Pulmonary Surfactant Surface Tension Influences Alveolar Capillary Shape and Oxygenation

Machiko Ikegami¹, Timothy E. Weaver¹, Shawn N. Grant¹, and Jeffrey A. Whitsett¹

¹Division of Pulmonary Biology, Cincinnati Children's Hospital Medical Center, University of Cincinnati, Cincinnati, Ohio

Alveolar capillaries are located in close proximity to the alveolar epithelium and beneath the surfactant film. We hypothesized that the shape of alveolar capillaries and accompanying oxygenation are influenced by surfactant surface tension in the alveolus. To prove our hypothesis, surfactant surface tension was regulated by conditional expression of surfactant protein (SP)-B in *Sftpb*^{-/-} mice, thereby inhibiting surface tension-lowering properties of surfactant *in vivo* within 24 hours after depletion of *Sftpb*. Minimum surface tension of isolated surfactant was increased and oxygen saturation was significantly reduced after 2 days of SP-B deficiency in association with deformation of alveolar capillaries. Intravascularly injected 3.2- μ m-diameter microbeads through jugular vein were retained within narrowed pulmonary capillaries after reduction of SP-B. Ultrastructure studies demonstrated that the capillary protrusion typical of the normal alveolar-capillary unit was reduced in size, consistent with altered pulmonary blood flow. Pulmonary hypertension and intrapulmonary shunting are commonly associated with surfactant deficiency and dysfunction in neonates and adults with respiratory distress syndromes. Increased surfactant surface tension caused by reduction in SP-B induced narrowing of alveolar capillaries and oxygen desaturation, demonstrating an important role of surface tension-lowering properties of surfactant in the regulation of pulmonary vascular perfusion.

Keywords: surfactant protein-B; transgenic mice; pulmonary blood flow; acute respiratory distress syndrome; pulmonary vascular perfusion

Pulmonary surfactant is a complex mixture of lipids and associated proteins that are required for formation and stability of surfactant film in the alveolus. After secretion from type II epithelial cells, surfactant forms a lipid rich film that covers the entire alveolar surface, reducing surface tension at the air/liquid interface from 70 mN/m to near 0 mN/m (1). The reduction of alveolar surface tension is required for the maintenance of alveolar surface area, upon which respiration depends. Surfactant function requires the presence of surfactant protein (SP)-B, a small hydrophobic protein that is tightly associated with surfactant phospholipids in the alveolus (1, 2). In humans and mice, SP-B deficiency or mutations in *SFTPB* cause respiratory failure and death in adults and neonates (3–5). Conditional reduction of SP-B for 4 days in adult mice causes respiratory failure associated with abnormal high surface tension of the surfactant present in bronchoalveolar lavage fluid (BALF) (6–8). This conditional SP-B mouse provides a useful model for study of the influence of surface tension on lung structure, function, and inflammation in adult lung *in vivo*. The loss of

surfactant surface activity influences the shape and function of cells beneath the surfactant film. For example, reduction in SP-B increases the surface tension of surfactant, influencing both cell shape and phagocytic activity of alveolar macrophages *in vivo*, providing support for the concept that surface tension influences cell shape and function in the alveolus (9).

Acute lung injury is a common cause of mortality and morbidity associated with high surface tension in the alveolus as a result of surfactant deficiency and dysfunction in both children and adults (10, 11). Concentrations of SP-B in BALF from patients with acute respiratory distress syndrome were decreased to 20 to 50% of normal (11). Pulmonary hypertension and intrapulmonary shunting is commonly associated with respiratory distress syndrome in neonates and adults, resulting in the impairment of ventilation perfusion or disrupting normal gas exchange.

The alveolar capillary unit consists of a region composed of both interstitial and epithelial components. In addition to alveolar pressure, other capillary compressive forces, including alveolar surface tension and tension in connective tissue fibers (12, 13), must be overcome for recruitment to occur. Because of the close apposition of the alveolar cell surfaces and epithelial component of vessels of the microcirculation, changes in surface tension may influence capillary blood flow via transmitted forces.

In the present study, we sought to test the hypothesis that decreased activity of pulmonary surfactant alters alveolar capillary shape that in turn influences alveolar capillary blood flow and gas exchange *in vivo*. For these studies, a mouse model in which the expression of SP-B in respiratory epithelial cells was conditionally controlled *in vivo* was used.

MATERIALS AND METHODS

Protocols were approved by Animal Care and Use Committee at the Cincinnati Children's Research Foundation.

Transgenic Mice

The conditional transgenic mice [CCSP-rtTA, (tetO)₇, *SFTPB/Sftpb*^{-/-}] were previously generated (7, 8) from two transgenic mouse lines (14, 15): (1) (CCSP-rtTA) mice expressing reverse tetracycline transactivator transcription factor (rtTA) under the control of a 2.3-kb element from the rat Clara cell secretory protein (CCSP) gene promoter that confers expression in both conducting epithelial cells and alveolar epithelial type II cells; and (2) [(tetO)₇, *SFTPB*] mice, in which the human SP-B cDNA was expressed under the control of the tetracycline response elements. CCSP-rtTA/(tetO)₇, *SFTPB* mice were bred with *Sftpb*^{+/-} mice to generate CCSP-rtTA, (tetO)₇, *SFTPB/Sftpb*^{-/-} mice (7, 8). Because *Sftpb*^{-/-} mice die perinatally unless the SP-B transgene is expressed, conditional transgenic mice were maintained on doxycycline-containing food from Embryonic Day 1 until 7 weeks of age. Doxycycline was removed for 4 days for the present studies. Control mice were maintained under continuous treatment with doxycycline and studied at 7 weeks of age. A separate group of 7-week-old animals, in which mice consumed no doxycycline (Off Dox) for 4 days and were then placed back on doxycycline (On Dox), were studied to assess the reversibility of SP-B-dependent changes. In these mice, SP-B was significantly decreased on Day 1 of withdrawal and was almost undetectable by Western blot analyses (8) 2 days after removal from doxycycline. Severe respiratory failure develops in these animals 4 days after removal from doxycycline,

(Received in original form September 16, 2008 and in final form January 16, 2009)

This work was supported by National Institutes of Health grants HL 061646 (to M.I., T.E.W., and J.A.W.) and HL56285 (to T.E.W.).

Correspondence and requests for reprints should be addressed to Machiko Ikegami, M.D., Ph.D., Professor of Pediatrics, Cincinnati Children's Hospital Medical Center, Division of Pulmonary Biology, 3333 Burnet Avenue, ML#7029, Cincinnati, OH 45229-3039. E-mail: machiko.ikegami@cchmc.org

Am J Respir Cell Mol Biol Vol 41, pp 433–439, 2009

Originally Published in Press as DOI: 10.1165/rcmb.2008-0359OC on February 6, 2009

Internet address: www.atsjournals.org

TABLE 1. SP-B-DEPENDENT SURFACE TENSION (mN/m) OF SURFACTANT

	Control	Off Dox			On Dox	
		1d	2d	4d	2d	7d
Equilibrium ST	20.5 ± 0.3	21.1 ± 0.4	39.1 ± 5.4*	36.7 ± 2.6*	21.2 ± 0.4	21.4 ± 0.6
Minimum ST	1.7 ± 0.4	8.9 ± 3.0*	18.4 ± 2.0*	16.2 ± 0.8*	8.2 ± 2.1*	1.9 ± 1.3
Relative SP-B in BALF†	1.0 ± 0.03	0.34 ± 0.05*	0.08 ± 0.05*	0.02 ± 0.01*	0.53 ± 0.15*	1.32 ± 0.03

Definition of abbreviations: BALF, bronchoalveolar lavage fluid; Dox, doxycycline; SP-B, surfactant protein B; ST, surface tension.

* $P < 0.05$ versus control ($n = 3$ /group).

† Data of Western Blot from reference 8.

a time at which SP-C, and surfactant phospholipid content and composition, are unaltered (7, 8).

Surfactant Surface Activity

Surface activity of isolated large aggregate surfactant was measured with a captive bubble surfactometer (16, 17). The concentration of each sample was adjusted to 7 nmol phospholipid/ μ L, and 3 μ L of the sample was applied to the air–water interface of a 25- μ L volume bubble by microsyringe. Equilibrium surface tension was measured at 300 s and then bubble pulsation was started. The minimum surface tension was measured after 80% bubble volume reduction at a rate of 12 cycles/minute.

Oxygen Saturation

Sequential measurements of percent oxygen saturation of arterial hemoglobin (SpO_2) were made in room air by using a small-animal pulse oximeter (MouseOx; Starr Life Sciences Corp., Allison Park, PA) according to the manufacturer's instructions.

Alveolar Capillary Size Determination by 3.2- μ m Microbead Infusion

To demonstrate the narrowed alveolar capillaries observed in the absence of SP-B associated with high surface tensions of surfactant *in vivo*, 3.2- μ m polystyrene yellow-green fluorescent-labeled microbeads (Molecular Probes, Eugene, OR) were prepared. After intraperitoneal injection of heparin (250 U/mouse), mice for the groups of 0 d control, 2 d Off Dox, 3 d Off Dox, and 4 d Off Dox were anesthetized with ketamine/xylazine (0.16 mg/g, 8 μ g/g body weight). Under additional local anesthesia with lidocaine, a 28-G venous catheter (Strategic Application, Inc., Libertyville, IL) was inserted into the jugular vein and 200 μ L PBS with 0.5% NP-40 (Sigma, St. Louis, MO) containing 1.5×10^5 of 3.2- μ m microbeads was infused by Auto Syringe Infusion Pump (Baxter, Deerfield, IL) at the rate of 20 μ L/minute. Anesthetized mice were killed by exsanguination 5 minutes after microbead infusion, and lung homogenate was incubated for 3 days in 4 M KOH with 0.05% Tween 20 (Sigma) at 37°C on a shaker to completely dissolve the tissue, followed by 20 minutes of centrifugation at $2,000 \times g$. The pellets were rinsed with 2 mL PBS containing 0.5% NP-40 and centrifuged three times to isolate microbeads (18). The number of microbeads isolated from lung homogenate and in the syringe remaining after the microbead infusion was counted using a hemacytometer under light microscopy (19), and was calculated as % microbeads in lung tissue relative to infused microbeads.

To distinguish the influence of hypoxia on pulmonary vasoconstriction from that of SP-B deletion, control mice were kept in the box in which O_2 was maintained at $16 \pm 2\%$ using mixture of air and N_2 flow. On Day 3, the pulmonary deposition of infused 3.2- μ m microbeads was studied as described above while placing the nose and mouth in 16% O_2 . SpO_2 was monitored during hypoxia and during the infusion of microbeads.

In addition to analyzing the percentage of recovery of microbeads in lung tissue, the presence of microbeads in alveolar capillaries was studied morphologically by electron microscopy and by light microscopy. For electron microscopy, tissue inflation-fixed at 25 cm H_2O with 4% formaldehyde (Electron Microscopy Sciences, Washington, PA) was post-fixed with Karnovsky fixative (20), stained, and embedded as previously described (21), and ultra-thin 90-nm sections were prepared. To prevent the microbeads from dissolving in xylene or being damaged by cutting, we used frozen fixed lung tissue and thicker sections for

light microscopy. For the frozen section study, inflation-fixed tissues were embedded in a compound for cryo-tissues (Tissue-Tek O.C.T.; Sakura Finetek, Torrance, CA) after dipping into a series of sucrose-PBS solutions. Tissues were snap-frozen at -70°C , sectioned to 10 μ m thickness, and stained by Diff-quick (Dade Behring, Newark, DE). Furthermore, the presence of fluorescently labeled microbeads in the entire lung was demonstrated by fluorescence microscopy (Ax 10 Plan 2; Zeiss, Göttingen, Germany).

Electron Microscopic Analysis

Intravascular blood coagulation was inhibited by an intraperitoneal injection of heparin (250 U/mouse) 30 minutes before anesthetization with intraperitoneal injection of pentobarbital sodium. The chest cavity was opened and an incision made on the auricle of the left atrium. A 20-gauge blunt needle was tied into the trachea, and the lung was slowly inflated to 25 cm H_2O as the total lung capacity. Inflation pressure was then reduced to 5 cm H_2O and maintained during perfusion fixation with 2.5% glutaraldehyde (glutaraldehyde, EM grade; Electron Microscopy Sciences) in PBS (9, 22, 23). The volume of the fixed lung was determined by the displacement method (13, 16). Fixed tissues were divided into six lobes and cut into 1 to 2 mm³ pieces for post-fixation in 2% osmium tetroxide in 0.1 M sodium cacodylate buffer with 1.5% potassium ferrocyanide and 4% uranylacetate solution, and then dehydrated by a series of ethanol solutions and propylene oxide. Two pieces of fixed tissue were blindly selected from each lobe by technical personnel who were not involved in this study. Fixed tissues were embedded in resin (Embed 812; Electron Microscopy Sciences). Ultrathin (90-nm) sections were prepared by ultra microtome (Ultra cut E; Reichert-Jung, Vienna, Austria) and examined by using an electron microscope (H7600; Hitachi High-technologies America, Pleasanton, CA).

Pressure–Volume Curve Analysis

Mice were injected with sodium pentobarbital (100 mg/kg, intraperitoneally) and placed in a container filled with 100% oxygen to ensure

Figure 1. Oxygen saturation (SpO_2). Oxygen saturation was measured in room air after removal of doxycycline from 7-week-old conditional surfactant protein (SP)-B mouse (Off Dox). Control mice were maintained under continuous treatment with doxycycline and studied at 7 weeks of age. Removal of doxycycline caused significant decreases in arterial oxygen saturation ($n = 10$ mice/group). * $P < 0.01$ versus control. †After 5 days of SP-B deficiency, mice die from respiratory failure.

Figure 2. Retention of fluorescent microbeads in the pulmonary vasculature during reduction of SP-B. Microbeads with 3.2- μ m diameter were infused through the jugular vein. (A) This size microbead was smaller than alveolar capillary diameter of control mice. Microbeads were nearly undetectable in the lung of control mice. In contrast, 25 to 60% of 3.2- μ m microbeads were recovered in the lungs during reduction of SP-B, 2 days, 3 days, and 4 days Off Dox group. Control mice in which SpO_2 was reduced to that in SP-B-deleted mice for 3 days (control 3 d hypoxia) did not retain the infused microbeads in the lung. Thus, retention of microbeads in the lung demonstrated in SP-B-deleted mice was not likely related to hypoxia-induced pulmonary vasoconstriction ($n = 4-6$ mice/group). * $P < 0.01$ versus control; ^t $P < 0.001$ versus 2 days and 3 days Off Dox; [#] $P < 0.05$ versus 2 days, 3 days, and 4 days Off Dox. (B–D) The location of microbeads in alveolar capillaries was studied by electron and light microscopy ($n = 6$ /group). No microbeads were detected in lungs of control mice (C). Numerous microbeads were detected in alveolar capillaries during 4 days of SP-B deficiency (B: *electron microscopy; D: arrow, light microscopy).

complete collapse of the alveoli by oxygen absorption. The mice were killed by exsanguination, and the trachea cannulated and connected by a syringe to a pressure sensor. After opening the diaphragm, lungs were inflated in 100- μ l increments every 10 seconds to a maximum pressure of 30 cm H_2O and deflated to generate pressure–volume curves (7).

Statistics

Data are expressed as mean $\pm$ SEM. Comparisons among groups were made by ANOVA with Tukey-Kramer multiple comparison test. Significance was accepted at the 5% level.

RESULTS

Relationships among SP-B, Surface Tension, Oxygen Saturation, and Alveolar Capillary Diameter

Deletion of the murine *Sftpb* causes respiratory failure at birth (3, 4) that can be rescued by the conditional expression of the human SP-B cDNA in alveolar epithelial cells (6–8). Removal of CCSP-rtTA, (tetO)₇, SFTPB/*Sftpb*^{−/−} mice (hereafter termed SP-B-deficient mice) from doxycycline (Off Dox) for 24 hours reduced in SP-B levels (8) and increased the minimal surface tension of surfactant isolated from their BALF (Table 1). While arterial oxygen saturation (SpO_2) was similar in wild-type *Sftpb*^{+/+}

and control mice during continuous treatment with doxycycline, removal of doxycycline significantly decreased SpO_2 (Figure 1). To assess whether depletion of SP-B altered alveolar capillary diameter *in vivo*, microbeads of 3.2- μ m diameter were infused through the jugular vein. These microbeads normally pass through alveolar capillaries and are not trapped in the lung. The fraction of microbeads trapped in lung tissue relative to that infused was calculated (Figure 2A). As expected, 3.2- μ m microbeads were barely detectable in the lungs of control mice. In contrast, 25% (2–3 d Off Dox) to 60% (4 d Off Dox) of the infused microbeads were trapped in the lungs of SP-B-deficient mice. While these small diameter microbeads exit the lung in control mice (Figure 2C), they were readily observed within the alveolar capillaries of the SP-B-deficient mice (Figure 2D). The 3.2- μ m microbeads trapped in the alveolar capillaries were detected by electron microscopy (Figure 2B). As shown in Figure 1, SpO_2 was 87% in mice 3 days after SP-B deletion. To see whether hypoxia induced pulmonary vasoconstriction might contribute to pulmonary vascular protrusion, deposition of infused 3.2- μ m microbeads was studied in the control mice during exposure to 16% O_2 (Figure 2A, control 3 d hypoxia). SpO_2 was kept at the targeted level and was $87.5 \pm 2.6\%$ on Day 3 in hypoxia. The recovery of microbeads was not significantly different from that of controls in room air and was significantly lower than

Figure 3. Distribution of fluorescent microbeads in the lung. Microbeads (3.2 μ m) injected in the jugular vein were detected in the lung by fluorescence microscopy. Few microbeads were in the lungs of control mice (A). In contrast, numerous fluorescent beads were observed 4 days after removal from doxycycline (B). Figure is representative of $n = 6$ /group. Scale bar = 100 μ m.

Figure 4. Influence of SP-B deficiency on alveolar capillary shape. Representative photographs of alveolar capillary from conditional SP-B mice are provided. Conditional SP-B mice were maintained on doxycycline (Control, A). After removal of doxycycline for 2 days, 3 days, and 4 days, alveolar capillaries were compressed and flattened in the alveolar septae (B–D). After resumption of doxycycline (On Dox 7 d [E] and 13 d [F]), alveolar capillaries protruded into the alveolar spaces, similar to that seen in controls (A) ($n = 4$ mice/group).

that in SP-B-deficient mice at 2, 3, and 4 days Off Dox. Thus, retention of microbeads in SP-B-deleted mice was not caused by hypoxia-induced pulmonary vasoconstriction. The presence of fluorescently labeled 3.2- μm microbeads in the lungs of SP-B-deficient mice was demonstrated by fluorescence microscopy (Figure 3B), being readily detected throughout the alveolar walls. In contrast, only a few microbeads were detected in the control mice lung (Figure 3A). Thus, the reduction of SP-B resulted in trapping of 3.2- μm microbeads in the alveolar capillaries.

SP-B Deficiency Influences Alveolar Capillary Shape

Electron microscopy of the lung demonstrated that reduction in SP-B was associated with changes in alveolar capillary shape (Figure 4). In control mice continuously treated with doxycycline (Figure 4A), or in mice rescued by resumption of doxycycline (Figures 4E and 4F), alveolar capillary walls protruded into the alveolar spaces. Flattening of alveolar capillaries was seen 2, 3, and 4 days after discontinuation of doxycycline (Figures 4B–4D), during which the surface tension of isolated surfactant was increased as assessed by captive bubble surfactometer. Because the apparent size of the alveolar capillaries varies under electron microscopy depending on where and with what angle the sections were made, the degree of alveolar capillary flattening (Figure 5A) was assessed by drawing a line between the two edge points of the capillary at the alveolar wall (*line a*). *Line b* was drawn at 90° from the midpoint, and the percentage of distance above the *line a* (*line c*), relative to the distance between capillary walls (*line b*), was determined ($c \div b \times 100$) for the first five capillaries detected in blindly selected areas. Four mice were studied for each group, and twenty capillaries were analyzed for each group. The extent of protrusion of capillaries into the airspace was significantly decreased 2 days after removal from doxycycline and continued to decrease thereafter. The observed compression of alveolar capillaries was reversed after resumption of doxycycline (Figure 5B). Thus, decreased SP-B reduces surfactant function and is temporally associated with compression of alveolar capillaries. Re-expression of SP-B reversed the abnormalities in both surfactant function and alveolar capillary shape.

Pressure–Volume Curves and Lung Edema Were Not Affected by a Short-Period SP-B Deficiency

Two days after removal of the conditional SP-B mice from doxycycline, lung pressure–volume curve was not significantly different from that of control mice (Figure 6). Three days after removal from doxycycline, lung volumes at maximum pressure (total lung volume) were similar to those of controls; however, lung volumes were significantly decreased on the deflation limb of the pressure–volume curve at this time, indicating changes in alveolar stability. Four days after removal from doxycycline,

total lung volumes were significantly decreased. These studies indicated that after reduction of SP-B there is a period during which surfactant surface activity was altered before significant changes in lung volumes or compliance were observed. During perfusion fixation for electron microscopic analyses, lungs were inflated and maintained at 5 cm H₂O airway pressure. The total lung volumes, measured after perfusion fixation by the displacement method, were similar in all the groups (Table 2). In our

Figure 5. Flattening of the alveolar capillary after reduction of SP-B. (A) Method for calculation of the extent of alveolar capillary protrusion. A line was drawn between the edges of the capillary from the alveolar wall (*line a*). Another line (*line b*) was drawn at 90° from the midpoint of *line a*. The distance between the inner capillary walls (*b*) and its distance above *line a* (*c*) was estimated. (B) The extent of alveolar capillary protrusion was estimated by calculating ($c \div b \times 100$). The degree of alveolar capillary protrusion was significantly decreased by removal of doxycycline. The shape of the alveolar capillary was reversed by resumption of doxycycline (7 d and 13 d On Dox). Alveolar capillaries were flattened *in vivo* by reduction of SP-B ($n = 4$ mice/group). Number of capillaries analyzed in randomly selected areas was 5/mouse and 20/group. ** $P < 0.001$ versus control, * $P < 0.03$ versus control.

Figure 6. Pressure-volume curves during reduction of SP-B. Each point of the pressure-volume curve represents the mean $\pm$ SE of all animals in each group of conditional SP-B mice ($n = 6/\text{group}$). SEs for some points are within the symbol mark. Total lung volumes at 30 cm H₂O were decreased 4 days after removal from doxycycline. Control mice were maintained under continuous treatment with doxycycline. * $P < 0.05$ versus control.

previous studies, lung edema in lung histology was observed 4 days after removal from doxycycline in this model (8). In the present study, removal from doxycycline for 2 to 3 days did not influence lung weight. The lung weight was significantly increased 4 days after removal from doxycycline (0.19 ± 0.01 g) compared with control (0.15 ± 0.01 g, $P < 0.05$). Likewise, the ratio of lung weight to body weight was significantly increased on Day 4 after deletion of *Sftpb* (Figure 7), likely representing changes related to pulmonary edema.

Alveolar Capillary Injury

In previous studies from this laboratory, inflammation and alveolar capillary protein leaks were not detected until 3 days after removal from doxycycline (8). To assess whether endothelial cell integrity was perturbed during the study period all the alveolar capillaries in randomly selected 200-mesh grid of 4 unit-square lung areas were examined by electron microscopy in a blinded fashion. No ultrastructural abnormalities were detected in the capillary endothelium 4 days after removal from doxycycline (data not shown).

DISCUSSION

The present study provides new insights into the pathogenesis of intrapulmonary shunting, commonly associated with both infantile and adult respiratory distress syndromes. Because of the remarkable structure of alveolar walls, pulmonary capillaries are located in close apposition to the surfactant film lining the respiratory epithelium. In the present study, decreased surfactant

activity was caused by conditional removal of SP-B, resulting in flattening of the pulmonary microvasculature and arterial oxygen desaturation at the time before onset of lung inflammation, protein leaks, lung edema, and alveolar surface area alterations. The present study supports the concept that the reduction of alveolar surface tension related to pulmonary surfactant plays a critical role in the maintenance of alveolar capillary shape, which in turn influences pulmonary blood flow. Physical forces caused by the increased surface tension related to surfactant dysfunction altered alveolar capillary shape and pulmonary blood flow, causing hypoxemia.

Pulmonary arteries supply the alveolar capillaries that drain into pulmonary veins. The diameter of the alveolar capillary segment is just sufficient for the passage of red blood cells. These microvascular segments are relatively short, forming a dense network that provides an almost continuous sheet of red blood cells coursing through the alveolar walls, as required for efficient gas exchange. The air-blood barrier includes a basement membrane, fibroblasts, and elastin, providing little structural support for the microvessels. Capillary walls come into close apposition to the airspaces, creating a distance of approximately 0.2 to 0.4 μm across which gas exchange occurs, as indicated by the present ultrastructural studies. In the absence of surface-active surfactant, the high surface tension at the air-liquid interface in the alveoli creates collapsing forces. A surfactant film covers the entire surface of the alveolus, lowering surface tension to nearly 0 mN/m (2). The present studies provide physiologic and ultrastructural evidence that reduction of SP-B decreases surfactant activity which likely influences alveolar surface tension. Reduc-

TABLE 2. VOLUME OF PERFUSION FIXED LUNG TISSUE AT AIRWAY PRESSURE 5 cm H₂O ARE NOT INFLUENCED BY SP-B DEFICIENCY

N = 4/group	Control	Off Dox			On Dox	
		2 d	3 d	4 d	7 d	13 d
Lung volume, ml	1.06 $\pm$ 0.05	1.03 $\pm$ 0.04	1.05 $\pm$ 0.06	1.03 $\pm$ 0.05	1.03 $\pm$ 0.06	1.05 $\pm$ 0.09

Definition of abbreviations: Dox, doxycycline; SP-B, surfactant protein B.

Figure 7. Lung weight (g)/body weight (kg) ratio. Four days after removal from doxycycline, lung weight/body weight ratio was increased 4 days after removal from doxycycline, consistent with pulmonary edema ($n = 6/\text{group}$). * $P < 0.05$ versus control.

tion of SP-B decreased surfactant activity, causing changes in alveolar capillary shape and size that, in turn, reduced oxygenation.

Excised perfused lung has been previously used to demonstrate the effects of surface tension on capillary resistance (24–26). In these studies, surface tension was altered by filling airways with saline or by nebulizing substances causing increased surface tension within the alveolus. Bachofen and Schurch demonstrated the influence of increased alveolar surface tension on alveolar capillary shape *in vitro* by lavaging airways with detergents (27). Hypoxemia occurs by multi-faced surface tension-related disease process, including decreased surface area (27) and lung edema, that might be difficult to segregate in other models. The conditional SP-B mice used in the present study have been previously well characterized, providing a model in which surfactant activity can be influenced *in vivo* (8). Genetic and biophysical studies support the important role of SP-B in surfactant function (1). *Sftpb*^{-/-} mice die of respiratory failure at birth (4), while deletion of SP-A (28), SP-C (29), and SP-D (30) (*Sftpa*^{-/-}, *Sftpc*^{-/-}, and *Sftpd*^{-/-} mice) does not alter perinatal survival in the mouse. Conditional replacement of SP-B in the *Sftpb*^{-/-} mice provides an adult mouse model in which surfactant function can be readily manipulated without disrupting phospholipid synthesis or altering other surfactant component composition (6–8). SP-B expression and lung function are rapidly controlled by the addition of or removal from doxycycline. In the present study, SP-B levels in BALF decreased to 35% of that in controls within 24 hours after removal from doxycycline, and were further reduced thereafter. Reduction in SP-B was associated with significant increases in minimum surface tension of isolated surfactant within 24 hours after removal from doxycycline in this model. Pressure–volume curves were not altered and lung inflammation was not detected 2 days after removal of doxycycline, at which time surface tension of the isolated surfactant was significantly increased. Thus, the observed changes in alveolar capillary shape and oxygenation occurred before the onset of decreased lung volume, compliance, or inflammation. Consistent with this observation, endothelial cell injury was not detected 4 days after discontinuation of doxycycline in the present model.

Since capillaries undergo compression without change in wall thickness when alveolar pressures increased (27), the caliber of the alveolar capillaries depends on the level of lung inflation. In the present study, airway pressure was maintained at 5 cm H₂O during perfusion fixation, a pressure known not to cause capillary flattening (31). The pressure–volume curves demonstrated that lung volume at 5 cm H₂O were 68% of total volume (or lung volume at 30 cm H₂O) in the control group, and

were decreased to 51% after discontinuance of doxycycline. Likewise, the volume of fixed lung determined by the displacement method was unchanged after deletion of SP-B, suggesting that the flattened alveolar capillary shape in SP-B–deleted mouse lung was not caused by lung over-expansion during perfusion fixation.

Acute lung injury is a common cause of mortality and morbidity in both neonates and adults and is associated with severe surfactant dysfunction. Surfactant SP-B levels in BALF are decreased in premature neonates with respiratory distress syndrome and in adult patients with acute lung injury. Surfactant activity is decreased during lung injury, leading to alveolar collapse and ventilation perfusion abnormalities. Abnormalities in surfactant composition, the presence of surfactant inhibitory proteins, and decreased abundance of large aggregate surfactant have been observed in both animal models and patients with acute lung injury (32, 33). Hypoxemia accompanies these disorders caused by decreased ventilation, as well as ventilation perfusion mismatches in which nonventilated areas of the lung are perfused (34). Likewise, ventilated areas with abnormal surfactant may be underperfused with resultant arterial hypoxemia. Severe hypoxia induces pulmonary vasoconstriction mediated by pulmonary arterial vascular small muscle contraction (35–37). Hypoxia induced vasoconstriction when oxygen saturation is under 50% in all species so far examined (36, 38, 39). The decreased alveolar capillary size presently observed in SP-B–deleted mice is distinct from that caused by hypoxic vasoconstriction. The present study demonstrates that surfactant dysfunction generates physical forces within the alveolar walls that alters alveolar capillary size and decreases alveolar capillary blood flow with resultant arterial hypoxemia.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

References

- Whitsett JA, Weaver TE. Hydrophobic surfactant proteins in lung function and disease. *N Engl J Med* 2002;347:2141–2148.
- Yu SH, Possmayer F. Adsorption, compression and stability of surface films from natural, lipid extract and reconstituted pulmonary surfactants. *Biochim Biophys Acta* 1993;1167:264–271.
- Nogee LM, DeMello DE, Dehner LP, Colten HR. Deficiency of pulmonary surfactant protein B in congenital alveolar proteinosis. *N Engl J Med* 1993;328:406–410.
- Clark JC, Wert SE, Bachurski CJ, Stahlman MT, Stripp BR, Weaver TE, Whitsett JA. Targeted disruption of the surfactant protein B gene disrupts surfactant homeostasis, causing respiratory failure in newborn mice. *Proc Natl Acad Sci USA* 1995;92:7794–7798.
- Nogee LM, Wert SE, Profit SA, Hull WM, Whitsett JA. Allelic heterogeneity in hereditary SP-B deficiency. *Am J Respir Crit Care Med* 2000;161:973–981.
- Melton KR, Nesselin LL, Ikegami M, Tichelaar JW, Clark JC, Whitsett JA, Weaver TE. SP-B deficiency causes respiratory failure in adult mice. *Am J Physiol* 2003;285:L543–L549.
- Nesselin LL, Melton KR, Ikegami M, Na CL, Wert SE, Rice WR, Whitsett JA, Weaver TE. Partial SP-B deficiency perturbs lung function and causes air space abnormalities. *Am J Physiol* 2005;288:L1154–L1161.
- Ikegami M, Whitsett JA, Martis PC, Weaver TE. Reversibility of lung inflammation caused by SP-B deficiency. *Am J Physiol* 2005;289:L962–L970.
- Akei H, Whitsett JA, Buroker M, Ninomiya T, Tatsumi H, Weaver TE, Ikegami M. Surface tension influences cell shape and phagocytosis in alveolar macrophages. *Am J Physiol* 2006;291:L572–L579.
- Pison U, Seeger W, Buchhorn R, Joka T, Brand M, Obertacke U, Neuhoof H, Schmit-Neuerburg KP. Surfactant abnormalities in patients with respiratory failure after multiple trauma. *Am Rev Respir Dis* 1989;140:1033–1039.

11. Gregory TJ, Longmore WJ, Moxley MA, Whitsett JA, Reed CR, Fowler AA, Hudson LD, Maunder RJ, Crim C, Hyers TM. Surfactant chemical composition and biophysical activity in acute respiratory distress syndrome. *J Clin Invest* 1991;88:1976–1981.
12. Gil J, Bachofen H, Gehr P, Weibel ER. Alveolar volume-surface area relation in air- and saline-filled lungs fixed by vascular perfusion. *J Appl Physiol* 1979;47:990–1001.
13. Weibel ER. Function morphology of lung parenchyma. In: Macklem PT, Mead J, editors. Handbook of physiology, section 3: the respiratory system. Volume III. Bethesda, MD: American Physiological Society; 1986. pp. 89–112.
14. Perl AK, Wert SE, Loudy DE, Shan Z, Blair PA, Whitsett JA. Conditional recombination reveals distinct subsets of epithelial cells in trachea, bronchi, and alveoli. *Am J Respir Cell Mol Biol* 2005;33:455–462.
15. Tichelaar JW, Lu W, Whitsett JA. Conditional expression of fibroblast growth factor-7 in the developing and mature lung. *J Biol Chem* 2000;275:11858–11864.
16. Ikegami M, Le Cras TD, Hardie WD, Stahlman MT, Whitsett JA, Korfhagen TR. TGF- α perturbs surfactant homeostasis in vivo. *Am J Physiol* 2005;289:L34–L43.
17. Schoel M, Schurch S, Goerke J. The captive bubble method for the evaluation of pulmonary surfactant: surface tension, area, and volume calculations. *Biochim Biophys Acta* 1994;1200:281–290.
18. Glenny RW, Bernard S, Brinkley M. Validation of fluorescent-labeled microspheres for measurement of regional organ perfusion. *J Appl Physiol* 1993;74:2585–2597.
19. Savai R, Wolf JC, Greschus S, Eul BG, Schermuly RT, Hanze J, Voswinkel R, Langheinrich AC, Grimminger F, Traupe H, *et al.* Analysis of tumor vessel supply in Lewis lung carcinoma in mice by fluorescent microsphere distribution and imaging with micro- and flat-panel computed tomography. *Am J Pathol* 2005;167:937–946.
20. Karnovsky MJ. Formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. *J Cell Biol* 1965;27:137A–138A.
21. Ikegami M, Na CL, Korfhagen TR, Whitsett JA. Surfactant protein D influences surfactant ultrastructure and uptake by alveolar type II cells. *Am J Physiol Lung Cell Mol Physiol* 2005;288:L552–L561.
22. Geiser M, Im Hof V, Siegenthaler W, Grunder R, Gehr P. Ultrastructure of the aqueous lining layer in hamster airways: is there a two-phase system? *Microsc Res Tech* 1997;36:428–437.
23. Benachi A, Delezoide AL, Chailley-Heu B, Preece M, Bourbon JR, Ryder T. Ultrastructural evaluation of lung maturation in a sheep model of diaphragmatic hernia and tracheal occlusion. *Am J Respir Cell Mol Biol* 1999;20:805–812.
24. Bruderman I, Somers K, Hamilton WK, Tooley WH, Butler J. Effect of surface tension on circulation in the excised lungs of dogs. *J Appl Physiol* 1964;19:707–712.
25. Sun RY, Nieman GF, Hakim TS, Chang HK. Effects of lung volume and alveolar surface tension on pulmonary vascular resistance. *J Appl Physiol* 1987;62:1622–1626.
26. Topulos GP, Brown RE, Butler JP. Increased surface tension decreases pulmonary capillary volume and compliance. *J Appl Physiol* 2002;93:1023–1029.
27. Bachofen H, Schurch S. Alveolar surface forces and lung architecture. *Comp Biochem Physiol A Mol Integr Physiol* 2001;129:183–193.
28. Korfhagen TR, Bruno MD, Ross GF, Huelsman KM, Ikegami M, Jobe AH, Wert SE, Stripp BR, Morris RE, Glasser SW, *et al.* Altered surfactant function and structure in SP-A gene targeted mice. *Proc Natl Acad Sci USA* 1996;93:9594–9599.
29. Glasser SW, Burhans MS, Korfhagen TR, Na C-L, Sly P, Ross G, Ikegami M, Whitsett J. Altered stability of pulmonary surfactant in SP-C deficient mice. *Proc Natl Acad Sci USA* 2001;98:6366–6371.
30. Korfhagen TR, Sheftelyevich V, Burhans MS, Bruno MD, Ross GF, Wert SE, Stahlman MT, Jobe AH, Ikegami M, Whitsett JA, *et al.* Surfactant protein-D regulates surfactant phospholipid homeostasis in vivo. *J Biol Chem* 1998;273:28438–28443.
31. Fu Z, Costello ML, Tsukimoto K, Prediletto R, Elliott AR, Mathieu-Costello O, West JB. High lung volume increases stress failure in pulmonary capillaries. *J Appl Physiol* 1992;73:123–133.
32. Lewis JF, Veldhuizen R, Possmayer F, Sibbald W, Whitsett J, Qanbar R, McCaig L. Altered alveolar surfactant is an early marker of acute lung injury in septic adult sheep. *Am J Respir Crit Care Med* 1994;150:123–130.
33. Gunther A, Siebert C, Schmidt R, Ziegler S, Grimminger F, Yabut M, Temmesfeld B, Walmrath D, Morr H, Seeger W. Surfactant alterations in severe pneumonia, acute respiratory distress syndrome, and cardiogenic lung edema. *Am J Respir Crit Care Med* 1996;153:176–184.
34. Hedenstierna G, Neumann P. Gas exchange in acute respiratory failure. *Minerva Anesthesiol* 1999;65:383–387.
35. Cotes JE. Distribution of ventilation and perfusion. In: Cotes JE, Leathart GL, editors. Lung function. Oxford: Blackwell Scientific Publications; 1993. pp. 179–212.
36. Dumas JP, Bardou M, Goirand F, Dumas M. Hypoxic pulmonary vasoconstriction. *Gen Pharmacol* 1999;33:289–297.
37. Wagner WW Jr, Latham LP. Pulmonary capillary recruitment during airway hypoxia in the dog. *J Appl Physiol* 1975;39:900–905.
38. Barman SA. Potassium channels modulate hypoxic pulmonary vasoconstriction. *Am J Physiol* 1998;275:L64–L70.
39. Haris P, Heath D. Influence of respiratory gases. In: Harris P, Heath D, editors. The human pulmonary circulation. London: Churchill Livingstone; 1986. pp. 456–483.