Very low surfactant protein C contents in newborn Belgian White and Blue calves with respiratory distress syndrome

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We have studied a respiratory distress syndrome (RDS) occurring in newborn calves of the Belgian White and Blue (BWB) breed that represents the large majority of beef cattle in Belgium. Pulmonary surfactant isolated from 14 BWB newborn calves that died from RDS and from 7 healthy controls was analysed for composition and surface activity. An extremely low content or, in some instances, an absence of surfactant protein C (SP-C) was detected in the RDS samples by Western blotting and differential amino acid analysis [$0.03 \pm 0.01\,\%$ (w/w) relative to total phospholipids, compared with $0.39 \pm 0.06\,\%$ for healthy controls (means $\pm$ S.E.M., $P < 0.001$)]. The contents of surfactant protein B (SP-B) were similar in RDS and control samples. The crude surfactant samples isolated from RDS calves had higher ratios of total protein to total phospholipid, altered phospholipid profiles and lower SP-A contents. Both crude and organic extracts of RDS surfactant samples showed increased dynamic surface tension compared with healthy controls when evaluated with a pulsating-bubble surfactometer. The addition of purified SP-C to organic extracts of RDS surfactant samples lowered surface tension. Strongly decreased levels of mature SP-C associated with fatal RDS and altered surface activity *in vitro* have, to the best of our knowledge, not been previously reported. The mechanisms underlying RDS and the decrease in SP-C in BWB calves remain to be established.

Key words: amino acid analysis, bronchoalveolar lavage, phospholipids, pulmonary surfactant, surface activity.

INTRODUCTION

A major role of pulmonary surfactant, the complex material that is synthesized by type II cells and lines the alveolar walls, is to decrease the surface tension at the alveolar air/liquid interface to prevent lung collapse at the end of expiration [1]. A high content of disaturated phosphatidylcholine (PtdCho), especially dipalmitoyl-D,L-α-phosphatidylcholine (DPPC) [2], and the presence of the two hydrophobic surfactant proteins B and C (SP-B and SP-C) [3] are thought to be essential for this surface activity. Two other surfactant-associated proteins, the hydrophilic SP-A and SP-D, are thought to be important in the innate defence mechanisms of the lung and in the secretion and recycling of surfactant [4,5]. SP-A is also involved in the formation of tubular myelin [6], in the enhancement of phospholipid adsorption in association with SP-B [7] and in the protection of surfactant against inactivation by plasma proteins, including fibrinogen [8]. SP-B and SP-C are synthesized as larger precursors (pro-SP-B and pro-SP-C) in alveolar type II cells; post-translational modifications and proteolytic cleavages produce mature proteins that are secreted into the alveolar lumen [9,10]. Both proteins might be important for enhancing the effective adsorption of phospholipids at the alveolar air/liquid interfaces during the respiratory cycles [3], but firm evidence in support of this is lacking.

Pulmonary surfactant deficiency states have been studied extensively for more than 40 years in humans, since the first convincing description of a lack of surface-active material in infants with hyaline membrane disease was published [11]. From that time, reports have focused on surfactant alterations not only in neonatal respiratory distress syndrome (RDS) but also in the adult form of acute respiratory distress syndrome (ARDS). Recently identified mutations in the SP-B gene, resulting in a deficiency of mature SP-B and altered pro-SP-C processing, have been shown to cause a lethal neonatal RDS in full-term infants (reviewed in [12]). Knock-out of the SP-B gene in mice [13] and blocking of SP-B with a monoclonal antibody [14] cause similar lethal postnatal respiratory failure and support the critical role of SP-B in the function of surfactant. Because the phenotype of SP-B deficiency is associated with lower levels of mature SP-C, unequivocal evidence for the individual functional importance of SP-B and SP-C is lacking.

Double-muscled cattle of the Belgian White and Blue (BWB) breed represent the large majority of beef cattle in Belgium. Physiological peculiarities in the oxygen transport chain have, at

Abbreviations used: ARDS, acute respiratory distress syndrome; BAL, bronchoalveolar lavage; BWB, Belgian White and Blue; DOPC, dioleyl-L-α-phosphatidylcholine; DPPC, dipalmitoyl-D,L-α-phosphatidylcholine; γ_{min}, surface tension at minimum bubble size; L-PtdCho, L-α-lysophosphatidylcholine; PL, total phospholipids; PtdCho, phosphatidylcholine; PtdGro, L-α-phosphatidyl-D,L-glycerol; RDS, respiratory distress syndrome; r_{max}, maximum bubble radius; SP, surfactant protein; TP, total protein.

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least in part, been implicated in the higher sensitivity of this breed to acute respiratory disorders [15]. Here we characterize the composition and activity of pulmonary surfactant isolated from newborn BWB calves that died after a clinical course of RDS. These calves were born at full term by caesarean section and showed, during the first day of life, clinical symptoms classically associated with respiratory failure: polypnoea, tachycardia, hypoxaemia, hypercapnia and a mixed metabolic and respiratory acidosis [16]. Despite intensive therapy with antibiotics, anti-inflammatory drugs and good nursing care, nearly 20 % of the affected calves died after a disease course of 1–5 days, with pulmonary autopsy findings characterized by atelectasis, emphysema, oedema, congestion and red hepatization.

The present paper reports an alteration in surface-tension properties and in the composition of crude surfactant isolated from bronchoalveolar lavage (BAL) fluids recovered from dead BWB calves with RDS. Alterations in the ratio of total proteins to phospholipids, the phospholipid profile, SP-A levels and extremely low or undetectable SP-C levels were found in these samples.

EXPERIMENTAL

Animals

Fourteen BWB calves with RDS [eight females aged (mean ± S.E.M.) 6.8 ± 3.6 days weighing 48.1 ± 3.8 kg and six males aged 2.6 ± 0.8 days weighing 51.5 ± 2.5 kg], six healthy BWB calves (five males and one female) aged 198 ± 8 days weighing approx. 230 kg, one 9-day-old 34 kg female BWB calf with neurological symptoms but without respiratory problems, and one premature female BWB calf born approx. 1 month before term by Caesarean section were involved in the study. It was not possible to obtain BAL fluid samples from younger healthy BWB calves because these animals are not slaughtered before 5–6 months of age and a BAL performed on live newborn healthy calves could not be considered because of the potential side effects on these high-value animals.

Materials

Phospholipids were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Amino acid standards were from Pharmacia Biotech, phenylisothiocyanate (Sequenal grade) from Rathburn, triethylamine (Sequenal grade) from Pierce, and acetonitrile from Baker or Rathburn. Acrylamide/bisacrylamide 30 % (w/v) solution was from Bio-Rad and nitrocellulose (pore size 0.45 μm) from Schleicher & Schuell. All other solvents and chemicals were of analytical grade.

Histopathology

Lung pieces, taken from different lobes after the death of BWB calves with RDS, were fixed in 10 % (v/v) buffered-neutral formalin and embedded in paraffin. Sections (6 μm thick) stained with haematoxylin and eosin were examined by light microscopy with particular reference to inflammatory changes, hyaline membranes and other evidence of lung injury.

Isolation of pulmonary surfactant

Pulmonary surfactant was isolated as described by Hawgood et al. [17] from BAL fluid recovered from BWB calves with RDS within 1–20 h of death, from healthy BWB calves within 2 h after slaughtering, from the 9-day-old live BWB calf, and from the premature BWB calf within 1 h of death.

Post-mortem BAL was performed on isolated lungs by instilling a total of 2 litres of buffer A [5 mM Tris/HCl/100 mM NaCl (pH 7.4)] via the trachea and recovering the lavage fluid by gravity flow after gentle massage of the lungs. In the live calf, BAL was performed after sedation by wedging a fibre-optic endoscope in a segment bronchus and by instilling five aliquots of 50 ml sterile saline, each of which was withdrawn after infusion. The lavages were immediately centrifuged at 150 $\boldsymbol{g}_{av}$ for 15 min (Sorvall RC26Plus, rotor SLA-1500) to remove cells and debris. After centrifugation of the supernatant at 20000 $\boldsymbol{g}_{av}$ for 2 h at 4 °C, the resulting pellet was equilibrated for 1 h in buffer A containing 1.64 M NaBr and centrifuged at 100000 $\boldsymbol{g}_{av}$ for 4 h at 4 °C (Beckman L7-65, rotor SW28). The pellicle recovered at the top of the centrifuge tube was resuspended in buffer A and centrifuged at 100000 $\boldsymbol{g}_{av}$ (Beckman L7-65, rotor SW60) for 1 h at 4 °C. The resulting pellet was resuspended in distilled water and is referred to as crude surfactant. The surfactant samples were stored at −20 °C.

To evaluate the possible changes occurring in surfactant composition during the post-mortem period, lungs from a healthy slaughtered calf were kept at room temperature and the lavage was performed after 24 h.

Total protein and phospholipid analysis

Total protein (TP) content was determined by the method of Lowry et al. [18] in the presence of 0.5 % SDS with BSA as a standard and related to the total phospholipids (PL) quantified from the lipid phosphorus content [19], assuming a mean surfactant phospholipid molecular mass of 775 Da. The individual phospholipids in organic extracts of crude surfactant were separated by two-dimensional TLC [20]. The lipids were detected under iodine vapour; each spot was then scraped off and assayed by lipid phosphorus determination [19]. The individual phospholipid content is expressed as a percentage of the total phospholipids present on the TLC plate.

The disaturated PtdCho level was determined in organic solvent extracts of surfactant with the OsO_4 method with neutral alumina chromatography and related to the total phospholipid content [21]. Different phospholipid mixtures containing DPPC, dioleyl-L-α-phosphatidylcholine (DOPC), egg L-α-phosphatidyl-D,L-glycerol (PtdGro), sphingomyelin, L-α-phosphatidyl-L-serine, L-α-phosphatidylethanolamine and L-α-lysophosphatidylcholine (L-PtdCho) were used to evaluate the method. The DPPC level in a mixture with the seven phospholipids in equal amount (200 μg of each) was underestimated by approx. 4 %. DPPC levels in mixtures of DPPC/DOPC or DPPC/DOPC/PtdGro (200 μg of each) were overestimated by approx. 15 %.

Determination of SP-A content

The SP-A level was assessed by a sandwich ELISA technique [22] and related to the total phospholipid amount. In brief, microtitre plates were incubated overnight at 4 °C with an IgG fraction of a polyclonal antiserum raised in rabbit against pig SP-A. The wells were then allowed to incubate with 1 % (w/v) BSA for 1 h, washed three times, then exposed for 1 h to purified bovine SP-A [17] or various dilutions of the samples in the presence of 1 % (w/v) BSA. After washing, incubation with biotinylated anti-(porcine SP-A) polyclonal rabbit antibodies for 1 h was followed by a washing step, the addition of streptavidin-poly-peroxidase and incubation for 1 h. Finally, after extensive washing, phosphate/citrate buffer, tetramethylbenzidine and H_2O_2 were added for colour formation. The reaction was stopped by the addition of H_2SO_4, and A_{450} was read. Control measurements

with purified bovine SP-A indicated that the assay was capable of accurately measuring SP-A at concentrations of 10–50 ng/ml.

Determination of the SP-B and SP-C contents

Samples of crude pulmonary surfactant were extracted with chloroform/methanol (1:1, v/v) [23]. Phospholipid concentrations in the organic extracts were determined from their lipid phosphorus contents [19].

The SP-B and SP-C contents can be determined by amino acid analysis provided that no other proteins are present in the preparations [24]. The presence of other proteins in organic solvent extracts of crude surfactant was checked by SDS/PAGE [25]. For this purpose the samples were dried under nitrogen, resuspended in a sample buffer and run on a 16% (w/v) polyacrylamide gel (500 μg of phospholipid in each lane). Staining with silver [26] did not reveal any contaminating proteins (results not shown). From this result only the presence of low-molecular-mass hydrophobic proteins cannot be completely ruled out, because the detection of such proteins on the gel might have been impaired by the large quantity of lipids. To exclude the possibility of contaminating hydrophobic peptides in the RDS samples, the ratio between hydrophobic (Thr, Tyr and Phe) and hydrophilic (Glu and His) residues present only in SP-B was determined for the RDS and control samples. The ratios for the RDS and control samples were 1.9 and 2.1 respectively. From all results combined, the presence of proteins other than SP-B and SP-C in the organic extracts is highly improbable.

Acid hydrolysis of organic solvent extracts of the crude surfactant was performed in 6 M HCl/0.5% phenol in evacuated glass tubes at 110 °C for 20–24 h. The hydrolysed samples were treated with phenylisothiocyanate. The phenylthiocarbamyl derivatives obtained were separated by reverse-phase HPLC (C_{18} RP Spherisorb S3ODS2 column, 4.6 mm × 100 mm) with an acetonitrile gradient, and quantified by measurement of A_{254}. Amino acid residues present only in SP-B (Thr, Glu, Gln, Tyr, Phe and His) were first used to quantify the amount of this protein. Residues present in SP-B and SP-C (Asn, Pro, Gly, Ala, Met and Arg) were chosen to quantify the total amount of both proteins. The SP-C content was obtained after subtraction of the contribution of SP-B from the total amount [24]. Molecular masses of 8.7 and 4.2 kDa were used for the SP-B and SP-C polypeptide chains respectively.

Western blotting of SP-C

Crude surfactant samples (50 μg of phospholipid each) were resolved by SDS/PAGE in a 16.5% (w/v) total polyacrylamide/bisacrylamide/6% (w/v) bisacrylamide gel with a Tris/tricine buffer system [27]. The gel was transferred electrophoretically to a nitrocellulose membrane in 100 mM Tris/16 mM glycine/20% (v/v) methanol at 40 mA for 10 h. After the membrane had been blocked with skimmed milk, immunodetection was performed by incubation for 16 h at 4 °C with anti-(human recombinant SP-C) rabbit serum (provided by Byk Gulden, Konstanz, Germany) at a titre of 1:1000 followed by incubation with goat anti-(rabbit-IgG) antibodies conjugated with horseradish peroxidase (Boehringer Mannheim, Germany). Bands were detected by enhanced chemiluminescence with an ECL® kit (Amersham Life Science, Little Chalfont, Bucks., U.K.).

Surface-tension measurements

The surface-tension properties of the samples were assessed by means of a pulsating-bubble surfactometer (Model ASC; Surfactometer International, Toronto, ONT, Canada) [28]. In brief, a bubble of ambient air drawn through a small tube was formed in a chamber containing the surfactant suspension. After 2 min of equilibration at maximum radius (r_{max}), the bubble was pulsated at 37 °C at a rate of 40 cycles/min between a maximum (0.55 mm) and a minimum (0.4 mm) radius, resulting in a 50% change in surface area. The bubble diameter was adjusted under visual control through a microscope. A pressure transducer connected to the chamber measured the pressure gradient (ΔP) across the bubble wall. The surface tension (γ, in mN/m) can be calculated by the law of Laplace ($\Delta P = 2\gamma/r$). The pulsator was stopped after 5 min at r_{max} without volume adjustment of the bubble and the evolution of ΔP was followed for 1–2 min to evaluate the rate of respreading of surface-active material. All crude surfactant samples were assayed at total phospholipid concentrations of 2 and 10 mg/ml. In a second series of measurements, organic solvent extracts of the surfactant samples were evaporated to dryness under nitrogen, resuspended in normal saline by vortex-mixing with glass beads, and analysed at 2 and 10 mg/ml.

To analyse whether the high surface tension of organic extracts of RDS samples compared with healthy controls was caused by the lack of SP-C, purified pig SP-C was added to organic extracts of RDS surfactant samples ($n = 4$). For these experiments pig SP-C and the organic extracts were recombined in chloroform/methanol (1:1, v/v), the mixture was dried under nitrogen and resuspended as above. Samples containing 0.4%, 1% and 2% (w/w) pig SP-C were analysed at total phospholipid concentrations of 2, 5 and 10 mg/ml.

Analysis of SP-C amyloid fibrils in RDS surfactant samples

SP-C can form amyloid fibrils on incubation in solution; such fibrils have been detected in a patient with pulmonary alveolar proteinosis [29]. Amyloid fibrils were looked for in the crude RDS surfactant samples by electron microscopy and SDS/PAGE of 100000 ***g*** insoluble material after repeated extractions with 1% (w/v) SDS [29].

Statistics

Values are expressed as means ± S.E.M. and medians. Comparisons between groups were performed by analysis of variance followed by Student's two-tailed t test for unpaired data.

RESULTS

Histopathology

Lung sections from RDS BWB calves showed various combinations of alveolar collapse, congestion, haemorrhagic intra-alveolar oedema, hyaline membranes and recruitment of granulocytes to the airspaces (Figure 1). The lesions were in many aspects similar to the post-mortem pathological features observed in ARDS in adult human patients [30].

Total protein and phospholipid analysis

The mean protein content of crude surfactants isolated from BAL fluids was expressed as a weight percentage of total phospholipids (TP/PL). TP/PL was significantly increased in the RDS BWB calves samples in comparison with healthy animals ($P < 0.05$) (Table 1).

The phospholipid profile assessed by TLC of organic solvent extracts of crude surfactant showed various differences between the two groups of calves (Table 2). The PtdGro content was significantly lower in the RDS animals than in the healthy group, whereas the levels of minor phospholipids (PtdIns, L-PtdCho,

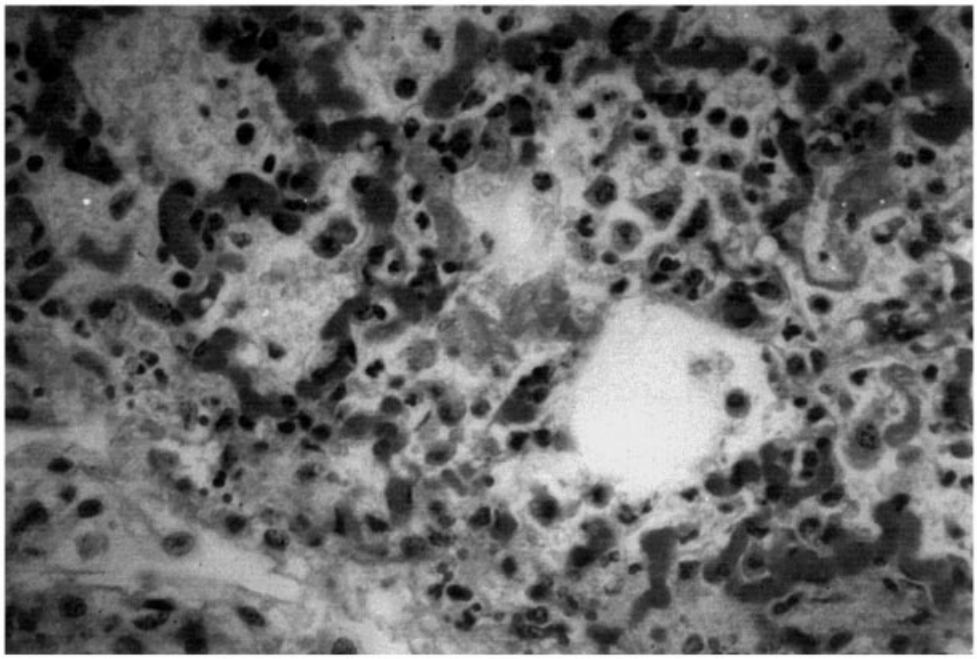

Figure 1 Lung section of a 5-day-old BWB RDS calf

The lesions observed are intense congestion, alveolar collapse, intra-alveolar oedema and acute inflammatory reaction. Haematoxylin and eosin stain. Magnification $\times 200$.

Table 1 TP/PL (percentages by weight) and ratio of SP-A to PL (ng of SP-A/nmol of PL) in pulmonary surfactant isolated from healthy and RDS BWB calves

$^{*}P < 0.05$; $^{**}P < 0.01$.

	TP/PL (% by weight)		SP-A/PL (ng/nmol)	
Source	Mean ± S.E.M.	Median	Mean ± S.E.M.	Median
Healthy BWB ($n = 7$)	12.8 ± 1.2	12.0	55.9 ± 21.3	45.9
RDS BWB ($n = 14$)	18.6 ± 6.9*	18.0	30.6 ± 16.7**	26.0

sphingomyelin and phosphatidylethanolamine/cardiolipin) were statistically higher in the surfactant of RDS animals. Interestingly, the contents of the major surfactant phospholipid species, PtdCho, and in particular disaturated PtdCho, which is considered to be the principal surface-tension-reducing agent [1,2], were similar in the two groups.

SP-A content

Sandwich ELISA assay was used for the quantification of SP-A. The ratio of SP-A to PL was significantly lower in crude surfactant samples from RDS than in healthy BWB calves (Table 1).

SP-B and SP-C contents

Organic solvent extracts of pulmonary surfactant were analysed by the phenylthiocarbamyl amino acid analysis [24]. Individual SP-B and SP-C contents calculated relative to the total phospholipid amount in pulmonary surfactant of RDS and healthy BWB calves are presented in Figure 2(A). The major difference between the two groups of animals is the very low or undetectable content of SP-C in the RDS BWB calves. In a large proportion of these samples (8 out of 14) not even a trace of SP-C could be detected. The difference in average SP-C contents between RDS and healthy calves [0.03 ± 0.01 compared with $0.39 \pm 0.06\,\%$ (w/w) respectively ($P < 0.001$)] is outstanding, whereas no significant difference in the SP-B levels was detected [0.52 ± 0.07 compared with $0.63 \pm 0.08\,\%$ (w/w) respectively] (Figure 2B).

Western blotting of SP-C

Rabbit polyclonal anti-(human recombinant SP-C) serum was used for SP-C immunodetection in crude surfactant samples of ten RDS calves (Figure 3; samples numbered as in Figure 2A). The intensities of the bands revealed by chemiluminescence confirm the SP-C contents given above; SP-C was detected in samples 5 and 12 but not in samples 2, 6, 10, 11, 13 and 14. Samples 3 and 7 exhibited very faint bands corresponding to SP-C, but no peptide was detected by amino acid analysis. A healthy BWB control sample (Figure 3, lane H) gave a distinct band, confirming the efficiency of the immunodetection procedure.

Surface-tension measurements

Experiments with the pulsating-bubble surfactometer revealed significant functional defects in crude surfactant from RDS BWB calves. Three different pieces of information were obtained from these measurements. First, the adsorption of surface-active

Table 2. Phospholipid analysis of the surfactant samples

Phospholipid distribution (percentage by weight relative to the total phospholipid amount recovered from the TLC plates) and ratio of disaturated PtdCho to PL (percentage by weight) in pulmonary surfactant isolated from BWB calves. Abbreviations: PtdSer, phosphatidylserine; SM, sphingomyelin; PtdEtn/CL, phosphatidylethanolamine/cardiolipin; disat. PtdCho, disaturated phosphatidylcholine; 24 h, sample taken 24 h after death of healthy control; n.d., not determined. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

Source	*n*	Proportion compared with PL (% by weight)							Disat. PtdCho/PL (% by weight)
		PtdSer	PtdIns	L-PtdCho	SM	PtdCho	PtdGro	PtdEtn/CL	
Healthy BWB	7	1.9 ± 0.4	2.6 ± 0.2	0.9 ± 0.2	1.0 ± 0.2	79.5 ± 0.7	9.7 ± 1.5	4.5 ± 0.3	50.5 ± 3.3
RDS BWB†	12	1.8 ± 0.3	4.4 ± 0.3**	1.9 ± 0.3**	2.0 ± 0.3*	80.2 ± 1.4	4.5 ± 0.7***	5.8 ± 0.4*	53.0 ± 6.0
24 h BWB	1	2.2	2.4	1.5	1.7	77.3	9	5.9	53.3
Premature BWB	1	1.2	5.9	1.3	1.1	81.9	4.4	4.2	n.d.

† RDS BWB compared with healthy BWB.

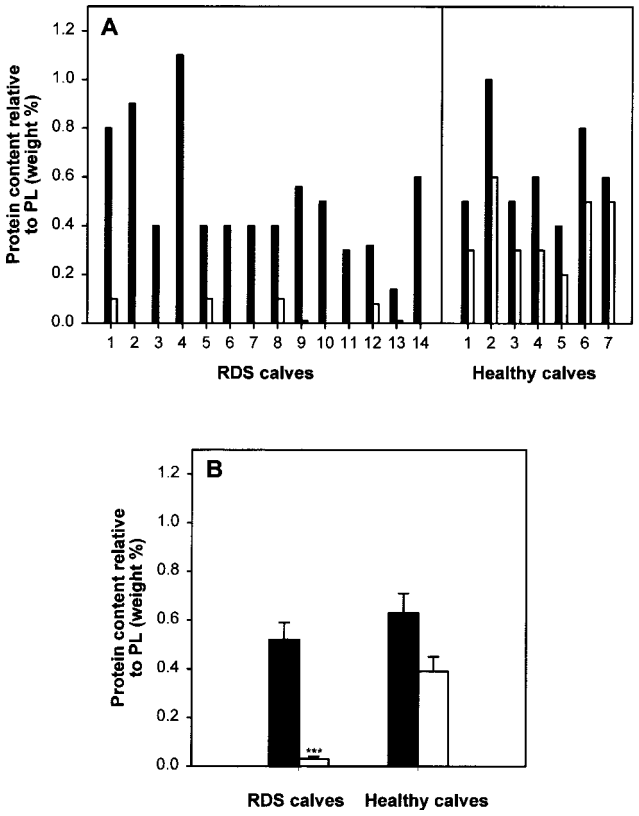

Figure 2 Quantification of SP-B and SP-C

Individual (**A**) and average (means ± S.E.M.) (**B**) contents of hydrophobic proteins SP-B (filled columns) and SP-C (open columns) in organic extracts of pulmonary surfactant isolated from RDS and healthy BWB calves, including the 9-day-old animal (sample 1 of healthy calves). $^{***}P < 0.001$). Values are expressed as percentages by weight relative to PL.

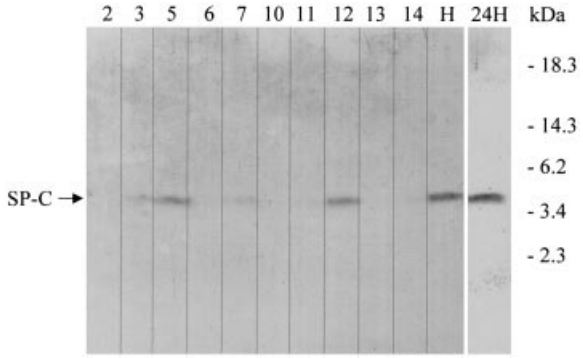

Figure 3 Western blot analysis of crude surfactant samples for native SP-C

Electrophoresed samples (50 μg of phospholipids applied in each lane) from RDS BWB calves [nos 2, 3, 5, 6, 7, 10, 11, 12, 13 and 14; samples numbered as in Figure 2(A)], a healthy BWB calf (lane H) and from a healthy slaughtered animal taken 24 h after death (lane 24H) were probed with anti-(human recombinant SP-C) and revealed by enhanced chemiluminescence.

material to the air/liquid interface of the bubble was significantly impaired for surfactant isolated from RDS calves compared with healthy animal samples, as reflected by the higher static surface tension measured initially at equilibrium (γ_{eq}) (Figure 4, values at

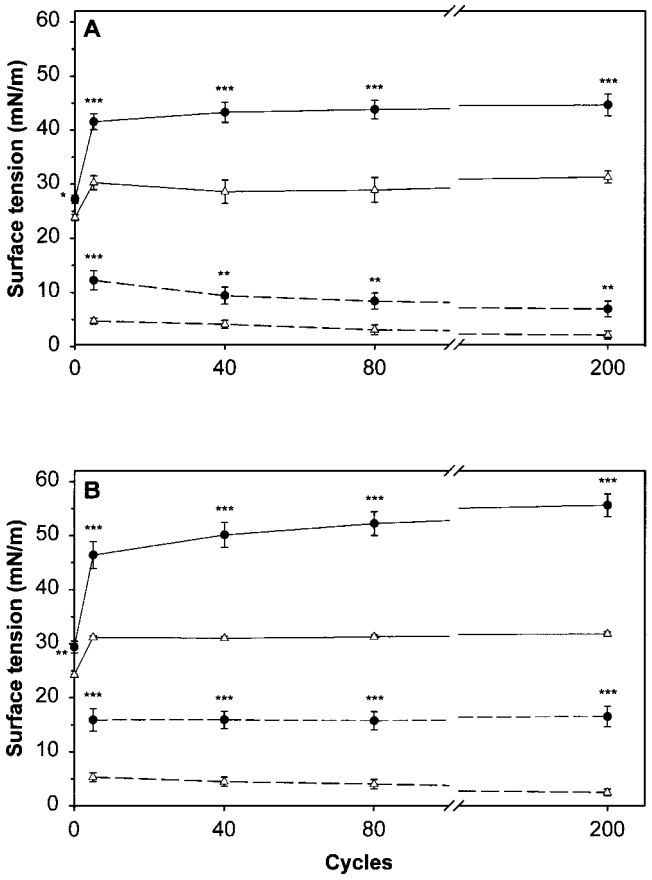

Figure 4 Dynamic surface properties of the crude surfactant samples

Equilibrium and dynamic surface tension during the bubble pulsation cycles in healthy (△) and RDS (●) BWB surfactant samples. Values (means ± S.E.M.) recorded before pulsation (initial data points) and subsequently at minimum (broken lines) and maximum (solid lines) bubble radii are shown for samples at phospholipid concentrations of 10 mg/ml (**A**) and 2 mg/ml (**B**). $^{**}P < 0.01$; $^{***}P < 0.001$ compared with healthy samples.

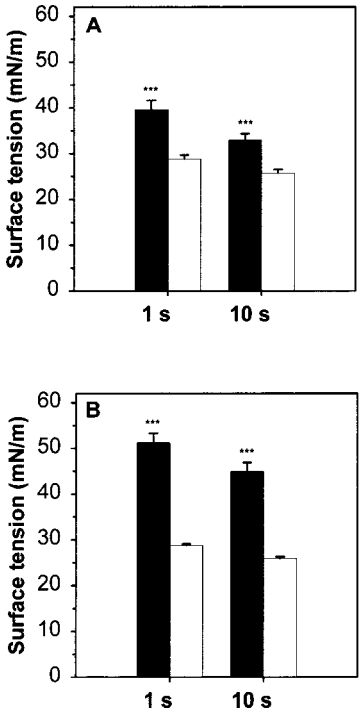

Figure 5 Static surface properties of the crude surfactant samples

Static surface tension (means ± S.E.M.) 1 and 10 s after stopping the bubble pulsation cycles in RDS (filled columns) and healthy (open columns) BWB surfactant samples. Measurements were made at phospholipid concentrations of 10 mg/ml (**A**) and 2 mg/ml (**B**). $^{***}P < 0.001$ compared with healthy samples.

the origin). Secondly, both surface tension at maximum bubble size and at minimum bubble size (γ_{min}) were increased in RDS calves throughout the bubble pulsation cycles, and the differences from healthy calves were even more pronounced at the lower phospholipid concentration (2 mg/ml; Figure 4). Thirdly, surface tensions, measured at r_{max} 1 and 10 s after the bubble pulsation cycles were stopped, were also significantly higher in the RDS group at both surfactant concentrations (Figure 5), indicating slower replenishment of surface-active material at the air/liquid interface.

To discriminate between the impact of the high TP/PL and the low concentration of SP-C on the surface activity of RDS calf surfactant, we compared the surface properties of organic solvent extracts (which contained only the lipophilic proteins SP-B and SP-C and in which the only protein abnormality was the low SP-C content; see Figures 2 and 3) with those of the corresponding crude surfactants (which contained, in addition to SP-B and SP-C, SP-A and other water-soluble proteins). Under the static conditions as described above, i.e. before and after the cycles of bubble pulsation, mean surface tensions measured at r_{max} were slightly higher in crude surfactant samples than in organic extracts, reaching statistical significance at 10 mg/ml (Figures 6A and 6C). These results could reflect the inhibition of surface activity by proteins present in the crude surfactants. In contrast, the high γ_{min} values recorded at the end of the bubble pulsation cycles were in the same range in crude and organic extracts of surfactant samples at both concentrations (Figure 6B), suggesting that the lack of SP-C has a major role in the altered dynamic surface properties of RDS calf surfactant. Furthermore, the addition of 2% (w/w) pig SP-C to an SP-C-deficient sample analysed at 10 mg/ml total phospholipid concentration decreased γ_{min} at 5 min from 18 to 11 mN/m. The addition of 1% (w/v) pig SP-C to a sample analysed at a total phospholipid concentration of 5 mg/ml gave a decrease in γ_{min} from 47 to 24 mN/m at cycle 5, and from 43 to 22 mN/m after 5 min of pulsation. The addition of 0.4% SP-C gave no detectable improvement in samples analysed at a total phospholipid concentration of 2 mg/ml.

Organic extracts from healthy BWB surfactant samples behaved like the corresponding crude surfactants, reaching very low surface tensions at the end of the bubble pulsation cycles (results not shown).

Controls

Because the BAL procedure on RDS animals was performed between a few minutes and 20 h after death, we evaluated the possible changes occurring in surfactant composition during this post-mortem time period. Lungs from a healthy slaughtered calf were lavaged after 24 h. The TP/PL determined was similar to that obtained for the healthy calves [11.4% (w/w)], indicating no

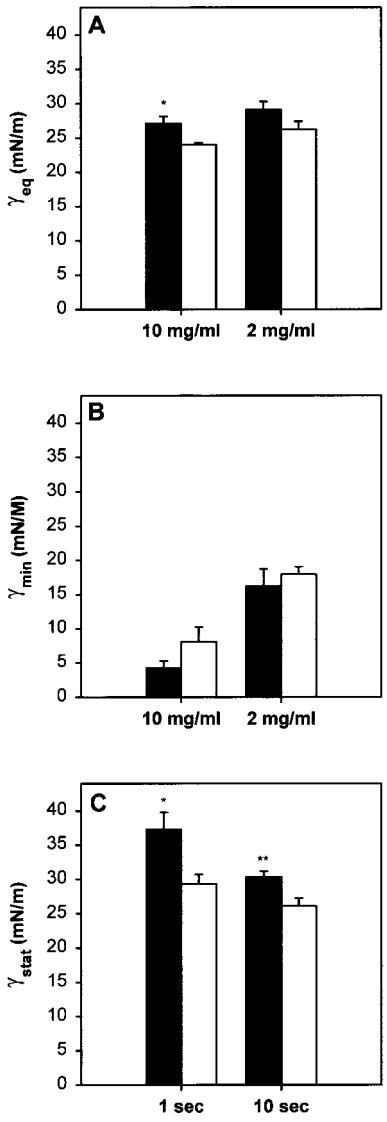

Figure 6 Comparison of surface properties between crude and organic extracts of surfactant samples

Surface tension (means ± S.E.M.) of crude surfactant (filled columns; $n = 9$) and organic extracts of surfactant (open columns; $n = 9$) from RDS calves was evaluated by measurements with a pulsating-bubble surfactometer. (**A**) Equilibrium surface tension (γ_{eq}) 2 min after bubble formation at 10 and 2 mg/ml phospholipid. (**B**) Minimum surface tension (γ_{min}) after 5 min of bubble pulsation at 10 and 2 mg/ml phospholipid. (**C**) Static surface tension (γ_{stat}) 1 and 10 s after stopping the bubble pulsation cycles at 10 mg/ml phospholipid. $^{**}P < 0.01$; $^{*}P < 0.05$ compared with organic extracts.

significant protein leakage into the alveoli 24 h after death. The contents of the negatively charged phospholipids (PtdGro and PtdIns) for the 24 h control and healthy BWB samples were similar (Table 2). The SP-A content measured for the control sample was 90.3 ng of SP-A/nmol of PL, which does not indicate the degradation of surfactant proteins in the alveoli of the RDS calves up to 24 h after death. Finally, the percentage of SP-C was 0.6 % (w/w) relative to total phospholipids, clearly different from the low values found in RDS. A distinct band obtained by Western blotting confirmed the presence of SP-C (Figure 3).

A 9-day-old calf included in the healthy group had a hydrophobic surfactant protein composition [0.5 % and 0.3 % (w/w) SP-B and SP-C respectively; sample 1 in Figure 2(A)] comparable to the values reported for older healthy calves.

A surfactant sample originating from the BAL fluid of a premature BWB calf born by Caesarean section 1 month before term was also used as an additional control. As expected, the phospholipid profile of this premature calf (Table 2) was mainly characterized by an increase in the ratio of PtdIns to PtdGro compared with healthy mature calves, and the hydrophobic protein composition [0.5 % and 0.2 % (w/w) SP-B and SP-C respectively] was different from that in the RDS calves.

DISCUSSION

The present study demonstrates striking abnormalities in the pulmonary surfactant of BWB calves deceased from RDS. BWB calves are high-value double-muscled animals genetically selected for their production performance and beef quality. However, these calves are more susceptible to acute respiratory diseases than are calves from other breeds. The RDS calves studied here were all born at term by Caesarean section performed at the beginning of calving, a common practice for the double-muscled BWB cattle breed.

Our major findings are the extremely low or even undetectable SP-C levels in surfactant samples from RDS BWB calves, determined by differential amino acid analysis and Western blotting, associated with abnormal surface activity. Although undetectable immunostaining for pro-SP-C in a group of full-term or near-term infants with respiratory failure has been reported recently [31], to the best of our knowledge this is the first time that altered mature SP-C levels combined with normal SP-B levels have been reported in association with fatal RDS. Other diseases with modifications of surfactant protein concentrations include the human hereditary SP-B deficiencies, which are characterized by the absence of SP-B, small amounts of SP-C and the accumulation of a processing product of pro-SP-C [32]. ARDS, in contrast, is associated with a down-regulation of SP-A and SP-B due to generalized lung cell injury [33], whereas *Pneumocystis carinii* pneumonia leads to lower SP-B levels with unchanged SP-A levels [34].

Among the other modifications encountered in the surfactant of RDS BWB calves, the increased TP/PL probably reflects transudation of plasma components into the alveolar lumen owing to increased alveolo-capillary permeability, accumulation of inflammatory proteins, or increased hydrostatic pulmonary vascular pressure. We have demonstrated that this higher TP/PL was not related to an accumulation of SP-A in the alveoli, as reported for the alveolar proteinosis disease [35], because a significantly decreased ratio of SP-A to PL was measured in samples isolated from RDS calves. A low SP-A level is characteristic of acute lung injury [35], which agrees with the prominent changes found in the histopathological sections of RDS calf lungs, and could be due either to decreased synthesis or secretion by type II cells, degradation within the alveoli, or leakage into the circulation.

The relevance of our results is supported by the comparison of the data obtained for the control healthy BWB calves to previously published values. The mean TP/PL in crude surfactant from healthy BWB calves is similar to that reported for freshly slaughtered cows [36], and the mean ratio of SP-A to PL is close to that reported for rat surfactant with a similar ELISA procedure [37]. The levels of PtdCho and disaturated PtdCho determined here for RDS and healthy BWB calves are close to the 79.2 ± 1.6 % and 52.9 ± 2.5 % (w/w) reported for healthy bovine samples [36]. The total SP-B and SP-C contents in surfactant

samples reported in the literature range from 1 % to 2 % (w/w, relative to total phospholipids) [38,39], with a value of 1.2 % for calf lung surfactant extract [40]. The values determined here for healthy BWB calves are in this range and are comparable to the values determined for 37 pig samples with the same procedure as used in this work (SP-B, 0.7 ± 0.1 %; SP-C, 0.6 ± 0.1 %) [24].

The RDS calves were born at full term as calculated from the dates of artificial insemination and confirmed by their high birth weight. Although mature SP-C has never been quantified in the surfactant of newborn mammals, the presence of mRNA encoding pro-SP-C and the 21 kDa SP-C precursor protein have been demonstrated early in the gestation period of different animals [41]. Here SP-C was present in a premature calf born 1 month before term in a higher amount than the levels recorded for the RDS calves. This provides a strong argument against the possible influence of lung immaturity on the low SP-C content of RDS animals. For practical reasons we compared the surfactant of RDS calves with that of older healthy animals. This is validated by the similar values found in this healthy group and a 9-day-old healthy calf close in age to the RDS animals. Taken together, these control results rule out a significant influence of age on the very low SP-C content found in the RDS animals. Furthermore, the post-mortem delay preceding the BAL procedure in the RDS calves did not explain the lower levels of SP-A and SP-C. Indeed, the surfactant isolated from a healthy calf lung left at room temperature for 24 h had normal TP/PL and high levels of SP-C and SP-A.

The surface activity of surfactant isolated from RDS calves was significantly impaired in comparison with that from healthy BWB animals. Protein accumulation in the alveoli produces a dose-dependent functional inhibition of surfactant *in vitro* [42,43]. To distinguish between the effect of the large amount of total proteins and the low SP-C content in the RDS group, we used a pulsating-bubble surfactometer to compare crude surfactant samples and their organic extracts (containing only lipids and hydrophobic proteins). The abnormally high γ_{min} values for crude surfactant and organic extracts of samples from RDS BWB calves indicate comparable alterations of surface activity in the presence or absence of contaminating water-soluble proteins. Notably, the addition of SP-C improves, but does not normalize, the γ_{min} of organic extracts of RDS samples, suggesting that additional factors, such as changes in phospholipid composition or the presence of unknown non-protein inhibitors, contribute to the lower surface activity. Furthermore, the higher surface tensions measured at stable r_{max} in the crude than in the extracted RDS surfactant samples suggest that the higher TP/PL could retard both the adsorption and respreading of tensioactive compounds under static conditions. Finally, the lower ratio of SP-A to PL in RDS BWB calves could contribute to surfactant dysfunction, as SP-A is probably important in the resistance of surfactant to inhibition by plasma proteins [8,44].

The presence in the alveoli of plasma-derived surfactant inhibitors might thus have contributed to RDS in BWB calves. In ARDS, abnormalities in surfactant composition have been reported but are believed to be the consequence of non-specific lung injury rather than primary pathogenic factors. The reported surfactant modifications in ARDS include a higher protein content, an altered phospholipid profile (lower contents of PtdCho, disaturated PtdCho and PtdGro; higher contents of PtdIns, phosphatidylethanolamine, L-PtdCho and sphingomyelin), lower SP-A and SP-B concentrations and important alterations in the surface activity 33,45–47]. Interestingly, in contrast to the results reported for human ARDS, we observed no modification of the SP-B levels or of the relative PtdCho and disaturated PtdCho contents in the RDS compared with healthy BWB calves. Thus the more tensioactive phospholipid fraction is present in an apparently normal amount in RDS calves.

The reason for the absence, or very small amounts, of SP-C in RDS BWB calves in association with a normal SP-B level is unknown. Aggregation of SP-C in the alveoli, analogous to amyloid fibril formation observed in human alveolar proteinosis [29] can be ruled out, because electron microscopy and SDS/PAGE of RDS surfactant failed to detect any amyloid fibrils (results not shown). At least two possible explanations can currently be considered: the lack of mature SP-C in pulmonary surfactant could be due to a mutation or deletion in the gene encoding pro-SP-C, or to the inhibition of one or several steps in the intracellular enzymic processing of pro-SP-C to the mature SP-C peptide [9]. Experiments aiming at distinguishing between these alternatives are currently in progress.

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