

# Moderate tidal volumes and oxygen exposure during initiation of ventilation in preterm fetal sheep

Noah H. Hillman<sup>1</sup>, Timothy J. Moss<sup>2</sup>, Ilias Nitsos<sup>2</sup> and Alan H. Jobe<sup>1,2</sup>

**BACKGROUND:** Preterm infants often receive mechanical ventilation and oxygen at birth. Exposure to large tidal volumes ( $V_T$ s) at birth causes lung inflammation, and oxygen may amplify the injury. We hypothesized that normal  $V_T$  ventilation at birth causes lung injury that is exacerbated by 95% oxygen.

**METHODS:** The head and chest of anesthetized preterm fetal sheep (129 ± 1 d gestation) were surgically exteriorized while maintaining the placental circulation. Fetuses were randomized to four groups with either  $V_T$  ventilation to 6 ml/kg or continuous positive airway pressure of 5 cm H<sub>2</sub>O, and either 95%O<sub>2</sub>/5%CO<sub>2</sub> or 95%N<sub>2</sub>/5%CO<sub>2</sub>. Age-matched fetuses were used as controls. After a 15-min intervention, the fetal lamb was returned to the uterus for 1 h 45 min.

**RESULTS:** In ventilated lambs,  $V_T$  was 6.2 ± 0.4 ml/kg at 15 min. Ventilation increased proinflammatory cytokines as compared with controls and lambs on continuous positive airway pressure, with recruitment of primarily monocytes to bronchoalveolar lavage fluid. Early response protein 1 was activated around the bronchioles in  $V_T$ -ventilated animals. The 15-min oxygen exposure did not change inflammatory mediators or other markers of lung and oxidative stress.

**CONCLUSION:** A  $V_T$  of 6–7 ml/kg at birth increased early markers of injury and lung inflammation. Brief exposure to 95% oxygen did not alter lung inflammation.

The initiation of ventilation at birth is unique because the fetal lung must rapidly transition from fluid-filled airspaces to gas exchange to sustain life (1). Approximately 10% of newborns need some assistance with this transition, and the majority of preterm infants ≤1,500 g receive some assisted ventilation (2,3). Many preterm infants are exposed to positive-pressure ventilation with large tidal volumes ( $V_T$ s) and oxygen, and this can cause airway epithelial injury and lung inflammation (4–6). Recent clinical studies of resuscitation demonstrate that clinicians are not able to regulate positive end expiratory pressure (PEEP), peak inspiratory pressures, or  $V_T$  given to infants (or in simulation) reliably, and the delivery of high  $V_T$  is frequent (6,7). The initiation of ventilation in preterm sheep with escalating  $V_T$  to 15 ml/kg stretches the airways and causes airway epithelial injury and diffuse lung inflammation (8–10). Large  $V_T$  ventilation at birth also permits plasma and interstitial

proteins to move into airspace, which decreases the response to subsequent surfactant treatment (11). The path to bronchopulmonary dysplasia may begin with the initiation of ventilation because large  $V_T$  at birth increases acute-phase response genes involved in inflammation, angiogenesis, vascular remodeling, and apoptosis within the lung (10). It is hypothesized that lower  $V_T$  during resuscitation, similar to the normal  $V_T$  of 5 ml/kg of spontaneously breathing infants, would cause less injury.

The 2010 International Liaison Committee on Resuscitation guidelines recommend that term infants be resuscitated with room air instead of oxygen (2) given that resuscitation with 100% oxygen can delay the first spontaneous breath and may increase mortality as compared with room air resuscitation (12). Unfortunately, preterm infants often do not respond to resuscitation with room air, with the majority of preterm infants requiring oxygen concentrations of about 40% to maintain appropriate oxyhemoglobin saturations and heart rates during the first minutes of life (13,14). Extremely preterm infants resuscitated with 90% oxygen had increased markers of oxidative stress, inflammation, and risk of bronchopulmonary dysplasia as compared with infants resuscitated with 30% oxygen (15). Exposure to high oxygen concentrations may also have a direct effect on the gene expression patterns within the epithelial cells (16). It is still unclear if a brief exposure to oxygen during resuscitation can contribute to lung inflammation and alter gene expression in preterm infants.

Because it is difficult to isolate the individual components of resuscitation from subsequent ventilation at birth, we have used a premature fetal sheep model to maintain placental support during ventilation to allow time for markers of injury to develop (8,10). Although we previously used an escalating  $V_T$  to 15 ml/kg for 15 min to cause lung injury, Brew *et al.* recently demonstrated modest anatomic injury at 24 h following 2-h  $V_T$  ventilation to 5 ml/kg with repair by 15 d (17). To test the hypothesis that initiation of ventilation with a lower  $V_T$  would not injure the preterm lung, we have evaluated a  $V_T$  below the normal  $V_T$  of 8 ml/kg of spontaneously breathing preterm lambs on continuous positive airway pressure (18). We asked if the lower  $V_T$  caused activation of acute-phase responses and inflammation. In addition, we tested whether exposure to 95% oxygen for 15 min changed lung inflammation and markers of oxidative stress.

<sup>1</sup>Division of Pulmonary Biology, Cincinnati Children's Hospital Medical Center, University of Cincinnati, Cincinnati, Ohio; <sup>2</sup>School of Women's and Infants' Health, University of Western Australia, Perth, Australia. Correspondence: Noah H. Hillman (Noah.Hillman@cchmc.org)

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RESULTS

All lambs ( $n = 5\text{--}6/\text{group}$ ) survived the fetal interventions and *in utero* recovery, and there were no differences in the venous blood gas values between ventilation groups before or after the intervention (Table 1). The target  $V_T$  of 6–7 ml/kg was achieved in the ventilated groups by 6 min ( $6.0 \pm 0.1$  ml/kg) but required the maximal peak inspiratory pressure of 40 cm H<sub>2</sub>O in all animals. The average  $V_T$  remained stable by 15 min at  $6.2 \pm 0.2$  ml/kg. Total protein levels from the bronchoalveolar lavage fluid (BALF) were low ( $19 \pm 2$  mg/kg) and similar among all ventilation groups and control animals. Injury scoring of hematoxylin and eosin–stained tissues demonstrated minimal epithelial sloughing, hemorrhage, and inflammatory cells for all groups

with no difference between groups (Table 1). Thus, the  $V_T$  of 6.2 ml/kg or the oxygen did not cause microscopic injury.

Lung Inflammation

Ventilation for 15 min increased mRNA for the proinflammatory cytokines (interleukin (IL)-1 $\beta$ , IL-6, IL-8, monocyte chemotactic protein (MCP)-1, and MCP-2) in the lung tissue (Figure 1). Oxygen exposure did not increase these cytokines. Exposure to a PEEP of 5 cm H<sub>2</sub>O with or without 100% oxygen did not increase cytokine mRNA as compared with controls. mRNA values for IL-1 $\beta$ , IL-8, and IL-6 in epithelium from the trachea did not change with ventilation or oxygen exposure (data not shown). MCP-1 protein was detected in the

Table 1. Birth weights,  $V_T$ , venous blood gases, and injury scores

Group	$n$	BW (kg)	$V_T$ at 15 min ml/kg	Before fetal maneuver—venous			After fetal maneuver—venous			Injury score (out of 6)
				pH	pCO <sub>2</sub> (mm Hg)	pO <sub>2</sub> (mm Hg)	pH	pCO <sub>2</sub> (mm Hg)	pO <sub>2</sub> (mm Hg)	
Controls	8	2.9 $\pm$ 0.1	—	—	—	—	—	—	—	1.8 $\pm$ 0.3
CPAP–N <sub>2</sub>	5	3.0 $\pm$ 0.1	No $V_T$	7.26 $\pm$ 0.05	62.4 $\pm$ 6.3	13.8 $\pm$ 2.2	7.22 $\pm$ 0.08	64.2 $\pm$ 10.8	15.7 $\pm$ 2.8	2.1 $\pm$ 0.2
$V_T$ –N <sub>2</sub>	6	3.5 $\pm$ 0.1	6.2 $\pm$ 0.4	7.22 $\pm$ 0.03	62.2 $\pm$ 3.9	13.2 $\pm$ 1.6	7.21 $\pm$ 0.03	60.8 $\pm$ 3.8	14.8 $\pm$ 1.8	2.0 $\pm$ 0.3
CPAP–O <sub>2</sub>	5	3.0 $\pm$ 0.1	No $V_T$	7.30 $\pm$ 0.01	55.0 $\pm$ 0.8	15.4 $\pm$ 1.2	7.32 $\pm$ 0.02	49.3 $\pm$ 2.7	15.2 $\pm$ 2.3	2.1 $\pm$ 0.4
$V_T$ –O <sub>2</sub>	6	2.8 $\pm$ 0.1	6.2 $\pm$ 0.4	7.25 $\pm$ 0.02	63.2 $\pm$ 5.2	13.4 $\pm$ 2.0	7.25 $\pm$ 0.05	60.0 $\pm$ 6.4	18.1 $\pm$ 1.2	2.3 $\pm$ 0.2

BW, birth weight; CPAP, continuous positive airway pressure;  $V_T$ , tidal volume.

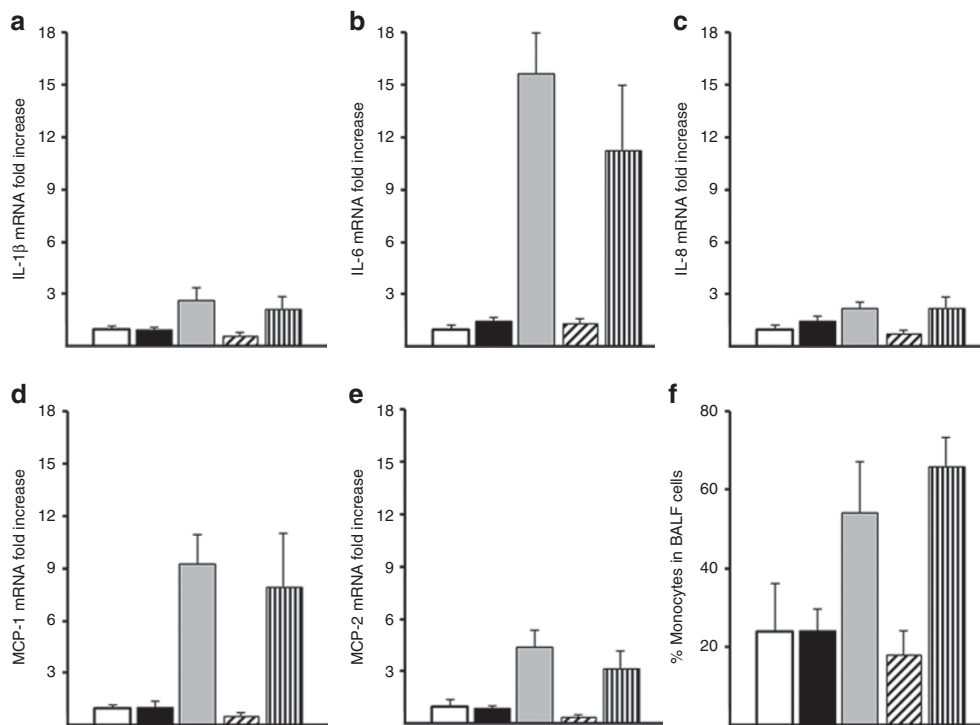


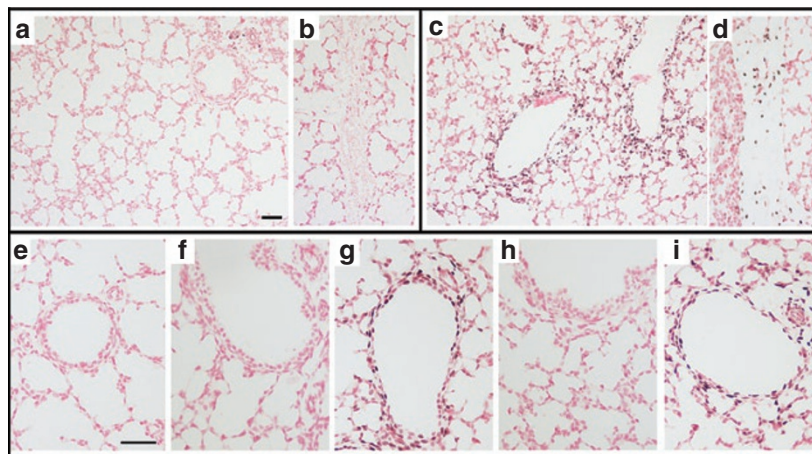
Figure 1. Tidal volume ( $V_T$ ) ventilation increased mRNA for proinflammatory cytokines. (a) Interleukin (IL)-1 $\beta$ , (b) IL-6, (c) IL-8, (d) monocyte chemotactic protein-1 (MCP-1), and (e) MCP-2 mRNA increased with mechanical ventilation ( $V_T$ ) ( $P < 0.05$  vs. controls and lambs receiving only continuous positive airway pressure (CPAP)). There were no additional effects of oxygen (O<sub>2</sub>) (vertical striped bar) on the increased mRNA from mechanical ventilation with nitrogen (N<sub>2</sub>) (gray bars). Animals receiving only CPAP of 5 cm H<sub>2</sub>O, with N<sub>2</sub> (black bars) or O<sub>2</sub> (dashed bars) did not have increased cytokine mRNA. The mRNA was measured from complementary DNA using RT-PCR and reported as fold increase over the mean of controls. (f) Monocytes in BALF, reported as percentage of total cells, increased in lambs receiving mechanical ventilation ( $P < 0.05$  vs. controls and lambs receiving only CPAP). White column, controls; black column, CPAP nitrogen; gray column,  $V_T$  nitrogen; diagonal stripes, CPAP oxygen; vertical stripes,  $V_T$  oxygen. BALF, bronchoalveolar lavage fluid.

cytoplasm of peripheral lung tissue cells in animals receiving  $V_T$  ventilation (data not shown). Inflammatory cells increased in the BALF of lambs receiving mechanical ventilation, with an overall increase in the percentage of monocytes (Figure 1). As compared with the other cytokines, the induction of IL-8 was modest (two- to threefold), and the neutrophil recruitment to the BALF was surprisingly low and occurred in lambs with the higher IL-8 mRNA values. Although occasional CD3<sup>+</sup> cells were present in the lung tissue, there was no increase in recruitment of these T cells to the lung with ventilation or oxygen exposure.

#### Acute-Phase Response Genes

Early growth response protein 1 (Egr-1) activation in control lambs and lambs receiving a PEEP of 5 cm H<sub>2</sub>O was limited to occasional staining within the blood vessels (Figure 2a,e,f,h). Egr-1 increased in the cells surrounding the smaller airways and the connective tissue with mechanical ventilation (Figure 2c,d) as compared with controls (Figure 2a,b), and the pattern of increased expression was not altered by exposure to oxygen (Figure 2e-i). Blinded scoring confirmed

increased Egr-1 protein in airways and connective tissue with mechanical ventilation (Table 2). There was little to no Egr-1 activation within the distal lung parenchyma (staining score:  $0.13 \pm 0.04$ ) (Figure 2c). Although the Egr-1 protein is abundantly present at 2h after intervention, the mRNA levels of transcription factors downstream of Egr-1 (platelet-derived growth factor A, fibroblast growth factor 2, arachidonate 5-lipoxygenase, epithelial growth factor receptor) were not altered by ventilation; similar effects were found in the case of mRNA for the counter-regulatory protein (NGFI-A binding protein 2) (Table 2). HSP70 mRNA was localized to the bronchial epithelium of surgical controls and fetal lambs receiving only PEEP but was lost from the epithelium of lambs receiving mechanical ventilation. HSP70 mRNA was not increased in the airway smooth muscle in lambs receiving ventilation with a  $V_T$  of 6 ml/kg. Heme oxygenase 1 (HO-1) mRNA from lung tissue did not change in these lambs as compared with that of the surgical controls (Table 3). HO-1 protein was localized within the cytoplasm of occasional cells within the distal parenchyma, but the distribution and number of cells did not change between intervention groups.



**Figure 2.** Early response protein-1 (Egr-1) protein increased around bronchioles with mechanical ventilation. (a,b,e) Control lambs had only occasional Egr-1 staining in the (a) vessels and none was found in (b) intralobar connective tissue (20× original magnification). (c–d) Egr-1 signal increased with mechanical ventilation with nitrogen (N<sub>2</sub>) and was localized to (c) cells around bronchioles and (d) in cells within connective tissue (20× original magnification). There was minimal staining in the distal epithelium. (e–i) Higher-power images of Egr-1 staining. Lambs receiving  $V_T$  ventilation with (g) N<sub>2</sub> and (i) O<sub>2</sub> demonstrate increased Egr-1 surrounding small airways. Continuous positive airway pressure exposure, with (f) N<sub>2</sub> or (h) O<sub>2</sub> did not increase staining. Scale bar = 50 μm.

**Table 2.** Egr-1 protein expression and mRNA for Egr-1-responsive transcription factors

	Egr-1 protein expression			Lung mRNA levels (fold increase over control)				
	+Cells/LPF	Bronchioles (out of 2)	CT (out of 2)	ALOX5	EGFR	FGF2	PDGFA	NAB2
Controls	8 ± 2	0.03 ± 0.03	0.4 ± 0.2	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
CPAP–N <sub>2</sub>	13 ± 3	0.07 ± 0.03	0.5 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	1.3 ± 0.3	1.0 ± 0.1	1.2 ± 0.1
$V_T$ –N <sub>2</sub>	196 ± 19*	1.86 ± 0.07*	1.7 ± 0.1*	0.9 ± 0.1	1.0 ± 0.1	1.2 ± 0.2	1.0 ± 0.1	1.4 ± 0.3
CPAP–O <sub>2</sub>	15 ± 2	0.08 ± 0.05	0.6 ± 0.2	0.8 ± 0.1	0.9 ± 0.2	0.9 ± 0.1	0.9 ± 0.1	0.7 ± 0.1
$V_T$ –O <sub>2</sub>	209 ± 23*	1.83 ± 0.09*	1.7 ± 0.1*	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.0 ± 0.2	1.2 ± 0.1

ALOX5, arachidonate 5-lipoxygenase; CPAP, continuous positive airway pressure; CT, connective tissue; EGFR, epithelial growth factor receptor; Egr-1, early response protein-1; FGF2, fibroblast growth factor 2; LPF, low-power field; NAB2, NGFI-A binding protein 2; PDGFA, platelet-derived growth factor A;  $V_T$ , tidal volumes.

\* $P < 0.05$  vs. control and CPAP animals.

**Table 3.** Heme-oxidase 1 and superoxide dismutase 1 in lung

	Lung tissue			BALF
	HO-1 mRNA (fold increase)	HO-1 protein (cells/LPF)	SOD1 mRNA (fold increase)	SOD activity (U/ml)
Controls	1.0 ± 0.12	28 ± 6	1.0 ± 0.19	0.19 ± 0.05
CPAP-N <sub>2</sub>	1.0 ± 0.06	50 ± 11	1.0 ± 0.08	0.31 ± 0.07
V <sub>T</sub> -N <sub>2</sub>	0.8 ± 0.10	59 ± 15	0.9 ± 0.04	0.22 ± 0.01
CPAP-O <sub>2</sub>	0.9 ± 0.02	55 ± 10	0.8 ± 0.04	0.10 ± 0.04
V <sub>T</sub> -O <sub>2</sub>	1.0 ± 0.03	42 ± 11	0.9 ± 0.05	0.39 ± 0.08

BALF, bronchoalveolar lavage fluid; CPAP, continuous positive airway pressure; HO-1, heme oxygenase 1; LPF, low-power field; SOD1, superoxide dismutase 1; V<sub>T</sub>, tidal volume.

### Oxidative Response

There were no differences in the inflammatory and acute-phase responses when lambs were exposed to 95% oxygen for the resuscitation. Superoxide dismutase 1 (SOD1) mRNA from peripheral lung tissue did not change in any of the groups as compared with that of the surgical controls (Table 3). Total SOD activity also did not change in the BALF. Hif1 $\alpha$  and Hif2 $\alpha$  proteins are expressed in the fetal lung at 129 d in control animals, with staining in the epithelial cells surrounding the bronchioles and in cells with a distribution similar to type II cells. There was no difference in the expression pattern for Hif1 $\alpha$  and Hif2 $\alpha$  between intervention groups, with or without oxygen, and control animals. Arginase 1 protein also did not change in the epithelium or lung parenchyma with either ventilation or oxygen exposure.

### DISCUSSION

We found that, similar to large V<sub>T</sub> ventilation, ventilation with a V<sub>T</sub> of 6.2 ml/kg for 15 min caused increased expression of acute-phase response genes and lung inflammation that are localized primarily around the bronchioles. The low overall release of total protein into the BALF and lack of airway epithelial sloughing indicated less damage than previously reported for a higher V<sub>T</sub> of 15 ml/kg (5,8). We did not find additional inflammation or markers of oxidative stress when lambs were ventilated for 15 min with 95% oxygen instead of nitrogen.

Ventilation of the preterm lungs with normal V<sub>T</sub> at birth caused lung inflammation and monocyte recruitment to the bronchoalveolar space, which may have been a response to the cytokines MCP-1, MCP-2, and IL-6. Our previous fetal sheep experiments with larger V<sub>T</sub>s demonstrated increased mRNA for IL-1 $\beta$ , MCP-1, IL-8, and IL-6 at 45 min after injury, whereas by 3 h after injury the IL-6 and IL-8 mRNA levels had begun to fall and these returned to baseline by 24 h (8–10). In this study, we did not find an additive effect of oxygen on proinflammatory cytokines. We previously found a small, twofold increase in lung IL-1 $\beta$  mRNA with 100% oxygen use in near-term lambs (140 d gestational age) ventilated with a V<sub>T</sub> of 9 ml/kg for 3 h as compared with lambs ventilated with 21% oxygen, but IL-6 and IL-8 did not change with oxygen in those lambs (4). The addition of 100% oxygen for 30 min to newborn rats ventilated with large V<sub>T</sub>s of 25 or 40 ml/kg caused moderate and inconsistent changes in the mRNA expression of proinflammatory cytokines (19). Newborn rats exposed

to low V<sub>T</sub> ventilation (3.5 ml/kg) for 8 h had increased mRNA for IL-6 and CXCL-2 (homolog to IL-8), but IL-1 $\beta$  did not change (20). The addition of 50% oxygen to low V<sub>T</sub> ventilation caused a modest increase in the IL-6 and CXCL-2 mRNA levels (20). Due to their consistent increases with ventilation, IL-6 or MCP-1 may be appropriate targets for pharmacologic interventions. However, it should be noted that our previous attempts to inhibit inflammation at birth with corticosteroids or specific cytokine inhibitors have been unsuccessful (21,22). Our data demonstrate that inflammation is activated by low V<sub>T</sub> ventilation at birth and may be inevitable in the setting of positive-pressure ventilation.

Ventilation at birth with a low V<sub>T</sub> caused the activation of *Egr-1*, an acute-phase gene responsive to mechanical stretch (23). *Egr-1* is multifunctional and can activate genes ranging from growth factors to coagulation cascades (24). *Egr-1* plays an important role in the initiation of inflammation and mice deficient in *Egr-1* are more resistant to ventilator-induced lung injury (25). The *Egr-1* signal was intense surrounding the bronchioles and in the connective tissue, suggesting airway stretch. Unlike with larger V<sub>T</sub> ventilation or ventilation of newborn lambs after birth, there was little *Egr-1* activation in the peripheral lung tissue, suggesting lung injury was limited to the airways (5,21). *Egr-1* can be activated by hyperoxia, but we did not find activation in lambs receiving only oxygen without V<sub>T</sub> ventilation (26). Although the *Egr-1* protein showed nuclear staining, there were surprisingly no changes in the mRNA for downstream targets of *Egr-1* protein (platelet-derived growth factor A, fibroblast growth factor 2, arachidonate 5-lipoxygenase, and epithelial growth factor receptor). We also did not find changes in the mRNA for NGFI-A binding protein 2, the major counter-regulatory protein for *Egr-1* (24). The lack of these responses could be due to the timing of the intervention or it could also result from the overall immaturity of the fetal lung. We also did not find differences in HO-1, which responds like many acute-phase response elements and is inducible by a variety of stimuli from lipopolysaccharide to hyperoxia (27). We did, however, find a loss of HSP70 mRNA in the epithelium, similar to our previous findings with larger V<sub>T</sub> ventilation, giving additional support to airway stretch during initiation with normal V<sub>T</sub> (5).

The antioxidant enzymes normally increase two- to threefold in the final 10–15% of gestation in many species and parallels the development of the surfactant system (28). Lambs born at 129 d gestational age are just beginning lung maturation and surfactant production. Fetal sheep at 125 d gestational age can generate a modest increase in some markers of oxidative stress (protein carbonyls and myeloperoxidase activity) following exposure to 7 d of intra-amniotic lipopolysaccharide (29). Given that the inflammatory system is immature in preterm lambs, the lambs may not have generated a large response to the oxidative stress (30). Although we only measured jugular PvO<sub>2</sub> levels at the end of resuscitation, the lack of any increase would suggest maintenance of fetal circulation and shunting across the patent ductus arteriosus, thus limiting any systemic increase in PaO<sub>2</sub>.

The majority of animal models showing oxidative stress responses from resuscitation use acute hypoxia before ventilation with oxygen or room air (31). The lack of increased

oxidative responses in our study may result from the lack of preceding hypoxia/hypoperfusion. Resuscitation of newborn piglets with 100% oxygen after acute hypoxia increased MMP-2 activity in the brain as compared with resuscitation with 21% oxygen, but animals exposed to 30 min of 100% oxygen without previous hypoxia did not demonstrate these changes (32). Similar to our findings on SOD, there were no differences found in antioxidant enzymes (SOD, catalase, or glutathione peroxidase) between term lambs ventilated with oxygen and air (33).

We did not find changes in oxygen-sensitive proteins (Hif1 $\alpha$ , Hif2 $\alpha$ , or arginase 1) with a brief oxygen exposure. Hif1 $\alpha$  and Hif2 $\alpha$  are expressed in fetal pulmonary epithelial, smooth muscle, and endothelial cells (34), and help regulate the expression of vascular endothelial growth factor. Prolonged ventilation of premature baboons leads to decreased Hif1 $\alpha$  expression (35). Ventilation of surfactant-deficient very preterm lambs (gestational age 115 d) for 4 h with high oxygen concentrations decreased Hif1 $\alpha$  and Hif2 $\alpha$  protein and decreased vascular endothelial growth factor mRNA (36). Although in our study Hif1 $\alpha$  and Hif2 $\alpha$  were present in the fetal lung, we did not find consistent decreases with this brief period of normal ventilation or with the addition of oxygen. We previously have not seen differences in vascular endothelial growth factor mRNA or vascular endothelial growth factor receptor protein in lambs exposed to short periods of ventilation (5). Arginase 1 competes with nitric oxide synthase for L-arginine, and activation impairs airway muscle relaxation. In newborn rat pups exposed to 50% oxygen for 7 d, arginase 1 is increased in the airway epithelium (37). Short-term exposure to 95% oxygen during fetal ventilation did not affect arginase 1 expression. The lack of changes in markers of oxidative stress and oxygen-sensitive proteins suggests that short-term exposure of preterm infants to oxygen may not increase injury caused by ventilation.

There are several limitations of the study that should be noted. As with many large-animal studies, the number of animals per group was only five–six, so small differences between groups will not be detected. Although our focus was on the evaluations of inflammation, acute-phase genes, and some oxidative markers, other genes may have changed within the model. This fetal sheep model, which maintains placental blood flow and ductal shunting, may have limited the systemic effects of oxygen on the lungs, as the PvO<sub>2</sub> did not change. We used 5% CO<sub>2</sub> in these animals to maintain mild hypercapnia, and our PvCO<sub>2</sub> at end of 15-min ventilation (56  $\pm$  4 mm Hg) was similar to the initial PvCO<sub>2</sub> (58  $\pm$  3 mm Hg). The exposure time of 15 min may also not have been long enough to cause direct oxidant injury to the epithelial cells. We used high peak inspiratory pressures of 40 cm H<sub>2</sub>O and no PEEP to generate a V<sub>T</sub> of only 6–7 ml/kg. We recently demonstrated that PEEP use during resuscitation with high V<sub>T</sub> decreases proinflammatory cytokines twofold (9). PEEP use with normal V<sub>T</sub> at birth might lessen, but not abolish, the negative effects of ventilation.

## Conclusion

Ventilation of the preterm lung at birth with low V<sub>T</sub> leads to airway stretch and lung inflammation. The degree of lung injury is

less than what is seen with higher V<sub>T</sub> ventilation (15 ml/kg), and there is minimal protein leak and airway epithelial disruption. In these lambs, oxygen exposure did not alter the inflammatory response to ventilation. Our results support the avoidance of positive-pressure ventilation, even low V<sub>T</sub>, when possible in preterm infants at birth. Although clinicians should attempt to limit oxygen exposure when possible, the use of supplemental oxygen as needed in preterm infants at birth (2) is supported by a lack of short-term effects of oxygen.

## METHODS

The investigations were approved by the animal ethics committees of the Western Australian Department of Agriculture and Cincinnati Children's Hospital Medical Center.

### Maternal Anesthesia and Surgery

Date-mated Merino ewes with singletons or twins at 129  $\pm$  1 d gestation (early alveolarization stage, term is 150 d in sheep) received ketamine (5 mg/kg) i.m. and xylazine (0.5 mg/kg) i.m. for induction of general anesthesia before halothane (1–2%) anesthesia. Using aseptic techniques, a midline hysterotomy was performed. The head and chest of the preterm lamb were exposed, and the lamb was given 10 mg/kg ketamine i.m.

### Fetal Ventilation Procedure

The fetal lamb was intubated with a cuffed tube and fetal lung fluid was removed with a catheter using gentle suction (8). Each lamb was randomly assigned to four groups ( $n = 5$ –6/group) based on the type of ventilation and the inspired gas mixture. Lambs received either mechanical ventilation for 15 min with a rate 30 breaths/min, inspiratory time 1 s, PEEP 0 cm H<sub>2</sub>O (Bourns BP200 time-cycled, pressure-limited infant ventilator with 12 l/min flow, Bear Medical Systems, Riverside, CA) or continuous positive airway pressure of 5 cm H<sub>2</sub>O with no V<sub>T</sub> (under these conditions the fetal lambs do not breathe spontaneously). Our target V<sub>T</sub> was 6–7 ml/kg with a maximal peak inspiratory pressure limited to 40 cm H<sub>2</sub>O. An in-line flow sensor (Florian Infant Graphics Monitor, Acutronic Medical Systems, Hirzel, Switzerland) was used to continuously measure V<sub>T</sub> and pressures. Lambs were further randomized to one of two heated, humidified gases: oxygen, 95%O<sub>2</sub>/5%CO<sub>2</sub> or nitrogen, 95%N<sub>2</sub>/5%CO<sub>2</sub>. After the 15-min intervention, the fetus was returned to the uterus, the uterus and maternal abdomen were closed, and the ewe recovered from general anesthesia. Two hours after the intervention, the ewe was humanely killed, followed by immediate surgical delivery of the fetus, which was euthanized with pentobarbital (100 mg/kg i.v.). Jugular venous blood gases were measured before the ventilation intervention and after the intervention. Cord blood gases were measured at delivery.

### Lung Processing and BALF Analysis

At autopsy, a deflation pressure–volume curve was measured after air inflation to 40 cm H<sub>2</sub>O pressure (38). BALF of the left lung was collected by repetitive saline lavage. BALF was used for measurement of total protein and differential cell counts (39). Total SOD activity was measured in BALF with a colorimetric activity assay (Arbor Assays, Ann Arbor, MI). Tissues from the right lower lung and tracheal epithelial scrapings were snap frozen. The right upper lobe was inflation fixed with 10% formalin at 30 cm H<sub>2</sub>O and then paraffin embedded (40).

### Quantitative RT-PCR

mRNA was extracted from tissue from the right lung and trachea (5). Complimentary DNA was made using Verso cDNA kit (ThermoScientific, Waltham, MA). Custom Taqman gene primers (Applied Biosystems, Carlsbad, CA) were designed from ovine sequences for arachidonate 5-lipoxygenase, Egr-1, epithelial growth factor receptor, fibroblast growth factor 2, HO-1, IL-1 $\beta$ , IL-6, IL-8, MCP-1, MCP-2, NGFI-A binding protein 2, platelet-derived growth factor A, and superoxide dismutase 1. Quantitative RT-PCR was performed on a 7,300 RT-PCR machine and software (Applied Biosystems). 18S primers were used for

internal loading control, and results are reported relative to the mean for control animals.

### Immunohistochemistry/*In Situ* Hybridization

Immunostaining protocols used 5  $\mu$ m paraffin sections of formalin-fixed tissues (8). Primary antibodies included antihuman early response protein 1 (Santa Cruz Biotechnology, Santa Cruz, CA, 1:250), Hif1 $\alpha$  (internal, 1:500), Hif2 $\alpha$  (internal, 1:1,000), CD3 (Dakocytomation, Glostrup, Denmark, 1:100), or arginase 1 (Abcam, Cambridge, MA, 1:150). *In situ* localization of mRNA was performed with digoxigenin-labeled antisense and sense sheep riboprobes for HSP70 (Roche, Indianapolis, IN) (5). Random, blinded hematoxylin and eosin-stained sections ( $\times$ 10/slide) were scored (0–2) based on hemorrhage, inflammatory cells, and epithelial sloughing (total six points) (5). Random sections ( $\times$ 10/slide) of blinded Egr-1 slides were scored (0–2 for staining around the airways, connective tissue, and peripheral lung tissue). Cells positive for Egr-1 and HO-1 were counted per low-power (10 $\times$ ) field.

### Data Analysis and Statistics

Results are shown as mean (SEM). Statistics were analyzed using InStat (GraphPad, La Jolla, CA) with Student's *t*-test and Mann–Whitney non-parametric tests as appropriate. Significance was accepted as  $P < 0.05$ .

### STATEMENT OF FINANCIAL SUPPORT

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