Function and inhibition sensitivity of the N-terminal segment of surfactant protein B (SP-B1-25) in preterm rabbits

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Abstract
Background—Surfactant protein B (SP-B) is an essential component of pulmonary surfactant, but shorter SP-B sequences may exert equivalent surface activity.
Methods—We synthesised a peptide based on the amino-terminal domain of SP-B (SP-B1-25), a full length SP-B1-78, and a full length palmitoylated SP-C peptide (SP-C1-35) and compared the in vivo function and sensitivity to plasma inhibition of preparations consisting of mixtures of phospholipids with SP-B1-25 or SP-B1-78 and/or SP-C1-35 to Survanta. Preterm rabbits born at 27 days of gestation were treated at birth with surfactant and ventilated for 60 minutes. At 15 minutes half of them received plasma intratracheally. Dynamic compliance was monitored every 15 minutes and postmortem pressure-volume curves were measured to define lung mechanics.
Results—Dynamic compliance and postmortem lung volumes were highest after treatment with a surfactant consisting of an SP-B peptide and SP-C1-35 or Survanta. Plasma instillation decreased dynamic compliance and lung volumes sharply, but the most effective activity was prior instillation of surfactants containing SP-B1-25.
Conclusion—These experiments suggest that the N-terminal domain of SP-B (SP-B1-25) exhibits in vitro and in vivo surface activity and is relatively insensitive to plasma inhibition.

Keywords: surfactant protein B; surfactant inhibition; lung function

Pulmonary surfactant is composed of 80% phospholipids (PL), 10% neutral lipids (cholesterol), and 10% proteins. Surfactant is synthesised and secreted by alveolar type II cells and reduces the surface tension at the air/water interface in the alveoli. Surfactant deficiency in preterm infants with respiratory distress syndrome (RDS) is now routinely treated with intratracheal instillation of an exogenous surfactant preparation. Although dipalmitoyl phosphatidylcholine (DPPC) and phosphatidylglycerol (PG) constitute the main phospholipid components in clinical surfactant, its biophysical activity depends to a large extent on the presence of the hydrophobic surfactant protein B (SP-B), a 79 amino acid homodimer of approximately 18 kDa. SP-B is an essential component of surfactant as mutations within the SP-B locus result in lethal respiratory failure in newborn infants. Isolation of surfactant from animal and human sources has been instrumental in the development of effective surfactant replacement therapy. An alternative that has gained increasing attention is the development of a synthetic surfactant composed of DPPC, PG, and palmitic acid (PA) plus synthetic peptides that resemble the functional domains of SP-B and SP-C. We have shown the functional activity of the synthetic full length SP-B1-78, based on the human sequence, and SP-BR236C, a mutant SP-B based on the sequence from an infant with lethal RDS who had a point mutation in exon 7 of the SP-B gene allele resulting in a cysteine for arginine substitution. However, as shorter SP-B peptides can exert surface activity in vitro and, for pragmatic reasons, we have shifted our focus to amphipathic peptides of the amino-terminal segment of surfactant protein B (SP-B1-25), known as SP-B1-25, which contains four positively charged residues. SP-B1-25 adds surface activity to a standard phospholipid mixture, but has not been tested in animal models with RDS and/or surfactant inactivation.

To investigate its in vivo activity we compared the effect of SP-B1-25 on lung function with that of SP-B1-78 in ventilated preterm rabbits treated with surfactant at birth. Because clinical surfactant preparations contain both SP-B and SP-C, these measurements were done with SP-B peptides alone or in combination with synthetic full length and palmitoylated SP-C1-35. To gain insight into the sensitivity of these synthetic surfactant peptides to protein inactivation, these lung function measures were also obtained in preterm rabbits in which surfactant treatment was followed by instillation of plasma.

Methods
Materials
Peptide synthesis reagents were purchased from Applied Biosystems (Foster City, CA, USA), high performance liquid chromatography solvents from Fisher Chemical Co (Pittsburgh, PA, USA), and all other chemicals from Sigma Chemical Co (St Louis, MO, USA) and Aldrich Chemical Co (Milwaukee, WI, USA). Dipalmitoyl phosphatidylcholine (DPPC) and 1-palmitoyl-2-oleoyl phosphatidylglycerol (POPG) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Survanta (Barrington, PA, USA), and all other chemicals from Sigma Chemical Co (St Louis, MO, USA) and Aldrich Chemical Co (Milwaukee, WI, USA). Dipalmitoyl phosphatidylcholine (DPPC) and 1-palmitoyl-2-oleoyl phosphatidylglycerol (POPG) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Survanta (Barrington, PA, USA), and all other chemicals from Sigma Chemical Co (St Louis, MO, USA) and Aldrich Chemical Co (Milwaukee, WI, USA). Dipalmitoyl phosphatidylcholine (DPPC) and 1-palmitoyl-2-oleoyl phosphatidylglycerol (POPG) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Survanta (Barrington, PA, USA), and all other chemicals from Sigma Chemical Co (St Louis, MO, USA) and Aldrich Chemical Co (Milwaukee, WI, USA). Dipalmitoyl phosphatidylcholine (DPPC) and 1-palmitoyl-2-oleoyl phosphatidylglycerol (POPG) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Survanta (Barrington, PA, USA), and all other chemicals from Sigma Chemical Co (St Louis, MO, USA) and Aldrich Chemical Co (Milwaukee, WI, USA).
practice, was obtained from Ross Laboratories (Columbus, OH, USA). Human fresh frozen plasma contained approximately 50 g/l plasma proteins. All surfactant preparations were used at a lipid concentration of 25 mg/ml in both the in vitro and in vivo studies. Date-mated pregnant New Zealand White rabbits were obtained from IFPS (Norco, CA, USA).

SYNTHESIS AND PURIFICATION OF SURFACANT PEPTIDES SP-B1–78, SP-B1–25, AND SP-C1–35

Full length oxidised SP-B1–78, the N-terminal domain of SP-B (SP-B1–25), and palmitoylated SP-C1–35 were each synthesised on a 0.25 mmol scale with a model 431A peptide synthesiser (Applied Biosystems-Perkin Elmer, Foster City, CA, USA) using FastMoc chemistry,14 purified with HPLC, and their molecular weight was determined by mass spectrometry. The synthesis, purification, and sequence confirmation of the SP-B1–78 and palmitoylated SP-C1–35 peptides, both based on the human sequence peptide, have been described previously.7,11 The N-terminal sequence of SP-B (SP-B1–25) was synthesised, purified, and its sequence characterised using the same methodology as for SP-B1–78.11 SP-B1–25 was based on the human sequence with one variation—cysteine 11 was replaced with alanine (Cys-11>Ala-11 variant monomer). Its
Experimental Surfactant Preparations

The experimental surfactant preparations were prepared by mixing synthetic peptides into a standard phospholipid mixture (PL) consisting of DPPC, POPG, and PA (69:22:9 wt/wt/wt) to create: PL, PL + SP-B1–78 (B1–78), PL + SP-B1–25 (B1–25), PL + SP-C1–35 (C), PL + SP-B1–78 + SP-C1–35 (B1–78 + C), and PL + SP-B1–25 + SP-C1–35 (B1–25 + C). SP-B1–78 was added in at 3% weight and SP-C1–35 at 1% weight, but SP-B1–25 was added in an amount equimolar to 3% full length SP-B1–78 or about 1% on a weight basis. In previous experiments we found 3% SP-B to provide an optimal response.

The phospholipids and synthetic peptide(s) stored in chloroform were freeze dried, added together, and the mixture was then rehydrated in 0.15N NaCl over 48 hours with gentle stirring at 37°C. All surfactant preparations were prepared at a concentration of 25 mg phospholipids/ml and administered in a dose of 100 mg phospholipids/kg body weight.

In Vitro Surface Activity

Changes in surface tension were measured during compression on unbuffered 0.9% NaCl at room temperature in a modified Langmuir–Wilhelmy balance (KimRay, Greenfield Surfactometer, Oklahoma City, OK, USA).11 17 Samples containing 50 µg phospholipids were loaded on a saline subphase in a 51.5 cm² rectangular Teflon trough. Compression of the surface film from 100% to 20% of the total area was carried out with a cycle time of 90 seconds. To study the effect of plasma, the surfactant surface layer was cycled three times, a 5 µl aliquot of plasma was applied on the surface, and four more compression cycles were obtained. The isotherms thus obtained were monitored using both minimum and maximum surface tension and to calculate the area of the hysteresis. Four measures were performed for each data point.

Animal Protocol

Thirty six pregnant New Zealand White rabbits were premedicated at 27 day gestational age with 50 mg/kg ketamine and 5 mg/kg acepromazine by intramuscular injection and then given general anaesthesia with 50 mg/kg ketamine intravenously prior to undergoing a caesarean section. Fetuses were sequentially delivered, weighed, and anaesthetised with an intraperitoneal injection of a mixture of 10 mg/kg ketamine and 0.1 mg/kg acepromazine. The median litter size was 10 (range 6–13) and median birth weight was 33.0 g (range 18–45). The trachea of each newborn was exposed through a small incision in the anterior neck and a short tube made from an 18-gauge needle was tied in the trachea. Each rabbit was ventilated with 100% oxygen using a anaesthesia bag and manometer for about five breaths and transferred to a temperature controlled ventilator-platthysmograph system that permits the simultaneous ventilation of 10 newborn rabbits.7 18 The platthysmographs are a series of 10 clear Plexiglas boxes, temperature controlled at 37°C, connected to 10 rebreathing circuits containing soda lime and driven by a Sechrist infant ventilator (Sechrist Industries, Anaheim, CA, USA). The rabbits were ventilated with 100% oxygen at a rate of 40 breaths/minute.
min with a 1:1 inspiratory-to-expiratory time ratio. No positive end expiratory pressure was used to avoid the air trapping that seems to occur in preterm rabbits. The initial peak inspiratory pressure was 30 cm H₂O and peak pressure was adjusted individually to achieve a tidal volume of about 10 ml/kg as measured with a pneumotachometer (Validyne, Northridge, CA, USA) and a multichannel recorder (Gould Inc, Cleveland, OH, USA). Peak inspiratory pressure was limited at 35 cm H₂O to avoid pneumothorax. Dynamic compliance was calculated by dividing tidal volume by peak inspiratory pressure and the body weight in kg (ml/kg/cm H₂O) and monitored every 15 minutes.

Except for a control group of 12 untreated preterm rabbits (no treatment, NT), all preterm rabbits were treated with 100 mg/kg of an experimental surfactant after insertion of the endotracheal tube and prior to bagging. Surfactant treatments were given in a randomized way. After 15 minutes of ventilation, half of the rabbits received 2 ml/kg of 1:1 diluted (with distilled water) plasma (~50 mg proteins/kg) by intratracheal instillation. After 60 minutes of ventilation each rabbit was killed by an intrathecal injection of lidocaine and disconnected from the ventilator. Pressure-volume curves were measured in situ to define lung mechanics. Lungs were inflated and deflated using a bidirectional Harvard pump coupled to a 10 ml glass syringe and pressure was continuously recorded on a Gould multichannel recorder. Each pressure-volume curve was corrected for the compliance of the system by subtracting the pressure-volume curve of the pump/syringe unit carried out before each curve. Verification that lung volumes changed the pump/syringe unit carried out before each curve. Verification that lung volumes changed was performed, the lungs were lavaged five times with 1.5 ml 0.9% NaCl warmed to body temperature. The protein content in the lung lavage material was assayed by a modified Lowry assay using bovine serum albumin as a standard, and protein recovery was calculated as mg lavage protein/kg body weight. Treatment groups consisted of 15 preterm rabbits. All experiments were performed humanely and with the approval of the Animal Care and Use Committee.

STATISTICAL ANALYSIS
All values are expressed as mean (SE). Between-group comparisons were done by one way analysis of variance followed by the Student-Newman-Keuls multiple comparison procedure. The t test was used for comparisons with control values. A p value of <0.05 was considered to indicate a significant difference.

Results
All surfactant preparations reached minimum surface tensions of <10 mN/m on the Wilhelmy balance. With the exception of the PL preparation in which plasma improved hysteresis, addition of plasma overall decreased the area of the hysteresis, although the effect was minimal with SP-B1–78 and SP-B1–25 (fig 1). Minimum surface tension was not affected by addition of plasma, except for PL where the minimum rose slightly from 3.9 (0.4) to 8.2 (2.4) mN/m.

Preterm rabbits (15 per group) treated with B1–25 + C, B1–78 + C, and Survanta had the highest mean dynamic compliance during the 60 minute ventilation period, with intermediate values for B1–78, B1–25, and C surfactants, and lowest values for PL surfactant (fig 2). Postmortem pressure-volume curves showed that lung volumes at 30 cm H₂O pressure were highest for preterm rabbits treated

Intratracheal plasma instillation 15 minutes after surfactant instillation resulted in a sharp and rapid decline in dynamic compliance which was largest (with a mean decline of 33–36%) in preterm rabbits treated with PL, B1–78, or C surfactants, intermediate (24–30%) in those treated with B1–78 + C surfactant or Survanta, and smallest (11–12%) after treatment with B1–25 or B1–25 + C surfactants (fig 4). The postmortem pressure-volume curves show that plasma instillation 15 minutes after surfactant treatment led to a generalised reduction in lung volume at 30 cm H\textsubscript{2}O pressure in all surfactant groups (figs 3 and 5). Pretreatment with B1–25 + C or B1–78 + C was more effective in preserving lung volume than pretreatment with Survanta, B1–25, or B1–78, and lung volumes were lowest for C and PL surfactants (fig 5).

Lavage proteins of preterm rabbits treated with both surfactant and plasma were higher than in rabbits treated with surfactant only (fig 6); the difference was indicative of the amount of plasma proteins instilled intratracheally. Under both conditions, lavage proteins in preterm rabbits treated with PL and, to a lesser extent, C surfactant were higher than in rabbits which received surfactant with an SP-B analogue.

Discussion

In these experiments, surfactant preparations containing two different surfactant protein B sequences were tested on the surface balance and in ventilated preterm rabbits in the absence and presence of plasma, a known strong surfactant inhibitor. The results show that surfactant consisting of the N-terminal domain of SP-B (SP-B1–25) in a standard phospholipid mixture exhibits in vitro and in vivo surface activity and is also relatively insensitive to plasma protein inhibition in ventilated preterm rabbits. Addition of palmitoylated SP-C1–35 to this surfactant improved the positive effects on lung compliance but did not affect the resistance against plasma inactivation.

Previous work by our group has shown the functionality of the synthetic peptides SP-B1–78 and SP-C1–35 in surfactant deficient lavaged rats, a model for adult RDS (ARDS).\textsuperscript{7,8} We recently tested SP-B1–78, SP-BR236C, and SP-C1–35 in 28 day gestation ventilated preterm rabbits given albumin intratracheally and rescued with surfactant.\textsuperscript{7} However, at 28 days gestational age, rabbits develop only mild to moderate RDS and can be easily stabilised in hyperoxia.\textsuperscript{10} We chose 27 day gestation preterm rabbits for these studies because they are almost completely surfactant deficient\textsuperscript{20} and develop severe RDS soon after birth. They cannot be stabilised without immediate surfactant treatment and starting mechanical ventilation at birth. Since the effect of surfactant treatment on lung function plateaus 15 minutes after birth, this time point was chosen for plasma instillation. Pulmonary surfactant is sensitive to functional inhibition by plasma proteins, which invade the alveolar space during acute lung injury.\textsuperscript{21–24} Among the plasma proteins, albumin,\textsuperscript{25,30} haemoglobin,\textsuperscript{31} C-reactive protein,\textsuperscript{35} and fibrinogen and fibrin monomers\textsuperscript{25,30} have proven surfactant inhibitory properties in vitro with the following rank order: fibrin monomer>fibrinogen>haemoglobin>albumin. Fresh frozen plasma was chosen for this study for its strong inhibiting effect. We used 5\textmu l plasma for the inhibition studies on the Wilhelmy balance—which amounts to the addition of 250 \textmu g of protein to 50 \textmu g of phospholipids—to check the effect of a high protein:phospholipid ratio (5:1 wt:wt). However, the newborn rabbits did not tolerate such a high protein:phospholipid ratio. In the preliminary experiments the instillation of 100 mg/kg plasma proteins was almost always fatal and we had to reduce the plasma protein dose to 50 mg/kg. Intratracheal instillation of this plasma protein dose was accompanied by a sharp reduction in dynamic lung compliance, which was used as a measure for in vivo inhibition resistance.

This study in 27 day preterm rabbits shows that the presence of SP-B1–25 in a standard phospholipid mixture resulted in a surfactant preparation which was highly effective in vitro and in vivo and that SP-B1–25 behaves in a functionally similar way to standard full length SP-B1–78. Surfactant with SP-B1–25 was less sensitive to inhibition by plasma proteins than surfactant containing SP-B1–78. Likewise, the combination of SP-B1–25 and SP-C1–35 (B1–25 + C surfactant) was functionally equivalent to B1–78 + C surfactant and Survanta, but was more resistant to protein inhibition. We still lack an explanation for the mechanism by which SP-B1–25 confers more resistance to protein inhibition than SP-B1–78, but speculate that this may be due to structural differences which shield relevant areas of these peptides from interaction with inhibitory proteins. We have previously tested short SP-B sequences and found them to be effective in vitro,\textsuperscript{11–13} but this is the first time that we show...
their functionality in an animal model with RDS. In these studies we used Survanta since it is generally used as a clinical surfactant in very preterm infants with RDS in neonatal intensive care units but, because of its relatively low SP-B content, it is probably not an ideal gold standard.

Positive end expiratory pressure (PEEP) was not used in the rabbit experiments as is usual with this model. PEEP enhances the in vivo surfactant function by increasing lung volume. Omission of PEEP generally dampens the response to surfactant treatment and results in an underestimation of in vivo surfactant function. However, it is not unlikely that, in these preterm animals, ventilation for 60 minutes without PEEP is sufficient to injure the lung and cause a loss of lung volume. Lavage protein values suggest that this is especially true for untreated controls and rabbits treated with PL and C surfactant.

These experiments in ventilated 27 day gestation preterm rabbits show that the two synthetic surfactant preparations B1–78 + C, and B1–25 + C were functionally similar to Survanta, a clinical surfactant extracted from bovine lungs. Since B1–25 + C surfactant was less sensitive to inhibition by plasma proteins than B1–78 + C surfactant and Survanta, the SP-B1–25 peptide may be important for the formulation of a new generation of clinical surfactant preparations.

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