Surfactant-Associated Protein B Kinetics *In Vivo* in Newborn Infants by Stable Isotopes

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ABSTRACT

Surfactant-associated protein B (SP-B) is critical to the biophysical function of pulmonary surfactant. No information is available on SP-B synthesis and kinetics in humans. We administered a 24-h i.v. infusion of ^{13}C-valine as metabolic precursor of SP-B to six newborn infants (weight 3.5 ± 0.5 kg; age 12 d, range 1–43 d). Three of the study infants also received i.v. ^{2}H-palmitate to label surfactant disaturated phosphatidylcholine (DSPC). SP-B and DSPC were isolated from tracheal aspirates, and their respective ^{13}C and ^{2}H enrichments were measured by gas chromatography–mass spectrometry. SP-B kinetics was measured successfully in all six infants. SP-B median (range) fractional synthesis rate was 30% per day (20–78% per day), secretion time was 4.5 h (1–9 h), time to peak was 24 h (12–36 h), and half-life was 21 h (8–35 h). The ascending part of the SP-B kinetic curve was similar to the DSPC curve, suggesting similar secretion pathways. SP-B half-life seemed to be shorter than DSPC half-life. These results agree with existing animal data. We conclude that the measurement of SP-B kinetics is feasible *in vivo* in humans by stable isotope technology. (*Pediatr Res* **57: 519–522, 2005**)

Abbreviations

DSPC, disaturated-phosphatidylcholine
FSR, fractional synthesis rate
SP-B, surfactant-associated protein B
SP-C, surfactant-associated protein C
ST, secretion time
TA, tracheal aspirate

Surfactant-associated proteins B and C (SP-B and SP-C, respectively) represent <5% of surfactant by weight, but they play a pivotal role in maintaining surfactant function (1). Surfactant deficiency is the hallmark of respiratory distress syndrome in preterm infants. In respiratory distress syndrome, there is a reduced amount of phospholipids (2) and of SP-B and SP-C (3). The key role of SP-B became evident in congenital SP-B deficiency, which is characterized by severe respiratory failure leading ultimately to death unless lung transplant is performed (1,4). To date, there are no data on SP-B kinetics in humans, but information is available in adult and newborn animals, in which radioactive labels can be used. These studies showed differences in SP-B clearance and kinetics depending on animal species and postnatal age (5–7).

We recently described methods based on stable isotopes to measure surfactant disaturated-phosphatidylcholine (DSPC) kinetics in humans (8,9). The main objective of this study was to demonstrate the feasibility of measuring SP-B kinetics *in vivo* in newborn infants by means of stable isotopes.

METHODS

SP-B kinetics was studied in six infants (Table 1) who were admitted to the neonatal intensive care unit, Department of Paediatrics, University of Padua. Inclusion criteria were *1)* gestational age >37 wk, *2)* respiratory failure requiring endotracheal intubation for an estimated length of at least 48 h, *3)* arterial and venous lines placed for clinical monitoring, and *4)* written parental consent. Exclusion criteria were congenital chromosomal abnormalities and exogenous surfactant given at <48 h before study start. The local Ethics Committee approved the study protocol.

All patients received a 24-h constant i.v. infusion of 2 mg/kg/h 1-^{13}C valine (Mass Trace, Woburn, MA), dissolved in normal saline, while they were on a lipid-free i.v. infusion. In three infants, we also infused i.v. 0.4 mg/kg/h 16,16,16 ^{2}H palmitic acid (CIL, Andover, MA), associated with human albumin (10).

Blood (0.6 mL) was drawn at time 0, 5:30, 6, 12, 18, and 24 h from the start of the study to determine the isotopic enrichment of plasma valine and of palmitic acid at plateau. Blood samples were placed in tubes that contained

Table 1. *Clinical characteristics of the study infants*

Patients	Sex	Birth weight (kg)	Gestational age (wk)	Weight (kg)	Age (d)	Intubation (d)	Ventilator mode	Main diagnosis
1	M	3.4	39	3.8	43	6	SIMV	Arnold Chiari
2	M	4.1	39	4.2	4	8	HFOV	Pneumonia
3	M	2.7	39	2.7	1	5	HFOV+NO	Pneumothorax
4	M	3.4	40	3.4	1	5	SIMV	Asphyxia
5	M	3.7	39	3.6	4	4	SIMV	Abdominal mass
6	M	2.9	41	3.2	20	4	SIMV	Neck lymphangioma
Mean		3.4	39.5	3.5	12	5.3		
SD		0.5	0.8	0.5	17	1.5		

EDTA and centrifuged immediately at $1300 \times g$, and plasma was stored at $-20°C$.

Tracheal aspirates (TAs) were performed according to a standardized procedure as previously described (9), and they were obtained before the start of the isotope(s) infusion, every 6 h, until 72 h and every 12 h until extubation or until study day 7. TAs were centrifuged at $150 \times g$ for 10 min, and supernatant was stored at $-80°C$ until analysis.

Production of antiserum anti–SP-B. Surfactant that was obtained from a patient with alveolar proteinosis (6.8 mg phospholipids) was extracted according to Bligh and Dyer (11). Aliquots that contained 0.5 mg of phospholipids were suspended in chloroform and applied onto columns Bond Elute NH_2 (500 mg of resin per column; Varian, Harbor City, CA), and each column was eluted sequentially with 6 mL of the following mixtures made of chloroform:methanol:acetic acid: 100:0:0, 20:1:0, 9:1:0, 4:1:0, 4:1:0.025, 3:2:0, 1:4:0, and 1:9:0. The 4:1:0 eluates, which contained pure SP-B, were combined and dried under nitrogen. One fourth of SP-B obtained by the method described above was suspended in 0.25 mL of saline solution by sonication, combined with 0.25 mL of complete Freund's adjuvant, and injected s.c. into an adult rabbit under light anesthesia. Boosting injections of SP-B in incomplete Freund's adjuvant were done after 1, 2, and 3 mo. Serum was obtained by exsanguination after 4 mo. This antiserum recognizes SP-B and Pro-SP-B but does not recognize SP-C. Isolation of DSPC from TA was performed as previously published (9).

Isolation of SP-B from TA. SP-B was isolated with Bond Elute NH_2 columns that contained 100 mg of resin (Supelco, Milan, Italy). TA supernatant was extracted according to Bligh and Dyer (11). The chloroform phase was dried under nitrogen, resuspended in chloroform, and applied to a Bond Elute NH_2 column that was preconditioned with 3–5 mL of chloroform. After loading, the columns were eluted sequentially with 3 mL of the following chloroform/methanol/acetic acid mixtures: 20:1:0, 9:1:0, 4:1:0, 4:1:0.025, 3:2:0, 1:4:0, and 1:9:0. For studying the elution of surfactant proteins, eluates were dried under nitrogen, solubilized, reduced, separated by PAGE (12), transferred on nitrocellulose, and probed with the antiserum anti-human SP-B and an antiserum anti–SP-C (Fig. 1). Antibodies anti–SP-C were provided by Altana Pharma AG (Konstanz, Germany). Most of SP-B eluted with the mixture 4:1:0, whereas SP-C started to be recovered with the mixture 3:2:0. In a separate experiment, in which the material eluted with the mixture 4:1:0 was separated by PAGE and stained with Coomassie stain, we found only a protein band corresponding to mature SP-B (data not shown).

SP-B eluted from the Bond Elute NH_2 column mainly with the 4:1:0 mixture and to a smaller extent with the 4:1:0.025 and 3:2:0 mixtures (Fig. 1). For the purpose of the present study, we analyzed only SP-B eluted by the mixture 4:1:0, because this fraction is not contaminated by SP-C (Fig. 1).

To measure the recovery of SP-B in fraction 4:1:0, we administered into the trachea of a 3-d-old rabbit 250 μCi of Trans ^{35}S Label (ICN Biomedicals, Irvine, CA). After 16 h, the alveolar surfactant was isolated and extracted according to Bligh and Dyer (11). The extract then was fractionated by Sephadex LH60 chromatography (1×80 cm column, eluents: chloroform:methanol:0.1 N HCl [47.5:47.5:5]) to isolate ^{35}S-labeled SP-B and SP-C. ^{35}S-labeled SP-B was dried down, suspended in chloroform, mixed with 150 μg of nonlabeled rabbit surfactant extract, and subjected to Bond Elute NH_2 chromatography. We recovered in the 4:1:0 eluate 40% of applied radioactivity. In a companion experiment, only traces of radioactivity were recovered in the 4:1:0 eluate, when ^{35}S-labeled SP-C was applied to a Bond Elute NH_2 column.

For studying the incorporation of ^{13}C valine into SP-B (*i.e.* SP-B kinetics in infants), the SP-B fraction had to be hydrolyzed to free amino acids by 0.5 mL of 6 N HCl for 24 h at 110°C under vacuum condition. Amino acids from acid hydrolysis of SP-B were derivatized into their N-acetyl-n-propyl derivatives (13).

Plasma valine enrichment. Fifty milliliters of plasma EDTA was deproteinized with sulfosalicylic acid (6% wt/vol), and plasma amino acids were
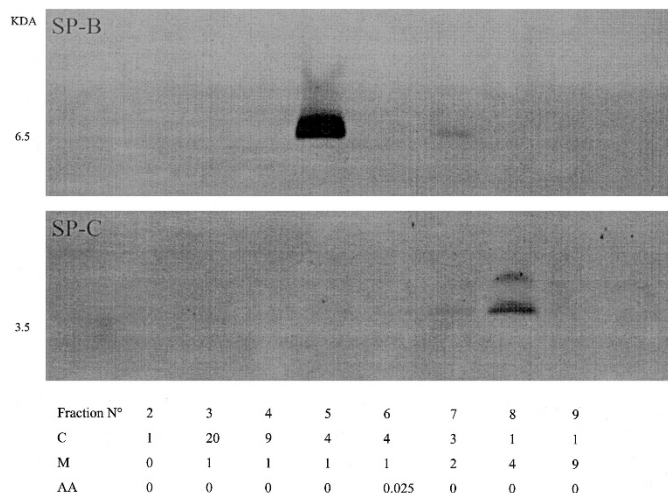

Figure 1. Isolation of SP-B. The Bligh and Dyer (12) extract of a BAL sample that contained 150 μg of phospholipids was dissolved in chloroform, applied to 100 mg of Bond Elute NH_2 column, and eluted with mixtures of chloroform:methanol:acetic acid. Eluates were dried, reduced, separated by PAGE, transferred onto nitrocellulose, and probed with antiserum anti–SP-B (top) or anti–SP-C (bottom).

Fraction N°	2	3	4	5	6	7	8	9
C	1	20	9	4	4	3	1	1
M	0	1	1	1	1	2	4	9
AA	0	0	0	0	0.025	0	0	0

derivatized according to Husek (14). Free plasma valine enrichment was measured by gas chromatography–mass spectrometry (Voyager, Thermoquest, Milan, Italy).

SP-B valine and DSPC palmitate enrichments. The enrichment of SP-B valine and DSPC palmitate was measured by gas chromatography–isotope ratio–mass spectrometry (8,10). Valine was injected in split less mode and separated on a Ultra2, J&W capillary column (25 m $\times$ 0.20 mm $\times$ 0.33 mm film thickness; J&W, Agilent Technologies Italia S.P.A, Cernusco sul Naviglio, Milan, Italy). DSPC palmitate was separated as previously published (8). Results were expressed as mole percentage excess and referred to a calibration curve for both ^{13}C valine and ^2H palmitic acid with enrichment values ranging from 0 to 5% mole percentage excess (8). Each sample was analyzed in triplicate.

DSPC amounts from TA. DSPC amount from TA was determined by gas chromatography as previously described (10).

Calculations. Tissue-bound and alveolar surfactant were regarded as one pool.

Kinetic parameters. Fractional synthesis rate (FSR), secretion time (ST), peak time, and half-life were calculated by using both ^{13}C-valine and ^2H-palmitate as previously published (8,10). Briefly, FSR was calculated by dividing the slope of the linear increase of the enrichment of DSPC and SP-B by the plasma steady-state enrichment of free ^2H-palmitate and ^{13}C-valine.

ST of DSPC and SP-B was defined as the time lag between the start of the infusion of the precursor and the appearance of the enriched product. This was calculated by plotting the regression line for the linear-increasing part of the enrichment *versus* time curve and extrapolating it to baseline enrichment.

Peak time is the time of maximum enrichment of surfactant DSPC and SP-B after the start of the isotope infusion. Half-life of DSPC and SP-B was calculated by exponential curve fitting at the final mono-exponential part of the down slope of the enrichment *versus* time curve.

Clinical variables are expressed as individual values and as mean ± SD. Kinetic data are presented as individual values and group median and range.
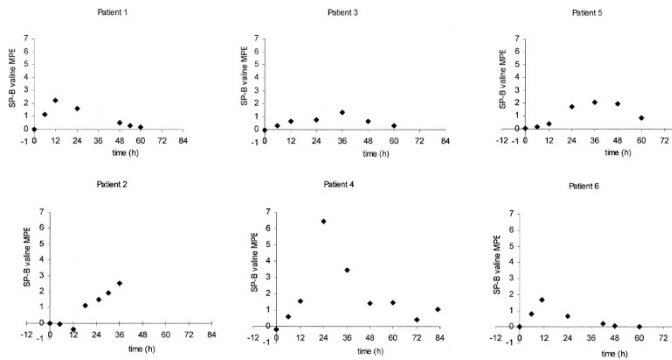
Figure 2. SP-B kinetic curves after a 24-h infusion of ^{13}C valine in six newborn infants on mechanical ventilation.

RESULTS

Characteristics of study infants are reported in Table 1. Patient 2 was intubated at birth for neonatal pneumonia that required fraction of inspired oxygen >0.40, high-frequency ventilation, and 100 mg/kg exogenous surfactant given 96 h before study start. Patient 3 presented with birth asphyxia, pneumothorax, and pulmonary hypertension that required high-frequency ventilation and nitric oxide for 4 d. All other infants had no lung disease, and they were intubated for major surgery (patient 5 and patient 6) or for perinatal asphyxia leading to neurologic failure (patient 4) or for vocal cord paralysis (patient 1). SP-B valine enrichment was measurable in all study infants and in all samples with the exception of patient 2 from 36 h onward. Figure 2 reports all six SP-B kinetic curves. Individual and mean SP-B kinetic parameters are reported in Table 2.

Median (range) DSPC FSR was 25% per day (20–28% per day), ST was 25 h (25–35 h), peak time was 42 h (36–42 h), and half-life was 35 h (25–96 h). Figure 3 depicts the enrichment curves of SP-B and DSPC in one study infant. The ascending parts of the SP-B and DSPC curves were similar, whereas SP-B half-life was shorter than that of DSPC in all three patients who received both tracers.

DISCUSSION

General considerations. In this pilot study, we report for the first time data on SP-B synthesis and kinetics measured by stable isotopes *in vivo* in humans. Because of the pivotal role played by SP-B in maintaining surfactant function in healthy and diseased lungs (1), we believe that this method will be proved to be important to the understanding of the role of SP-B in human lungs. Our preliminary results indicate that SP-B has a short half-life of ~20 h. This could be due to a rapid turnover, to a less extensive recycling in comparison with surfactant lipids, or to a combination of these mechanisms. If this finding were to be confirmed in a larger number of patients, then we hypothesize that SP-B concentrations may rapidly become critically low in case of reduced synthesis or of accelerated catabolism.

Analytical considerations. SP-B and SP-C are highly hydrophobic proteins, and their analytical separation is difficult. Their quantitative measurements are troublesome, especially in samples from newborn infants, in whom concentrations could be low. Immunoblotting techniques (3) are not suitable for isotopic enrichment determination because of the possible amino acid contaminations. We therefore chose to isolate SP-B from SP-C and the lipids by sorbent chromatography (15). Besides patient 2, in whom the descending part of the SP-B kinetic curve was not measurable (Fig. 2), we found a coefficient of variation of <3% for each SP-B sample measured in triplicate.

We did not find measurable contamination of SP-B by SP-C 1) by immunoblotting (Fig. 1) or 2) by mixing surfactant extracts with SP-C labeled *in vivo* with ^{35}S and then performing Bond Elute NH_2 chromatography (see "Methods"). The main disadvantage of our method is the time involved in measuring each TA, which is ~2 d. With this limitation in mind and considering results from animal work (5–7) and from our own studies, we decided to process only seven to eight time points for each individual patient. Having obtained data, as depicted in Fig. 2, we suggest that future studies should analyze TA every 6 h for the first 48 h and every 12 h until 72 h.

Our findings compared nicely with animal data. Previous work in mice and rabbits (5,6,16) showed that secretion rate of SP-B was similar to that in DSPC, but clearance rate was different. All of these studies used either i.v. and/or intratracheal radioactive precursors (5–7,15). The longest duration of published studies was 48 h, whereas we studied our patients for up to 5 d. This allowed us to use only one tracer.

Clinical relevance. Low levels of mature SP-B lead to impaired lung function and severe respiratory failure (1,4,17–

Table 2. *SP-B and DSPC kinetics after 24-h i.v. infusion of 1-^{13}C valine and ^2H palmitate*

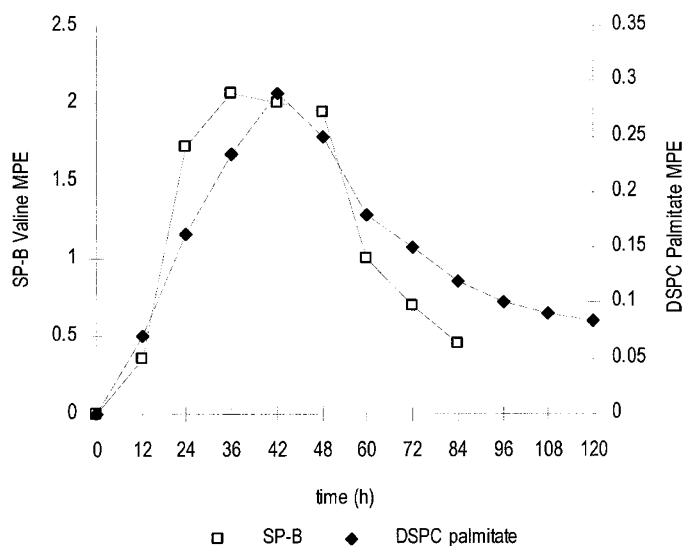| | SP-B | | | | DSPC | | | | |
Patients	FSR (%/d)	ST (h)	Time to peak (h)	Half-life (h)	FSR (%/d)	ST (h)	Time to peak (h)	Half-life (h)	DSPC amount from TA (mg/mL)
1	52	1	12	21					0.22 ± 0.43
2	28	9			28	25	42	25	0.14 ± 0.17
3	27	4	24	8	25	25	36	35	0.21 ± 0.17
4	78	8	24	29					0.11 ± 0.11
5	20	5	36	35	20	35	42	96	0.24 ± 0.20
6	33	1	12	8					0.14 ± 0.04
Median	30.5	4.5	24	21	25	25	42	35	0.18
Range	20 –78	1 –9	12 –36	8 –35	20 –28	25 –35	36 –42	25 –96	0.19

Figure 3. SP-B and DSPC kinetic curves after the simultaneous i.v. administration of ^{13}C valine (□; left *y* axis) and ^{2}H palmitate (◆; right *y* axis) in study infant 5.

20). Thus, measuring SP-B kinetics *in vivo* in humans will be an irreplaceable tool for identifying clinical situations in which SP-B synthesis may be reduced or its catabolic rate accelerated.

Limitations of the study. The number of patients is definitely too small to make any meaningful clinical consideration. The main message of this article is that SP-B kinetics can be measured *in vivo* in humans using stable isotope technology and that SP-B and DSPC kinetic studies can be obtained in the same patient. Because Clara cells from the conductive airways are able to synthesize SPs (21,22), further studies will be needed to ascertain differences between surfactant samples from TA and alveolar surfactant. Several investigators found similar surfactant composition in TA and bronchoalveolar lavage fluid (23,24), but SP-A, SP-B, and SP-C were found to be lower in TA than in lung lavage fluid (23). Of note, surfactant analysis from TA is currently the standard method for the study on surfactant in human research (3).

CONCLUSION

In conclusion, we describe here a method to measure SP-B kinetics *in vivo* in humans by stable isotope technology. We were able to obtain kinetic curves in all study infants, including those with severe lung disease. We speculate that this method could be a valuable tool to better understand the role of SP-B in human lung disease.

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