essential for converting I^- into hypoiodous acid, the extent of expression of these genes during ontogeny was measured by RT-qPCR using total RNA isolated from lung tissues collected from lambs at various

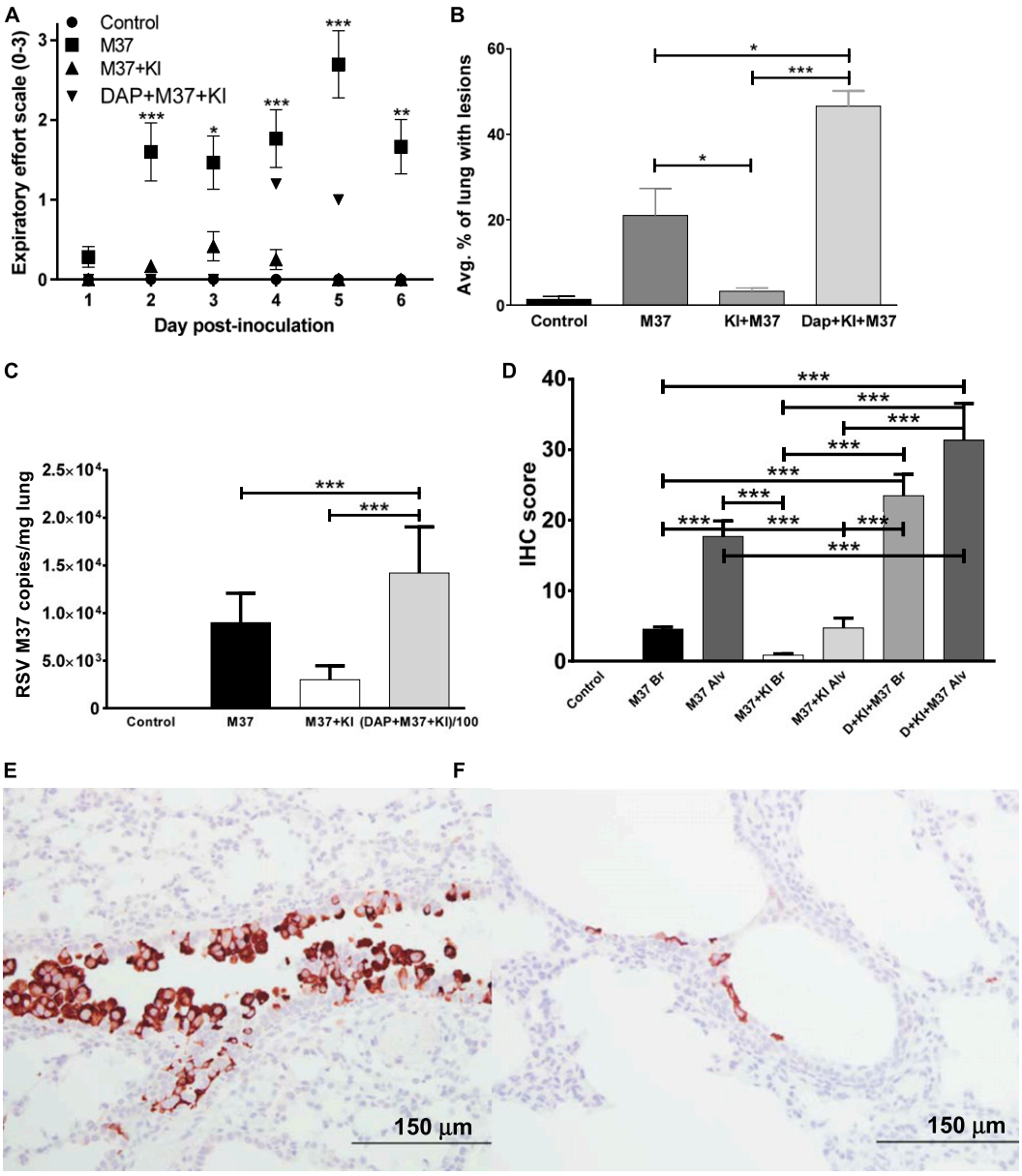

Figure 3. The effect of KI on RSV M37 infection newborn (2- to 3-d-old) lambs. (*A*) Enhanced expiratory effort (forced expiration). Lambs inoculated with RSV M37 and receiving KI had significantly reduced expiratory efforts at Days 3, 4, and 5 after inoculation compared with RSV-inoculated lambs lacking KI and control lambs. Control group, $n = 6$; M37 group, $n = 11$; M37 + KI group, $n = 10$. *Error bars* = SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (*B*) Lambs receiving KI had significantly reduced gross lesions compared with RSV-inoculated lambs lacking KI and control lambs. RSV M37–infected lambs receiving KI and dapsone had significantly increased gross lesions compared with control and RSV-infected (no KI). Control group, $n = 14$; M37 group, $n = 11$; M37 + KI group, $n = 10$; M37 + KI + dapsone group, $n = 5$. *Error bars* = SEM. $^{*}P < 0.05$; $^{***}P < 0.001$. The minor control group lesions were found (by immunohistochemistry) to not be RSV. (*C*) Lambs receiving KI had a trend of reduced RSV M37 mRNA compared with lambs lacking KI, whereas lambs receiving RSV M37, KI, and dapsone had significantly increased RSV M37 mRNA levels compared with lambs treated with M37 alone and M37 + KI. *Error bars* = SEM. $^{*}P < 0.05$; $^{***}P < 0.001$. (*D*) Newborn lambs inoculated with RSV M37 and receiving KI had significantly reduced levels of viral antigen in alveolar regions compared with RSV-inoculated lambs lacking KI. In bronchioles, RSV M37 antigen was not significantly altered; control lambs lacked RSV antigen. RSV M37-infected lambs receiving KI and dapsone had significantly increased RSV antigen compared with control and RSV-infected (no KI). Control group, $n = 14$; M37 group, $n = 11$; M37 + KI group, $n = 10$; M37 + KI + dapsone group, $n = 5$. *Error bars* = SEM. $^{*}P < 0.05$; $^{***}P < 0.001$. (*E*, *F*). Lung from newborn lambs infected with RSV M37 and stained for RSV antigen. (*E*) Lung of lamb lacking KI administration in which there is abundant RSV antigen (*brown*) in most airway epithelial cells. (*F*) Lung of lamb that received KI; a few cells contain RSV antigen.

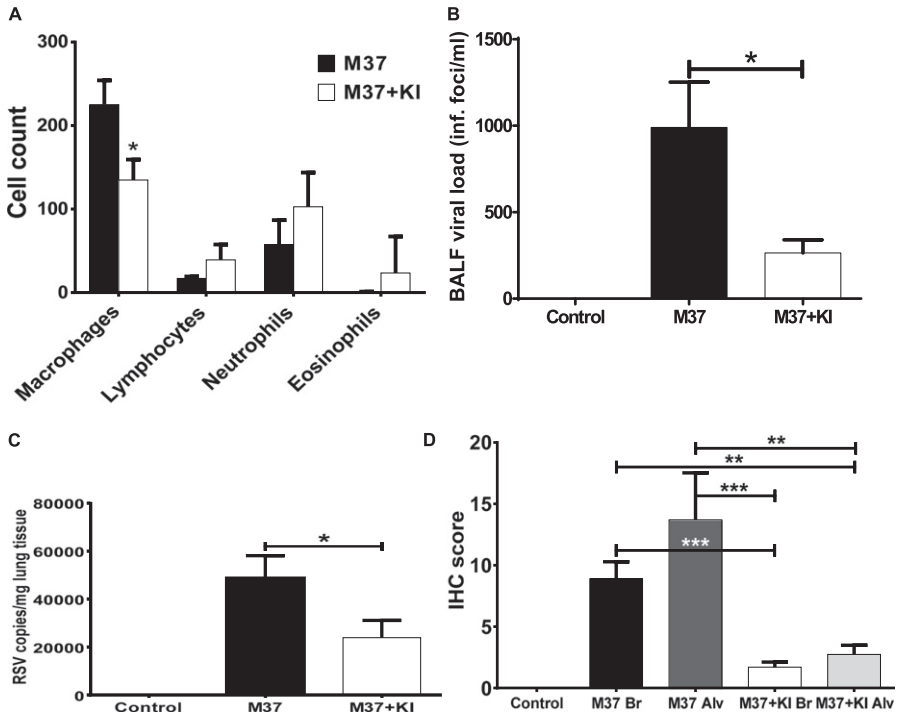

Figure 4. The effect of KI on RSV M37 infection in 3-week-old lambs. (*A*) Effect of KI treatment on the cellular composition of bronchoalveolar lavage fluid (BALF) in RSV-infected 3-week-old lambs. Cytospin preparations of BALF were stained with modified Wright's, and 300 cell differentials were performed to assess relative differences in populations of inflammatory cells. Values are expressed as mean ± SD. *$P < 0.01$ based on ANOVA followed by Tukey-Kramer multiple comparisons test. (*B*) (BALF) titers of RSV in 3-week-old lambs. Lambs receiving KI had significantly less viable RSV than lambs lacking KI. Control lambs lacked RSV titers. Control group, $n = 4$; M37 group, $n = 5$; M37 + KI group, $n = 5$. *Error bars* = SEM. *$P < 0.05$. (*C*) RSV M37 mRNA levels in lungs of lambs receiving KI trended toward significant reductions compared with RSV-inoculated lambs lacking KI; controls lack RSV mRNA. Control group, $n = 8$; M37 group, $n = 10$; M37 + KI group, $n = 10$. *Error bars* = SEM. *$P < 0.05$. (*D*) Three-week-old lambs receiving KI had significantly reduced levels of RSV antigen in bronchiolar and alveolar regions compared with RSV-inoculated lambs lacking KI; controls lacked RSV antigen. Control bronchioles and bronchi (Br), $n = 8$; control alveoli (Alv), $n = 8$; M37 Br, $n = 30$; M37 Alv, $n = 30$; M37 + KI Br, $n = 30$; M37 + KI Alv, $n = 30$. *Error bars* = SEM. **$P < 0.01$; ***$P < 0.001$. IHC, immunohistochemistry.

time points during gestation and compared with adult expression levels as described previously (41, 42). Expression of LPO and Duox1 was low preterm, at gestational Days 115 and 130, and at birth, whereas expression was markedly increased in adults (Figures 5A and 5B). Expression of Duox2 was increased at Day 115 of gestation, decreased progressively with gestational age, and was markedly increased in adults (Figure 5C). These data indicate that Duox1, Duox2, and LPO are expressed in the airways of newborn lambs, albeit at lower levels than in adult sheep.

Discussion

Viral respiratory tract infections are common and can be life threatening, and there are no fully effective therapies or approved vaccines. Overall our findings indicate that the use of high-dose KI supplementation *in vivo* lessens the severity of RSV infections in newborn and 3-week-old lambs through the augmentation of mucosal oxidative defenses.

There are several animal models of RSV infection, and each has unique features. Lambs, like infants, have submucosal glands, which produce LPO and express sufficient levels of Duoxs and halide transport systems (23, 24) to support formation of the pseudohypohalide $OSCN^-$ or, in the presence of sufficient I^-, HOI. Rodents lack significant submucosal gland formation in the intrapulmonary airways and therefore lack sufficient LPO production for a fully functional oxidative defense system within the airways (31). In addition, lambs are susceptible to infection by several strains of RSV, including hRSV M37, which was used in this study (27–30, 33–36, 41). Gerson and colleagues demonstrated that the ovine airway secretions contain LPO and that a reduction of LPO function by dapsone administration reduces antibacterial activity (23), thus demonstrating that the ovine airway has a functional LPO-dependent antibacterial system. The present study further demonstrates that the ovine ASL contains SCN^- and for the first time shows that intravenous or intragastric delivery of I^- increases ovine ASL [I^-]. The results also suggest that the upper airway of lambs recapitulates the halide-transporting activity of the human nasal mucosa. Furthermore, this study is the first to show reductions in RSV disease severity after KI supplementation. In addition to lacking significant submucosal glands, mice do not have sodium/iodide symporter (NIS) in the trachea (B. Banfi, unpublished observations) and thus would require enormous amounts of KI to reach the air surface liquid I^- concentrations attained in lambs and humans receiving much less.

Newborn lambs receiving KI exhibited reduced levels of expiratory effort, gross lesions, RSV antigen distribution, and a trend of reduced RSV N gene mRNA levels. KI treatment in the older, 3-week-old lambs was associated with reduced RSV titers in BALF together with reduced RSV antigen load by immunohistochemistry and reduced RSV nucleoprotein mRNA levels by RT-qPCR. Microscopic lesions (consolidation scores) were not significantly reduced with KI treatment in newborn or 3-week-old lambs, although there was a trend toward reduced lesions in the 3-week-old lambs. Thus, although not all measured parameters of RSV infection were altered by KI treatment, reductions in viral parameters (e.g., viral titer, antigen, and RNA levels) and clinical features (reduced expiratory effort) are of particular significance in lessening RSV disease severity. Mechanistically, administration of dapsone with RSV and KI in newborn lambs reversed the effects of the KI treatment and resulted in disease severity equal to and often significantly greater than M37 alone (no KI or dapsone). HOI and/or hypoiodite (OI^-) production is very difficult to measure *in vivo* because such reactive oxygen species have very short molecular half-lives in general (12, 14). Thus, the extent to which

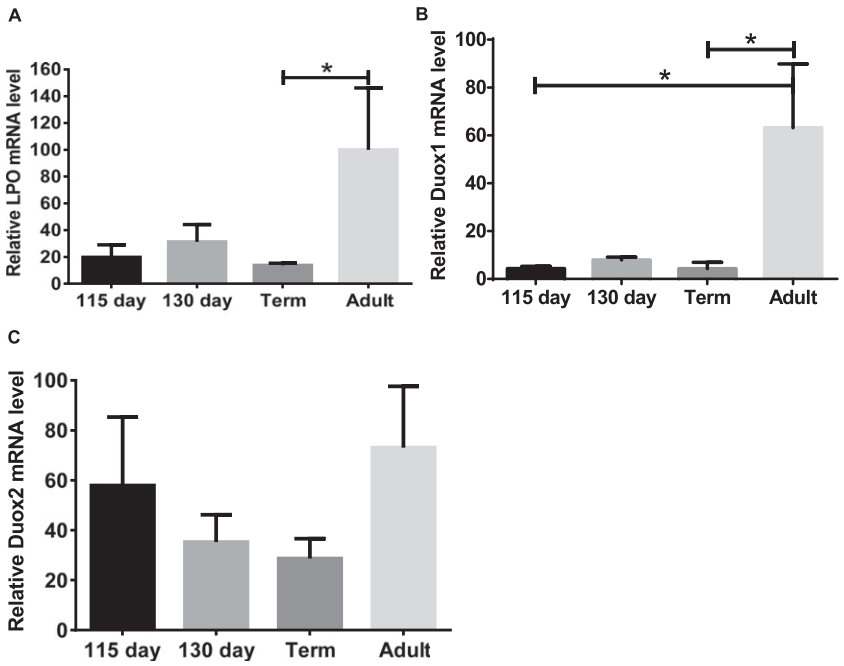

Figure 5. Ontogeny of lung expression of lactoperoxidase (LPO) and dual-functioning oxidases (Duox)1 and Duox2 in lambs as assessed by RT-qPCR. (*A*) Expression of LPO mRNA at various stages of lamb development: 115 days of gestation group, $n = 5$; 130 days of gestation group, $n = 4$; term lamb group, $n = 4$; adult lamb group, $n = 4$. *Error bars* = SEM. *$P < 0.05$. (*B*) Expression of Duox1 mRNA at various stages of lamb development. (*C*) Expression of Duox2 mRNA at various stages of lamb development: 115 days of gestation group, $n = 3$; 130 days of gestation group, $n = 3$; term lamb group, $n = 4$; adult lamb group, $n = 4$. *Error bars* = SEM. *$P < 0.05$.

HOI^- was formed in the ASL of the KI-treated lambs in this study is not known. However, because dapsone inhibits LPO and because LPO is essential for catalyzing the reaction of KI and H_2O_2 to HOI, these findings suggest that *1*) the Duox/LPO system is essential to the anti-RSV activity seen in these studies, and *2*) KI itself is not sufficient for the anti-RSV activity. These findings are consistent with *in vivo* studies that demonstrated significant anti-RSV activity by HOI and a lack of direct RSV killing by KI, LPO, or H_2O_2 individually (24). Dapsone has been used in sheep to inhibit LPO activity, which allowed increased colonization by a gram-negative bacterial pathogen in the respiratory tract (23). Also, lambs in this study demonstrated high ASL [I^-] levels when receiving daily KI. It is also possible that there are other nonoxidative properties of KI that result in anti-RSV activity, such as its efficacy as an expectorant agent (43), but expectorants are generally not effective in reducing RSV replication.

KI treatment reduced disease severity to a greater degree in 3-week-old lambs than in newborn lambs, as reflected by the large reductions in titer, antigen, and viral mRNA levels (viral mRNA levels trended lower in KI-treated 2- to 3-day-old lambs but were not significantly reduced despite a larger group number). Although the reason for this is not fully understood, we speculate that the respiratory tracts of 3-week-old lambs are more mature and have more fully differentiated epithelia than newborns and therefore have more available Duox/LPO expression and activity to convert KI to HOI than do newborn lambs. Alternatively, we have shown previously that younger lambs (especially preterm) exhibit increased disease severity compared with older lambs. Therefore, it could be that RSV disease severity was slightly less in the 3-week-old lambs as reflected by their lack of increased expiratory effort compared with the markedly enhanced expiratory efforts observed in the younger lambs. Thus, if RSV disease severity is less in older lambs than in newborn lambs, the effects of KI treatment may appear more pronounced in older lambs. It is also possible that older lambs may have enhanced innate and adaptive immune responses that synergize with KI treatment.

KI was delivered prophylactically in this study; thus, iodide levels in the ASL were high at the time of RSV nebulization. Because of this, it was suspected that nebulized particles would come into contact with HOI present on the ASL, inhibiting initial RSV attachment and infection. However, although prophylactic administration of KI reduced many parameters of RSV infection and RSV disease severity, it did not completely prevent RSV infection; KI-treated lambs developed some gross and histologic lesions, and viral RNA and antigen were present (albeit at significantly reduced levels) compared with untreated animals. It is possible that not all RSV virions were inactivated on initial contact with the respiratory mucosa during nebulization. More specifically, it is likely that HOI levels are higher in the nasal mucosa, trachea, and bronchi, all three of which are anatomical locations where submucosal glands (and LPO) are present, than in the distal bronchioles and alveoli (both lacking in submucosal glands). Thus, nebulized virions deposited onto the upper airways may be damaged or destroyed by HOI, whereas those virions deposited onto bronchioles or alveoli may have evaded HOI. Deposition and infection by RSV at these distal locations may allow some level of viral infection because the distal bronchioles and alveoli are important sites of RSV replication. In other words, there may be a mismatch between sites of HOI production and some areas of the airways and alveoli that are susceptible to RSV infection.

Because KI was delivered daily after initial infection, it is possible that HOI has effects on RSV virions produced in the lung after RSV nebulization. KI treatment may impair or damage virus released from respiratory mucosa during replication cycles several days after inoculation of RSV. Prophylactic protection by KI, as demonstrated in this study, might be effective in preventing the spread of RSV to noninfected individuals treated with KI. It is also possible that KI treatment might have some beneficial effect in individuals infected with RSV. Further studies are needed to optimize KI as a therapeutic, including determining its effectiveness with different routes of infection (e.g., intratracheal, intrabronchial, or aerosolized RSV), its activity against different viral and bacterial pathogens, how to optimize the dose and timing of KI administration,

and the effects of inhibition of various components of the Duox/LPO/halide system.

In a previous study, we showed that $[I^-]$ increased in the upper airway secretions of human subjects after oral intake of KI (24). In the current study, through the use of the lamb model, we determined that I^- was secreted not only in the upper airways but also in the trachea after I^- supplementation. In humans and lambs, the $[I^-]$ was found to be more than 20-fold higher in ASL than in serum. The mechanism responsible for secretion of I^- in the airways is not known. Fragoso and colleagues have reported that NIS is expressed in the submucosal glands of airways, where it is localized to the basolateral membrane of epithelial cells (16). On the apical side of the epithelium, I^- may be exported through I^-–permeable ion channels or anion transporters. Among the apical anion channels of airway epithelia, Ca^{2+}–activated Cl^- channels and the cystic fibrosis transmembrane conductance regulator are known to be I^- permeable (44). Furthermore, the anion transporter pendrin has been detected in the apical membrane of airway epithelial cells (20). NIS, cystic fibrosis transmembrane conductance regulator, Ca^{2+}–activated Cl^- channels, and pendrin have also been implicated in the secretion of SCN^-. The fact that the increased ASL $[I^-]$ is accompanied by reduced SCN^- levels supports the notion that the same ion transporters are involved in SCN^- and I^- secretion in the airways.

Previous studies have shown that lambs express all components of this mucosal oxidative defense system at levels sufficient for activity in the airways (23), and our results indicate that prophylactic administration of KI reduces RSV disease severity. These findings have implications for the use of KI in infants against RSV or other respiratory pathogens because KI is relatively inexpensive, is stable, can be readily transported, and does not require refrigeration or specialized storage. The extent to which KI treatment may reduce RSV disease severity in preterm and premature lambs and in lambs was not assessed in this study. RSV disease in humans often occurs in infants born prematurely and therapies are needed, but additional studies are required to better understand the ontogeny and activity of the components of the Duox/LPO/halide host defense system in the preterm lung. Overall our findings indicate that the use of high-dose KI supplementation *in vivo* lessens the severity of RSV infections, potentially through the augmentation of mucosal oxidative defenses. ■

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