Lung function in developing lambs: is it affected by preterm birth?

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De Matteo R, Snibson K, Thompson B, Koumoundouros E, Harding R. Lung function in developing lambs: is it affected by preterm birth? J Appl Physiol 107: 1083–1088, 2009. First published August 13, 2009; doi:10.1152/japplphysiol.00129.2009.—Children born before term often have reduced lung function, but the effects of preterm birth alone are difficult to determine owing to iatrogenic factors such as mechanical ventilation. Our objective was to determine the effects of preterm birth alone on airway resistance, airway reactivity, and ventilatory heterogeneity as an index of intrapulmonary gas mixing. Preterm birth was induced in sheep 12 days before term; controls were born at term (~147 days). Lung function was assessed at 8 wk postterm. To assess medium-large airway function we measured airway resistance and reactivity to carbachol. Multiple breath N2 washout (MBW) was used to assess ventilatory heterogeneity in conducting (Scond) and acinar (Sacin) airways. Baseline airway resistance and responsiveness to carbachol were similar in preterm and term lambs; for all animals combined, mean Sacin was 0.29 liter⁻¹ · s⁻¹ and Scond was 0.26 liter⁻¹ · s⁻¹. Males had significantly higher Sacin and Scond than females, indicating poorer gas mixing in small conducting airways; there was no sex difference in Sacin. We conclude that preterm birth per se in lambs does not affect baseline airway resistance, airway responsiveness, or ventilatory heterogeneity as measured by MBW. The observed sex-related differences in airway responsiveness and ventilatory heterogeneity in the conducting airways could help explain sex differences in lung function observed in humans.

Up to twelve percent of all births occur before 37 completed weeks of gestation and are considered preterm (3). Recent studies have shown that preterm birth, especially very preterm birth, increases the risk of having reduced lung function and respiratory symptoms later in life (5, 7). Respiratory effects observed in infants, children, and young adults born very prematurely include low forced expiratory flow rates, wheezing, and bronchial hyperreactivity. Exercise capacity may also be restricted (22, 31). The long-term effects are greatest in those who are born very preterm with prolonged ventilatory support and in those who develop chronic lung disease or bronchopulmonary dysplasia (BPD) (5, 7). Iatrogenic factors that can contribute to adverse lung development include mechanical ventilation, the use of high ventilatory pressures and/or volumes, and the use of high levels of inspired oxygen. Each of these factors may contribute to lung injury and altered lung development that could predispose individuals born preterm to later reductions in lung function (1). Although clinical and population data show that preterm birth can lead to adverse effects on lung function later in life, the underlying processes are unclear. Importantly, the separate roles of prematurity per se and iatrogenic factors associated with respiratory support of the preterm infant are difficult to determine in humans.

In order to identify the effects of preterm birth alone on key aspects of lung function, avoiding confounding effects associated with ventilatory support after birth (e.g., mechanical ventilation, pulmonary exposure to hyperoxic gas), we have developed an ovine model of preterm birth. In this model, referred to as moderate preterm birth, lambs are induced to be born at the earliest gestation age that is possible without the requirement of respiratory support (4, 25, 26). In humans, approximately 80% of preterm births can be considered as moderately severe in that birth occurs between 32 and 37 completed weeks of gestation (12). Using our sheep model of moderately severe preterm birth per se, in the absence of respiratory support, we have shown that in preterm lambs up to 6 wk of age there were transient changes in alveolar size and number, thicknesses of the alveolar septa and blood-gas barrier, and surfactant protein expression (4, 26); there was a persistent increase in the thickness of the epithelium in the alveoli and conducting airways (4) and a persistent change in the proportions of type I and type II alveolar epithelial cells (26). These studies have shown that preterm birth per se has the potential to alter lung development such that later lung function could be impaired. In adult sheep that were born preterm, with no respiratory support, we have shown that lung function and airway wall structure were not significantly altered as a result of preterm birth (24, 25), but there remained the possibility that lung function in younger, developing animals could be altered. The present study addresses the possibility that structural and biochemical changes that we previously observed in the lungs of developing lambs following preterm birth alone could translate into alterations in lung function.

In the present study we have focused on aspects of lung physiology that have the potential to provide new information on how preterm birth itself, in the absence of respiratory support, affects lung function during postnatal development. Our objective was to determine the effects of preterm birth per se on airway resistance, airway reactivity, and ventilatory heterogeneity in developing lambs, at an age that is analogous to human childhood. Our hypothesis was that developing lambs born before full term, with no ventilatory support, would have increased airway resistance and reactivity and would have altered air flow within the conducting and acinar regions of the lung. Owing to reports of poorer lung function (10, 27) and

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respiratory morbidity (9) in male preterm infants compared with females, we have interrogated our data for sex differences.

MATERIALS AND METHODS

Animal groups. The studies were conducted on lambs from dated Border Leicester × Merino ewes that either gave birth at term (control group) or were induced to deliver their lambs before term (preterm group). The term group (n = 13; 7 female, 6 male, 7 singleton, 6 twin) were born spontaneously and vaginally at 145 ± 0 days postmaturing. The preterm group (n = 14; 10 female, 4 males, 6 singleton, 8 twin) were also born vaginally. Preterm birth was induced by administration of Epostane (2α,4α,17-4,5-epoxy-17-hydroxy-4,17dimethyl-3-ketoandrostan-2-carbonitrile, Sanofi-Synthlabo; 50 mg iv) ∼36 h before the required time of preterm birth (133 days of gestation) (4, 26). A single, subclinical dose of betamethasone (3.0–5.7 mg intramuscular, Celestone Chronodose, Schering-Plough) was given to ewes delivering preterm lambs, 10 h before the administration of Epostane, to facilitate survival of lambs; we chose a dose that would enhance neonatal survival without inducing parturition. The gestational age for preterm birth (∼0.9 of term) was chosen because it is the earliest at which viable lambs could be born without the need for respiratory support. After birth, lambs were briefly removed from their mothers to be dried, warmed, and bottle fed colostrum; apart from suctioning of the oropharynx they received no respiratory support after birth. Lambs were raised with their mothers for the first 6–7 wk after birth in individual pens. After being separated from their mothers, lambs were fed a diet of lucerne chaff with free access to water and were weighed regularly. At 8 wk after term-equivalent age (TEA; defined as 145 days after mating), lambs underwent assessment of baseline airway resistance, bronchial responsiveness to inhaled carbachol, and ventilatory heterogeneity as detailed below. The age at which we studied lung function in these animals, 8 wk after TEA, was chosen to represent childhood in humans. All animal procedures were approved by the Monash University Animal Ethics Committee.

Airway resistance and responsiveness. We used established procedures involving the direct measurement of transpulmonary pressure and pulmonary airflow to obtain data on the resistance of large- and medium-size airways of lambs (25). Briefly, unsedated lambs were comfortably restrained in a canvas harness, and topical anesthesia was applied to the nasal passages (2% Lignocaine Gel, Orion, Australia). Esophageal pressure was measured with a balloon catheter, which was placed into the lower esophagus via the right nostril. Auffed endotracheal tube was inserted into the trachea via the left nostril under bronchoscopic guidance, and tracheal pressures were recorded with a side-hole catheter positioned with its tip 5 cm past the distal end of the endotracheal tube. Airflow measurements were obtained via a pneumotachograph attached to the proximal end of the endotracheal tube. Acquisition and analysis of pulmonary physiological data were performed using customized Labview software (National Instruments). This software analyzed flow and pressure data from an average of at least five breaths; it excluded data from breaths that differed by more than 20% in volume from the previous breath.

Following intubation, baseline airway resistance (RL) was calculated from 5–15 breaths. Airway responsiveness to the cholinergic bronchoconstricting agent carbachol was then assessed by sequentially administering aerosolized carbachol in a range of doubling doses (0.25–4% carbachol wt/vol). Each dose consisted of 10 breaths of aerosolized carbachol delivered at a tidal volume of 100 ml and a rate of 20 breaths/min to the mechanically ventilated lambs. The concentrations of administered carbachol were expressed in breath units (BU), with 1 BU defined as the dose received from one breath of 1.0% wt/vol carbachol aerosolized solution. Airway responsiveness was determined in each sheep by calculating the cumulative number of BUs of inhaled carbachol required to increase airway resistance by 200% above the baseline value. The dose given to each sheep to double the resistance was typically between 7 and 50 BU of carbachol.

Ventilatory heterogeneity. Ventilatory heterogeneity, which reflects the relative mixing of gas within the lung, was estimated using the multiple breath washout (MBW) test. Instrumentation, analysis, and underlying theory of MBW tests have been documented elsewhere (19). Briefly, the MBW using nitrogen measures the change in the phase III slope following repeated breathing of 20% oxygen and 80% argon; the slope values are normalized with respect to the mean expired N2 concentration. The N2 concentration was measured using a N2 analyzer (Medgraphics). As there is potential crosstalk between argon and nitrogen with this type of analyzer, the crosstalk was measured and accounted for as part of the calibration process. The normalized phase III slope of the first breath (SACIN) defines acinar heterogeneity and the increasing normalized phase III slope (SCOND) during rebreathing defines conductive heterogeneity proximal to the acinar entrance (Fig. 1).

MBW was measured during tidal breathing through a tight-fitting anesthetic mask. The lamb was allowed to breathe at its natural breathing rate and tidal volume. After a period of air breathing with stable end-expiratory lung volume at functional residual capacity (FRC), inspired air was switched to the test gas mixture (20% O2–80%
argon); tidal breathing continued until the exhaled concentration of N₂ was less than 2%. During each expiration, the N₂ phase III slope was computed and normalized. This leads to a normalized slope (Sn) that increases as a function of breath number or lung gas turnover; lung gas turnover is determined as the cumulative expired volume divided by ventilated functional residual capacity (FRCₑmbw). On theoretical grounds it can be shown that 1) the rate of rise of the Sn curve is due to the convective flow differences between lung units larger than acini, arising from heterogeneity in the conductive airways; and 2) the offset of the Sn curve is mainly determined by diffusion-convection-dependent heterogeneity generated in the acinar airways. Hence, Scond is simply computed as the rate of Sn increase as a function of lung gas turnover (exhaled volume divided by FRC), between 1.5 and 6 lung turnovers. Then Sact is computed as the Sn value of the first MBW expiration minus a correction term to remove any conductive zone contribution; this correction term equals the lung turnover corresponding to the first breath, multiplied by Scond.

At 9 wk post-TEA, lambs were humanely killed by an overdose of pentobarbital sodium (325 mg/ml iv). Major organs were collected and weighed.

**Data analysis.** Data are presented as means ± SE. Levene’s test for homogeneity of numerical data was used to determine that data were normally distributed. A two-way ANOVA was used to analyze the effects of prematurity (term vs. preterm) and sex (male vs. female) on airway resistance, airway responsiveness, and ventilatory heterogeneity. When appropriate, post hoc analysis, using the Student-Newman-Keuls method, was used. A Spearman rank order correlation was used to determine the relationship between Scond and baseline airway resistance. Significance was indicated by P < 0.05.

**RESULTS**

**Growth of lambs.** Preterm lambs were born at 133 ± 0 days after mating and weighed 3.3 ± 0.2 kg; control lambs were born at 145 ± 0 days after mating and weighed 4.2 ± 0.2 kg (P < 0.05, term vs. preterm for both gestational age and weight at birth). At the time of study, preterm lambs were aged 57 ± 2 days post-TEA (71 ± 2 days after birth) and weighed 16.1 ± 0.7 kg; term born lambs were studied at 58 ± 1 days after birth when they weighed 15.8 ± 0.9 kg. There was no significant difference in the postconceptional ages or body weights of preterm and term lambs at the time of study or in the body weights of males (16.9 ± 0.9 kg) and females (15.4 ± 0.7 kg).

**Baseline airway resistance (RL).** There was no significant difference between RL values in term (2.1 ± 0.3 cmH₂O·l⁻¹·s⁻¹, n = 12) and preterm lambs (2.4 ± 0.6 cmH₂O·l⁻¹·s⁻¹, n = 12). However, there was a strong tendency for RL to be higher in females (2.6 ± 0.3 cmH₂O·l⁻¹·s⁻¹, n = 10, 5 singleton, 5 twin) than males (1.7 ± 0.1 cmH₂O·l⁻¹·s⁻¹, n = 14, 7 singleton, 7 twin, P = 0.06, Fig. 2). There was no significant difference in tidal volume between preterm and term lambs, or between males and females (Fig. 2).

**Airway responsiveness to inhaled carbachol.** Analysis of the airway responsiveness data showed no differences between preterm (26.3 ± 4.7 BU, n = 11) and term groups (35.5 ± 5.7 BU, n = 12); however, females had significantly greater responsiveness than males (Fig. 2). The greater airway sensitivity of female lambs is indicated by the females needing a significantly smaller dose of carbachol to induce a 200% increase in resistance (females 23.0 ± 3.8 BU, n = 12, 5 singleton, 7 twin) compared with males (40.4 ± 5.6 BU, n = 11, 5 singleton, 6 twin, P < 0.05). Further analysis showed that male term lambs had a significantly lower responsiveness to carbachol than both female term lambs and male preterm lambs (Fig. 2).

**Ventilation heterogeneity.** Ventilation heterogeneity could only be measured in 11 animals (5 term, 6 preterm; 5 males, 6 females). There were no significant differences in either conductive or acinar ventilation heterogeneity between preterm and term lambs (Fig. 3). However, males had significantly higher conductive heterogeneity than females (0.28 ± 0.06 liter⁻¹ vs. 0.17 ± 0.05 liter⁻¹; P < 0.05) independent of gestational age at birth (Fig. 3). There was no difference in...
acinar heterogeneity between males and females. FRC did not differ between preterm or term lambs, or between males and females (Fig. 3). There was no correlation between baseline airway resistance and $S_{cond}$ ($r^2 = 0.374$, $P = 0.080$, Fig. 4).

**DISCUSSION**

The major finding of this study was that moderate preterm birth, in the absence of respiratory support after birth, did not result in altered lung function or bronchial reactivity in young lambs. The lack of altered respiratory physiology in this group of moderately preterm lambs suggests that impaired lung function seen in other studies after preterm birth may be due to either the severity of the preterm birth and/or the effects of mechanical ventilation and the development of lung disease. Studies in human survivors of very preterm birth show that infants who are ventilated for long periods, often developing chronic lung disease or BPD, have the most severe respiratory outcomes (5, 7); thus it is likely that prolonged mechanical ventilation of the very immature lung or prolonged exposure to high fractional inspired oxygen ($F_{O_{2}}$) induces structural or functional changes in the lung parenchyma or the pulmonary airways that reduce lung function later in life. The absence of any alteration in lung function in our preterm group and term lambs suggests that the lung fully recovers functionally from the effects of moderate preterm birth. The age at which our lambs were born (133 days; 0.9 of term) was the earliest that is compatible with survival without respiratory support; with regard to lung maturity and the need for ventilatory support at birth, this age is approximately equivalent to 30–32 wk of gestation in humans.

**Baseline airway resistance.** Our findings show that at 8 wk post-TEA there was no discernable difference between preterm and term lambs in baseline airway resistance when measured in a quiet resting situation. These data are consistent with our previous study that examined airway resistance in preterm lambs at TEA (2 wk after birth) and 6 wk post-TEA (4). In that study, while preterm lambs at TEA had significantly higher pulmonary resistances compared with newly born term lambs, the observed difference between lung function of term and preterm lambs was not present at 6 wk post-TEA (4). Our previous study (4) and the present study confirm that the early effects of mild preterm birth on lung function at rest are no longer present at 6–8 wk post-TEA. Children born with very low birth weight (<1,000 g) have near normal lung function by 8 yr of age (6). However, studies that have undertaken more extensive lung function testing indicate that at 10 yr of age, children born very preterm (born before 32 wk gestation and weighing <1,000 g) have significant impairment in exercise capacity, despite having only mild airway obstruction and gas trapping (22). A similar finding was reported in another followup study that examined lung function in young adults at 19 yr of age who were ex-preterm babies (31). Preterm birth has been linked to lower exercise capacities in young adults, although mean lung function parameters were within the normal range (31). Additional lung function testing in these young adults found that preterm birth resulted in lower total diffusion capacity of the lung for carbon monoxide ($D_{LCO}$), suggesting reduced oxygen uptake during exercise. The authors suggest...
that a lower $D_{lCO}$ may be due to decreased alveolar number or a thicker blood-air barrier (31). Interestingly, data from lambs born preterm support the notion that preterm birth is associated with increased thickness of the blood-air barrier, which is up to 26% thicker in preterm lambs compared with lambs born at term (4).

Airway reactivity. Bronchial hyperresponsiveness (BHR) is often observed in preterm infants who developed chronic lung disease (CLD) (14, 15, 17, 23). Even without CLD, BHR is increased in school age children who were born preterm (8, 17). In the present study, we found that there was no difference between preterm and term lambs at 8 wk post-TEA in airway responsiveness to carbachol. This suggests that moderately preterm birth per se is not sufficient to cause BHR. The development of BHR by the age of two years in ventilated preterm infants with CLD was associated with a decrease in lung compliance, but not airway resistance; lung function in these infants was measured by the single-breath occlusion technique (23). Presumably, in that study the combination of prematurity and mechanical ventilation caused sufficient trauma to the airway epithelium to induce later BHR (14, 23).

In general, BHR decreases with age in young children; thus it is possible that, in ventilated preterm infants, the normal postnatal airway maturation process is impeded, resulting in a higher BHR more typical of younger infants.

We found that the airways of female lambs as a group were more responsive to cholinergic stimulation than those of males. Differences in BHR between boys and girls have been reported but findings are not consistent. One study found that boys tended to have greater airways responsiveness to methacholine compared with girls (13) while another indicated that girls were more responsive than boys (16). After puberty, women have greater BHR compared with men, and not unexpectedly women have a greater incidence of asthma (18). There is still some debate whether BHR is intrinsically greater in women, or whether it is associated with the lower baseline forced expiratory volume in 1 s (FEV$_1$) observed in women. Further experiments are needed to identify the mechanisms underlying the sex effects on BHR.

Ventilatory heterogeneity. Despite increasing experimental and clinical data on the measurement of $S_{acinh}$ and $S_{cond}$ in humans, there are only limited published data in animals (19–21, 29–31). Animal studies using MBW analysis have often observed in preterm infants who developed chronic lung disease (CLD) (14, 15, 17, 23). Even without CLD, BHR is increased in school age children who were born preterm (8, 17). In the present study, we found that there was no difference between preterm and term lambs at 8 wk post-TEA in airway responsiveness to carbachol. This suggests that moderately preterm birth per se is not sufficient to cause BHR. The development of BHR by the age of two years in ventilated preterm infants with CLD was associated with a decrease in lung compliance, but not airway resistance; lung function in these infants was measured by the single-breath occlusion technique (23). Presumably, in that study the combination of prematurity and mechanical ventilation caused sufficient trauma to the airway epithelium to induce later BHR (14, 23).

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Although the measured airway resistance and $S_{cond}$ were not significantly correlated, airway resistance tended to be lower in our male lambs; it is expected that lower airway resistance will lead to a higher $S_{cond}$. A previous study in normal human subjects demonstrated that an increase in airway conductance (the reciprocal of resistance), measured at 5 Hz with the forced oscillation technique, was related to an increase in $S_{cond}$ following a challenge with methacholine (11); however, baseline $S_{cond}$ and conductance were not related. The relationship between the increase in $S_{cond}$ and the increase in airway conductance in that study (11) demonstrated that both of these parameters were sensitive to changes occurring within the same airway compartment.

Conclusions. We conclude that moderately preterm birth that does not necessitate respiratory support after birth does not affect baseline airway resistance, airway responsiveness, or ventilatory heterogeneity in small airways. The observed sex-related differences in airway responsiveness and ventilatory heterogeneity in small conducting airways could help explain sex-related differences in lung function observed in humans born preterm.

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