Role of vagal innervation on pulmonary surfactant system during fetal development

Luxmi Gahlot,1 Francis H. Y. Green,2 Anita Rigaux,1 Jennifer M. Schneider,1 and Shabih U. Hasan1

Departments of 1Pediatrics and 2Pathology and Laboratory Medicine, Institute of Child and Maternal Health, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada

Submitted 21 May 2008; accepted in final form 17 March 2009


Vagally mediated afferent feedback and compliant lungs (surfactant system) play vital roles in the establishment of adequate alveolar ventilation and pulmonary gas exchange at birth. Although the significance of vagal innervation in the establishment of normal breathing patterns is well recognized, the precise role of lung innervation in the maturation of the surfactant system remains unclear. The specific aim of the present study was to investigate whether vagal denervation compromises the surfactant system during fetal development. Experiments were performed on 12 time-dated fetal sheep: 8 underwent cervical vagal denervation, and 4 were sham operated. Vagal denervation was performed at 110–113 days gestation. Fetal lambs were instrumented in utero to record arterial pH and blood-gas tensions. The animals were delivered by cesarean section under general anesthesia between 130 and 133 days gestation (term ~147 days). Lung samples were collected for wet-to-dry ratios, light and electron microscopy, and overall lung morphology. In addition, total proteins, total phospholipids, and surfactant proteins A and B were analyzed in both lung tissue and bronchoalveolar lavage fluid. Vagal denervation had no effect on alveolar architecture, including type II cells or the morphology of lamellar bodies within them. Furthermore, surfactant proteins A and B and total phospholipids were similar in lung tissue and bronchoalveolar lavage fluid between the two groups. A significant correlation was observed between circulating cortisol concentrations and surfactant proteins in the bronchoalveolar lavage fluid and lung tissue. We provide definitive evidence that vagal innervation at midgestation is not required for maturation of the pulmonary surfactant system during fetal development.

one of the most vital and unique adaptations that occurs immediately after birth is the establishment of continuous breathing (19) and adequate pulmonary gas exchange, which requires vagally mediated volume feedback and compliant lungs (surfactant system). We and others have shown that vagal neural input is necessary for the establishment of alveolar ventilation and pulmonary gas exchange shortly after birth and during early postnatal life (15, 30, 32). Pulmonary surfactant, a developmentally regulated complex lipoprotein mixture, is synthesized by type II pulmonary epithelial cells. The surfactant is tightly packed in the lamellar bodies (120–180 per type II cell) from where it is secreted as tubular myelin to form a film at the alveolar air-liquid interface (3, 55). Lung surfactant plays a critical role in the transition from the fluid-filled fetal lungs to the aerated newborn lungs by maintaining alveolar expansion and stabilizing the alveoli (12, 26) and later in the maintenance of lung function (25) and immunoregulation (63). The mechanisms through which glucocorticoids and a number of other hormones and growth factors affect surfactant synthesis, secretion, and re-uptake have been extensively investigated (3, 7, 48, 57). Similarly, the importance of physical factors in the stimulation of lung growth (36) has been addressed by several elegant studies. However, very few studies have focused on the role of in vivo neural input on the development of the pulmonary surfactant system during fetal life.

An earlier study showed abnormal pulmonary ultrastructural changes and absence of lamellar bodies in vagally denervated fetal lambs (1), suggesting that vagal innervation plays an important role in the development of the surfactant system during the perinatal period. However, recent studies in fetal and neonatal lambs do not support such an inference (30, 62). At least three distinct possibilities exist that could explain such divergent results: 1) in the later studies, vagal denervation was performed in the intrathoracic (62) as opposed to the cervical region (1), thus sparing the upper airway motor control, which, along with the other physical factors, plays an important role in lung development (20, 27); 2) the denervation was performed late in the third trimester of pregnancy (62) at a time when the surfactant system was approaching maturity (11); and, finally, in the earlier studies, 3) the absence of lamellar bodies could have been due to suboptimal histological and staining techniques, because the predominantly lipid structure of the lamellar bodies is modifiable by conventional fixation techniques for electron microscopy (13, 45, 64).

In view of the critical role that surfactant plays in extraterine adaptation and pulmonary gas exchange, it is vital that the role of vagal innervation in the pulmonary surfactant system be investigated in a definitive and systematic manner. The specific aim of the present study was to investigate the relationship between vagal innervation and the surfactant system during fetal development. We tested the hypothesis that cervical vagal denervation performed in the developing fetus does not decrease the lamellar bodies in pulmonary epithelial cells, the total lung phospholipids, or surfactant proteins A (SP-A) and B (SP-B).

Materials and methods

Animal preparation. All surgical procedures were performed in accordance with the Canadian Council on Animal Care, and the study protocol was approved by the institutional animal care committee. Twelve fetal sheep underwent either vagal denervation in the cervical region (n = 8) or sham surgery (n = 4) at 110–113 days of gestation. Surgery was performed under general anesthesia using ketamine hydrochloride (8–9 mg/kg, Rogarsetic, Rogar/STB, London, Ontario)
and 4% halothane in oxygen for induction and 1.5–2.5% halothane for maintenance.

The fetal head and neck were partially exteriorized through midline maternal abdominal and uterine incisions. With the use of sterile techniques, a 4-cm ventral neck incision was made to expose the fetal carotid arteries, jugular veins, and vagosympathetic trunk. Carotid artery and jugular vein catheters (2.0 mm outer diameter and 1.0 mm inner diameter, Portex, Hythe, Kent, UK) were inserted and secured in place. The arterial catheter was used to draw blood for analysis of pH and blood-gas tensions, whereas the venous catheter was used to administer antibiotics. Vagal denervation was performed in the cervical area in six fetuses (denervated). Approximately 4–5 cm of the vagus nerve was cleared of the surrounding tissue and sectioned. The sectioned ends were treated with 4% phenol and further folded over and tied with 2.0 silk sutures to avoid nerve regeneration. The vagi were identified but not sectioned in four fetuses (sham operated). After the completion of instrumentation, the fetuses were returned to the uterine cavity, and all incisions were sewn in layers. The fetal vascular catheters were exteriorized through an incision in the left maternal flank and stored in a cloth pouch secured to the maternal abdominal wall. A polyvinyl catheter was placed in the maternal jugular vein for infusion of antibiotics and fluids (3 mm outer diameter, Tygon). The ewes were housed in large (1.8 x 2.4 m) custom-made individual holding pens with free access to food and water. Daily care included administration of 125 mg (for fetuses) and 375 mg (for ewes) of cefazolin sodium in saline (Ancef, SmithKline Beecham Pharma, Oakville, Ontario), 20 mg (for fetuses) and 80 mg (for ewes) of gentamicin sulfate (Garamycin Injectable, Schering-Plough), and 20–23 days postsurgery for any signs of infection or onset of labor. Furthermore, fetal blood was drawn on the last day of the experiment to determine plasma cortisol concentrations, whereas the venous catheter was used to monitor heart rate and blood-gas tensions, whereas the arterial catheter was used to determine arterial pH and blood-gas tensions (arterial PCO2 and arterial PO2). The arterial pH and blood-gas tensions were measured to ensure fetal well-being until the day of delivery. Furthermore, fetal blood was drawn on the last day of the experiment to determine plasma cortisol concentrations, and analysis was performed using competitive chemiluminescent immunoassays (Centaur, Bayer Diagnostics, Terrytown, NY).

Postmortem collection of samples. The ewes were monitored for 20–23 days postsurgery for any signs of infection or onset of labor. The fetuses were delivered via cesarean section performed under general anesthesia at 130–133 days gestation. Thereafter, the ewes and fetuses were euthanized using Euthanyl (pentobarbitone 240 mg/ml), according to the Canadian Council on Animal Care guidelines. The fetuses were weighed, and complete sectioning of the vagal nerves was performed in each case by identifying the cut and folded ends of the nerve. Location of the denervated vagi was identified by the silk sutures used for folding and securing the denervated ends. Similarly, the integrity of the vagal nerves was confirmed in the sham-operated animals. Lungs were exposed through a midline thoracotomy. The right main bronchus was tied off, and the right upper and lower lobes were removed and sampled for measurements of wet-to-dry weight ratio, electron microscopy, and SP-A and -B.

Bronchoalveolar lavage fluid. The left main stem bronchus was cannulated, and bronchoalveolar lavage was performed using chilled normal saline solution (0.9% NaCl). One hundred milliliters per kilogram body weight in four aliquots were slowly infused using a gravitational method to avoid fluid leaks, as previously described (30). Bronchoalveolar lavage fluid (BALF) was used for the assays of total phospholipids and SP-A and -B. Thereafter, the left lung was perfused (inflation fixed) with 10% freshly prepared formalin at 25 cmH2O for 24 h for routine light microscopy. BALF was stored at 4°C and centrifuged at 150 g for 10 min to remove cellular debris. The supernatant was frozen at −70°C for total phospholipids and SP-A and -B. Total phospholipids in the supernatant were assayed using the well-established methods of Bartlett (5). Total protein was measured as described by Lowry et al. (37). Measurement of the total protein in BALF was used to normalize the samples for variations in sampling methods. Some BALF samples may have more protein, resulting in higher levels of SP-A and -B that are unrelated to the experimental condition. Normalizing to total protein may help in accounting for some of the variations in the sample values. Furthermore, increased alveolar-capillary permeability may also occur during bronchoalveolar lavage that could, in turn, affect a number of other variables, e.g., lung wet-to-dry ratios.

Light microscopy. Samples of lung parenchyma of 1.0 x 1.0 x 0.3 cm were taken from each lobe. In addition, blocks of tissue were taken from the lobar bronchi perpendicular to their long axes. Five micrometer sections of the airways and the parenchyma were stained by hematoxylin and eosin and elastic trichrome.

Morphometric analysis. To investigate differences in the airways of the sham-operated and denervated animals, morphometric analysis was performed. Airways were analyzed if seen with cartilage and in true cross section. The boundary of the airway was determined by the surrounding lung parenchyma. The inner border of the basement membrane defined the internal perimeter of the airway. The lumen was defined as points falling internal to the basement membrane.

The area fractions of selected features in the airway wall profile were determined by a modified point-counting technique (59) using an Axioskop light microscope (Carl Zeiss model 451888), drawing tube, and square lattice grid containing 240 points, at x10 magnification. The point grid was superimposed onto a segment of the airway wall, and the number of points falling on each area of interest per grid was counted. The features that were quantified included interstitium, cartilage, mucous gland, nerve, smooth muscle, blood vessel, epithelium, and lumen. Using stereological principles (59), the area proportion occupied by the structures counted was calculated, using the following formula: area (\(\mu m^2\)) = \(Z^2n\), where \(Z\) is the distance between two points on the grid (magnification factor), and \(n\) is the number of points that land on the structure of interest. Luminal surface length (internal perimeter) was determined by using the following formula: luminal surface length (\(\mu m\)) = \(bmi \times Z \times m/4\), where \(bmi\) is the number of times the grid intersects with the basement membrane, and \(Z\) is the magnification factor (or the distance between two points on the grid). To determine the thickness (smooth muscle, cartilage), assuming the feature was uniformly distributed around the basement membrane, the following formula was used: thickness (\(\mu m\)) = area (\(\mu m^2\))/luminal surface length (\(\mu m\)).

Electron microscopy. Lung samples from the right upper and lower lobes were fixed using a nonaqueous fixation technique optimized for the preservation of mucoproteins and surfactant lipids (34, 51, 53). The samples were fixed with 1% osmium tetroxide (wt/vol) dissolved in fluorocarbon FC-77 fluid for 90 min and further postfixed in 2.5% glutaraldehyde solution containing 1% cetylpyridinium chloride in 0.05 M sodium cacodylate (CaCo) buffer, pH 7.3, for 90 min at room temperature. The tissues were then dehydrated in acetone, infiltrated with acetone/Epon 812, sectioned, stained with uranyl acetate/lead citrate, and examined with a Hitachi 7000 TEM electron microscope.

Lung wet-to-dry ratio. To determine the lung wet-to-dry-weight ratios, pieces of lung tissue from upper and lower lobes were tied and dissected from sham-operated and denervated animals, weighed, and dried in preweighed aluminum containers for 3 days at 120°C. SP-A and SP-B assays. SP-A and SP-B concentrations were determined as previously described, courtesy Dr. Jeffrey Whitsett and William Hull, Cincinnati, OH (2, 43). Briefly, wells were coated with 100 \(\mu\)l of 0.1 M sodium bicarbonate and left overnight at 4°C. The plates were washed with wash buffer solution, and 100 \(\mu\)l of 82b buffer with 5% goat serum for SP-A samples or 5% human albumin for SP-B samples were added. The wash buffer comprised 0.1 M Tris, pH 8.0, and 0.05% (vol/vol) Tween 20. The 82b buffer comprised 0.15 M NaCl, 0.01 M Tris, pH 7.4, and 5 mg/ml bovine serum albumin. For SP-A, 5% (vol/vol) of 25% human serum albumin was added, and, for SP-B, 2.5% (vol/vol) of 25% human serum albumin
and 2.5% (vol/vol) of goat serum was added. After 15 min, the buffer was removed, and 100 μl of standard at concentrations of 5, 10, 25, 50, 75, and 100 ng/ml were added and incubated for 1–2 h at 37°C. One hundred microliters of sample were added per well, diluted in phosphate-buffered saline containing 5% (vol/vol) Nonidet P-40. Serial dilutions of 1:10 and 1:4, resulting in the dilutions of 1:10, 1:40, 1:160, and 1:640, were used. The wells were washed three times with wash buffer, and 100 μl of horseradish peroxidase conjugate (goat anti-rabbit IgG), diluted 1:1,000 in buffer with 5% human plasma for SP-A samples and 5% human albumin for SP-B samples, were added and incubated at 37°C for 1 h. Before the addition of the horseradish peroxidase conjugate, antibodies specific to SP-A and SP-B were added in a 1:5,000 dilution. The wells were again washed with wash buffer, 100 μl of substrate solution were added, and color development was observed. Color development was stopped with 100 μl of 50% sulfuric acid. The absorption was read at an absorbance of 492 nm.

Statistical analysis. The gestational age at surgery and at delivery, fetal body weight at delivery, lung wet-to-dry ratios, plasma cortisol concentrations, total phospholipids in BALF and lung tissue, morphometric analysis, and SP in tissues and BALF were analyzed using an independent sample t-test. The effects of vagal denervation and sham surgery on arterial pH and blood-gas tensions between sham-operated and denervated animals were analyzed using two-way ANOVA for repeated measure. Spearman’s rank correlation was used to test the direction and strength of the plasma cortisol concentrations and SP-A and -B in BALF and lung tissue. Since no significant differences were observed between sham-operated and denervated animals in Spearman’s rank correlation analysis, data were combined to delineate the relationship between cortisol concentrations and SP-A and -B. All values are given as means ± SE, and P ≤ 0.05 was considered significant.

RESULTS

Arterial pH and blood-gas tensions. The arterial pH, arterial PCO2, and arterial PO2 (Torr) are given in Fig. 1. The pH and the blood-gas tensions decreased in sham-operated animals and denervated animals during the early postoperative period and gradually returned to normal range.

Gestational ages, body weight, lung wet-to-dry ratio, and plasma cortisol concentrations. The gestational age at surgery and at cesarean section was similar between the sham-operated and denervated groups. No difference was observed in body weight at delivery, lung wet-to-dry-ratio, and plasma cortisol concentrations between the two groups (Table 1). The sectioning and integrity of the vagi in the denervated and sham-operated animals, respectively was confirmed in each case.

Light and electron microscopy. The lungs of the sham-operated and denervated animals appeared grossly normal. Examples of the light microscopic appearances of the lung parenchyma and airways of sham-operated and denervated animals are shown in Figs. 2 and 3, respectively. The histological appearances of the lung parenchyma were characteristic of normal development for the gestational age of the fetal lambs in both groups. In both groups, mature type II cells were seen within the alveoli. The airways were also histologically normal, with no visible differences between the two study groups (Fig. 3).

Electron microscopy showed mature lamellar bodies in the type II cells in both the sham-operated and denervated animals (Fig. 4, A and B), respectively. The number and size of the

A

B

C

Fig. 1. Arterial pH (pHa; A), arterial PCO2 (PaCO2; B), and arterial PO2 (PaO2; C) at various gestations in sham-operated and denervated animals. No significant differences were observed between the two groups.
lamellar bodies in type II alveolar epithelial cells between sham-operated and denervated animals were similar by subjective assessment.

**Morphometric analysis.** The result of the morphometric analysis of airway features is illustrated in Fig. 5. There were no significant differences in the area fractions (expressed as a thickness in \( \mu \)m) of cartilage, smooth muscle, mucous glands, nerves, blood vessel, interstitium, and lumen in the sham-operated and denervated animals.

**Total phospholipids and total proteins.** The results of the total phospholipids and total proteins measured in BALF are shown in Fig. 6, A and B. Total phospholipids were 9.2 ± 1.58 and 6.8 ± 0.64 mg/kg in the denervated and sham-operated groups, respectively. Total proteins were 17.2 ± 1.6 and 14.1 ± 2.2 mg/ml in the denervated and sham-operated groups, respectively.

**SP-A and SP-B.** Surfactant-associated proteins measured in BALF and lung tissues are shown in Fig. 6, C and D, respectively. SP-A and SP-B measured in lung tissue and BALF were similar between the two study groups. However, a significant positive correlation was observed between circulating cortisol concentrations and SP-A and -B in BALF and lung tissue in both sham-operated and vagally denervated animals (Figs. 7 and 8, respectively).

**DISCUSSION**

The remarkable increase in the survival of very low birth weight infants (35) has largely been attributed to the widespread administration of maternal glucocorticoids during preterm labor to augment lung maturation (47) and postnatal treatment of infants with exogenous surfactant, which plays a critical role in reducing surface tension at the liquid-air interface (26). Although the subject of vagal innervation and its effects on the surfactant system have been debated for the past three decades with divergent results, the present study provides important and unequivocal evidence that vagal input at midgestation is not necessary for type II pneumocyte maturation. Electron microscopy of sham-operated and denervated animals revealed no differences in size or maturity of the lamellar bodies in type II alveolar epithelial cells between sham-operated and denervated animals were similar by subjective assessment.

**Table 1. Body weight, lung wet-to-dry ratio, and plasma cortisol concentrations at delivery**

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Denervated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age at surgery, days</td>
<td>110±0.0</td>
<td>110±0.3</td>
</tr>
<tr>
<td>Gestational age at cesarean section, days</td>
<td>131±0.3</td>
<td>131±0.4</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>3.63±0.1</td>
<td>3.51±0.2</td>
</tr>
<tr>
<td>Lung wet-to-dry ratio</td>
<td>7.5±1.4</td>
<td>7.8±0.3</td>
</tr>
<tr>
<td>Plasma cortisol concentrations, ng/ml</td>
<td>23±12</td>
<td>51±14</td>
</tr>
</tbody>
</table>

Values are mean ± SE.

![Fig. 2. Light microscopy of lung parenchyma in sham-operated (A and B) and denervated (C and D) animals. Both photomicrographs show a normal membranous bronchiole in the center of the field of view, surrounded by alveoli of approximately equal size. At higher magnifications (B and D), both groups showed mature type II cells (arrows) in the alveoli, with no apparent differences between sham-operated and denervated animals. A and C: original magnification \( \times 100 \); B and D: \( \times 400 \).](image-url)
bodies in type II pneumocytes. In addition, we provide important new data on total phospholipids and SP-A and SP-B in BALF and lung tissues. Kitterman et al. (28) have reported a correlation between plasma cortisol concentrations and saturated phosphatidylcholine (PC). Other studies have shown that administration of glucocorticoids leads to reversible increase in surfactant-associated proteins (54); however, to our knowledge, a correlation between circulating cortisol concentrations and surfactant proteins has not been investigated in the developing ovine fetus. Thus our study provides new data showing a strong correlation between circulating cortisol concentrations and SP-A and SP-B. We also show that motor innervation of the larynx does not appear essential for lung growth in utero, as shown by the normal lung histology, lung weights, and morphometric indexes of the airways in the denervated animals.

Vagal innervation, lamellar body formation, and maturation. Using the experimental protocol similar to ours, Alcorn et al. (1) reported normal lung development and architecture in their vagally denervated lambs. However, they also observed vacuole-like, membrane bound intra-cytoplasmic empty spaces within type II cells, which they interpreted as abnormal lamellar bodies. In contrast, we report mature lamellar bodies within the type II cells of our denervated lambs. Lamellar bodies are the intracellular storage sites for pulmonary surfactant, as they are deficient in a number of lysosomal enzymes necessary for the de novo synthesis of PC and phosphatidylglycerol. Thus the loss of lipid material from tissue sections may be an artifact of fixation, embedding, or sectioning. Phospholipids, the major component of lamellar bodies and plasma membranes, are particularly vulnerable to these artifacts, making them difficult to preserve for electron microscopy (13, 45, 64). The classic procedure of gluteraldehyde fixation followed by alcohol dehydration and epoxy resin embedding can result in the loss of 73–91% of lipids (38, 64). Furthermore, differences in the viscosity of the epoxy resin components could lead to organelle shrinkage during embedding. Spurr resin, which was used in the study by Alcorn et al. (1), is particularly vulnerable to this shrinkage effect (45). Yang et al. (64) describe atypical and empty lamellar bodies following conventional fixation of mouse lungs. Their illustration of abnormal lamellar bodies, resulting from fixation artifacts, are almost identical to those shown by Alcorn et al. (1) in both vagally denervated and sham-operated lambs, findings that suggest the empty vacuole-like spaces were the result of a fixation or processing artifact.

Several approaches for reducing loss of lipid material from cell membranes and lamellar bodies have been used, with
II cells contain receptors for both cholinergic and lung preparations (10, 16, 41, 46, 48). Although isolated type secretion of lung surfactant, both in vivo and in isolated perfused acetylcholine infusion, have been shown to stimulate the se-
anisms, including direct stimulation of the vagal nerve and
two groups.

Vagal innervation, surfactant secretion, and dysfunction. The role of vago-sympathetic innervation on surfactant synthesis, secretion, and dysfunction is another area that has long been debated (1, 41, 46). Cholinergic and β-adrenergic mechanism, including direct stimulation of the vagal nerve and acetylcholine infusion, have been shown to stimulate the secre-
tion of lung surfactant, both in vivo and in isolated perfused lung preparations (10, 16, 41, 46, 48). Although isolated type II cells contain receptors for both cholinergic and β-adrenergic agonists, it is only the β-adrenergic agonists that stimulate secretion of surfactant from the alveolar type II cells (10). A rapid increase in surfactant flux (12- to 13-fold) and pool size shortly before birth has been documented in fetal sheep and other species (6, 44). Since our experimental protocol was designed to deliver fetuses around 133 days gestation, it is possible that the effects of vagosympathetic denervation on a number of physiological and biochemical variables might have been different had the fetuses been delivered closer to term gestation. Sympathetic (α- and β-adrenergic) and cholinergic systems can have specific effects on surfactant synthesis and secretion. Schellenberg et al. (50) investigated the effects of chemical sympathectomy and β-adrenergic blockade on alve-
olar and tissue phospholipids and saturated PC. They showed that chemical sympathectomy suppressed the increase in alve-
olar phospholipids in response to thyrotropin-releasing hor-
more and cortisol administration (50). However, the lung tissue phospholipids and/or saturated PC increased in a manner similar to or higher than the values observed in nonsympath-
etectomized animals, suggesting that stimulation of pulmonary synthesis is not dependent on sympathetic innervation. Inhibi-
tion of surfactant secretion may be the reason for lower alveolar phospholipid values in sympathetectomized animals (50), indicating separate mechanisms for surfactant synthesis and secretion. It is possible that, if vagally denervated lambs had been delivered at term, they might have had intact lamellar bodies and lung tissue surfactant constituents, but lower alve-
olar phospholipids and saturated PC concentrations.

Direct mechanical stretching, due to lung inflation, of the
type II cells is believed to be the primary physiological stim-
ulus for the secretion of surfactant (49, 61). Stretching of the type II cells increases intracellular Ca²⁺ concentration and increases surfactant release (61). A number of studies have shown that air inflation of the lungs is a sufficient stimulus for surfactant release (23, 39, 46), and that the initial expansion of the newborn lung acts as the primary stimulus for surfactant release (33). In the present study, animals were delivered by cesarean section under general anesthesia, and the lungs were not inflated with air or subjected to spontaneous breathing, thus eliminating these as potential confounding factors for this study. Surfactant dysfunction after vagal denervation (8, 18, 29) could result from pulmonary edema secondary to upper airway obstruction (8, 18, 29) and increased intrathoracic pressure, leading to damaged alveolar-capillary membrane. Such change in permeability could allow serum proteins, hemoglobin, fatty acids, and cellular degradation products into the alveolar space, where they can impair the surface-active function of surfactant (24, 58). Vagal denervation performed in the intrathoracic region, which spares the recurrent laryngeal nerves, is not associated with a decrease in total phospholipids or to histological changes of respiratory distress (30, 32).

Fig. 5. Morphometric analysis of conducting airways in sham-operated and
denervated animals for interstitium, cartilage, and mucous gland (A) and nerve, smooth muscle (Ms), and blood vessels (Vs; B). Values are expressed as a thickness in millimeters. No significant differences were observed between the two groups.

Vagal innervation, surfactant secretion, and dysfunction. The role of vago-sympathetic innervation on surfactant synthesis, secretion, and dysfunction is another area that has long been debated (1, 41, 46). Cholinergic and β-adrenergic mechanisms, including direct stimulation of the vagal nerve and acetylcholine infusion, have been shown to stimulate the secretion of lung surfactant, both in vivo and in isolated perfused lung preparations (10, 16, 41, 46, 48). Although isolated type II cells contain receptors for both cholinergic and β-adrenergic agonists, it is only the β-adrenergic agonists that stimulate secretion of surfactant from the alveolar type II cells (10). A rapid increase in surfactant flux (12- to 13-fold) and pool size shortly before birth has been documented in fetal sheep and other species (6, 44). Since our experimental protocol was designed to deliver fetuses around 133 days gestation, it is possible that the effects of vagosympathetic denervation on a number of physiological and biochemical variables might have been different had the fetuses been delivered closer to term gestation. Sympathetic (α- and β-adrenergic) and cholinergic systems can have specific effects on surfactant synthesis and secretion. Schellenberg et al. (50) investigated the effects of chemical sympathectomy and β-adrenergic blockade on alve-
olar and tissue phospholipids and saturated PC. They showed that chemical sympathectomy suppressed the increase in alve-
olar phospholipids in response to thyrotropin-releasing hor-
mone and cortisol administration (50). However, the lung tissue phospholipids and/or saturated PC increased in a manner similar to or higher than the values observed in nonsympath-
etectomized animals, suggesting that stimulation of pulmonary synthesis is not dependent on sympathetic innervation. Inhibi-
tion of surfactant secretion may be the reason for lower alveolar phospholipid values in sympathetectomized animals (50), indicating separate mechanisms for surfactant synthesis and secretion. It is possible that, if vagally denervated lambs had been delivered at term, they might have had intact lamellar bodies and lung tissue surfactant constituents, but lower alve-
olar phospholipids and saturated PC concentrations.

Direct mechanical stretching, due to lung inflation, of the
type II cells is believed to be the primary physiological stim-
ulus for the secretion of surfactant (49, 61). Stretching of the type II cells increases intracellular Ca²⁺ concentration and increases surfactant release (61). A number of studies have shown that air inflation of the lungs is a sufficient stimulus for surfactant release (23, 39, 46), and that the initial expansion of the newborn lung acts as the primary stimulus for surfactant release (33). In the present study, animals were delivered by cesarean section under general anesthesia, and the lungs were not inflated with air or subjected to spontaneous breathing, thus eliminating these as potential confounding factors for this study. Surfactant dysfunction after vagal denervation (8, 18, 29) could result from pulmonary edema secondary to upper airway obstruction (8, 18, 29) and increased intrathoracic pressure, leading to damaged alveolar-capillary membrane. Such change in permeability could allow serum proteins, hemoglobin, fatty acids, and cellular degradation products into the alveolar space, where they can impair the surface-active function of surfactant (24, 58). Vagal denervation performed in the intrathoracic region, which spares the recurrent laryngeal nerves, is not associated with a decrease in total phospholipids or to histological changes of respiratory distress (30, 32).

However, absence of the afferent feedback, critically required for lung expansion at birth, could lead to an increase in the minimum surface tension of the lungs due to pulmonary atelectasis (62). Both overexpansion (40) and/or atelectasis (56), which can be associated with vagal denervation, can also lead to surfactant dysfunction. In summary, data from others and our laboratory suggest that vagal denervation does not play a direct role in surfactant dysfunction (8, 24, 30, 32, 58, 62).

Motor innervation of the upper airway and morphometric analysis. No significant differences were observed in the lung morphometric analysis between the two groups. Motor innervation of the upper airway, which is dependent on intact vagi and fetal breathing movements, plays important roles in the maintenance of functional residual capacity and lung development. Vagal denervation leads to paralysis of all laryngeal muscles, causing the vocal cords to lie in a cadaveric position (53a). During fetal apnea, the laryngeal adduction (thyro-aryte-
noid muscles) reduces the efflux of tracheal fluid, whereas, during fetal breathing movements, active phasic laryngeal dilation mediated via posterior crico-arytenoid muscles leads to high net tracheal efflux (21). Thus it is likely that vocal cord paralysis may allow increased efflux during fetal apnea (50% of the total time at 110 –125 days gestation), while absence of phasic dilation in conjunction with reduced lung recoil during fetal breathing movements (50% of the total time) may retard the tracheal fluid efflux, resulting in little net change in overall lung expansion. Furthermore, fetal sheep can defend its lung expansion by increasing lung liquid secretion (22). Finally, it is likely that vagal denervation performed at a relatively late gestation in terms of structural pulmonary development and for a short

![Figure 6](image6.png)

Fig. 6. A: total phospholipids (mg/kg body wt) measured in the BALF at 130–133 days gestation in sham-operated and denervated animals. B: total protein (mg/ml) measured in BALF at 130–133 days gestation in sham-operated and denervated animals. C: surfactant protein (SP)-A and -B measured in BALF (µg/ml). D: SP-A and -B in the lung tissue (µg/mg). No significant differences were observed between the sham-operated and denervated animals.

![Figure 7](image7.png)

Fig. 7. SP-A and SP-B in BALF (µg/ml) as a correlation to plasma cortisol concentrations (ng/ml). Significant correlation was observed between SP-A, SP-B, and cortisol concentrations ($P = 0.003$ and 0.012, respectively).

![Figure 8](image8.png)

Fig. 8. SP-A and SP-B in lung tissue (µg/mg protein) as a correlation to plasma cortisol concentrations (ng/ml). Significant correlation was observed between SP-A, SP-B, and cortisol concentrations ($P = 0.015$ and 0.017, respectively).
duration (2–3 wk) would have little effect on morphometric analysis. Since glucocorticoids play a critical role in maturation of the surfactant system (14, 44, 54) and may have been affected by fetal and maternal surgical procedures and sectioning of the vago- sympthetic trunk, we measured plasma cortisol concentrations at delivery of the animals. We found no statistically significant difference in plasma cortisol concentrations between sham-operated and denervated animals. Furthermore, these values are comparable with the concentrations obtained from control animals in our laboratory (35.01 ± 10.21; mean ± SE; unpublished observations). Also, these plasma cortisol values are similar to those reported by Schellenberg et al. (50) in control animals. The increase in surfactant proteins is reversible, despite repeated large doses of glucocorticoids (54). In our present study, we followed the experimental protocol, as described by Alcorn et al. (1), to clarify the role of vagal innervation in pulmonary surfactant during fetal development. However, there are clear differences in the presence of lamellar bodies between the two studies, which suggest that the differences in the presence of lamellar bodies between the two studies cannot be explained by the surgical stress alone and are the result of fixation artifact.

In conclusion, we provide definitive evidence that the maturation of the surfactant system during fetal development in sheep is not dependent on intact vagal innervation. Furthermore, the present study provides a number of gestation-specific important morphological and biochemical variables related to fetal lung development. Our findings do not contradict the studies that have established the important role of vagal innervation in the maintenance of normal breathing patterns and pulmonary gas exchange during the early neonatal period (17, 30, 31, 52). Instead, our findings strongly support the hypothesis that the effects of vagal denervation in the early neonatal period are secondary to the alterations in pulmonary mechanical forces induced by the denervation. Previous studies showing absence of lamellar bodies in vagally denervated fetal lambs were probably an artifact resulting from suboptimal lung tissue fixation techniques. In fact, a trend toward an increased plasma cortisol concentration, higher total phospholipids, and surfactant-associated proteins in the denervated animals further lend support to our hypothesis that vagal innervation plays little role in the maturation of the surfactant system during fetal development. Finally, it is important to note that our comprehensive study addresses and resolves the long-debated relation-
duration of the surfactant system during fetal development in human fetal lung development. Our findings do not contradict the observations). Also, these plasma cortisol values are similar to those reported by Schellenberg et al. (50) in control animals.

REFERENCES
