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Comparative Biochemistry and Physiology, Part A 141 (2005) 191 – 199

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The development of the pulmonary surfactant system in California sea lions

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Received 18 February 2005; received in revised form 9 May 2005; accepted 9 May 2005
Available online 16 June 2005

Abstract

Pulmonary surfactant has previously been shown to change during development, both in composition and function. Adult pinnipeds, unlike adult terrestrial mammals, have an altered lung physiology to cope with the high pressures associated with deep diving. Here, we investigated how surfactant composition and function develop in California sea lions (*Zalophus californianus*). Phosphatidylinositol was the major anionic phospholipid in the newborn, whereas phosphatidylglycerol was increased in the adult. This increase in phosphatidylglycerol occurred at the expense of phosphatidylinositol and phosphatidylserine. There was a shift from long chain and polyunsaturated phospholipid molecular species in the newborn to shorter chain and mono- and disaturated molecular species in the adult. Cholesterol and SP-B concentrations were also higher in the adult. Adult surfactant could reach a lower equilibrium surface tension, but newborn surfactant could reach a lower minimum surface tension. The composition and function of surfactant from newborn California sea lions suggest that this age group is similar to terrestrial newborn mammals, whereas the adult has a "diving mammal" surfactant that can aid the lung during deep dives. The onset of diving is probably a trigger for surfactant development in these animals.

Keywords: Cholesterol; Phosphatidylcholine; Phosphatidylglycerol; Phosphatidylinositol; Phosphatidylserine; Pinniped; SP-B; Surface activity

1. Introduction

The pulmonary surfactant system varies greatly between species and appears to be influenced by evolutionary selection pressures such as body temperature, lung structure and function, as well as metabolic strategies (Bernhard et al., 2001a; Orgeig and Daniels, 2001; Postle et al., 2001; Daniels et al., 2004). The surfactant system has also been shown to vary within a species under certain conditions, for example, when the animal is undergoing a physiological stress such as torpor (Langman et al., 1996; Codd et al., 2002; 2003). One of the most commonly studied changes in the surfactant system is during development. The composition (Benson et al., 1983; Burdge et al., 1993; Johnston et al., 2000; Bernhard et al., 2001b; Rau et al., 2004), function (Gluck et al., 1970; Stevens et al., 1987; Farrell et al., 1990) and control (Miller et al., 2001; Sullivan et al., 2001; 2002) of pulmonary surfactant have all been shown to change during development in many different types of vertebrates.

Adult pinnipeds spend a large amount of their lives under water, and hence, lung and surfactant function reflect the different requirements for oxygen delivery. However, unlike cetaceans, which are born in the water and spend their whole lives there, pinnipeds are born on land, and spend the majority of their development out of the water. A typical example of the developmental pattern of a pinniped is illustrated by the California sea lion. California sea lion pups do not leave the rookery until after

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1095-6433/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpb.2005.05.035

6 months of age (Melin et al., 2000). Before this time, they are nursed by their mothers, and there is no evidence that they follow the mothers on their foraging trips (Melin et al., 2000). Hence, pups less than 6 months old do not dive. Given this terrestrial nature in the first part of life, followed by an aquatic lifestyle after 6 months of age, we hypothesised that surfactant development (in terms of composition and function) will reflect this shift in life style. Hence, we hypothesised that California sea lion pups will have a similar surfactant system to terrestrial mammals until they first start to forage for themselves. Once they enter the water, we hypothesised that they will develop an adult-type surfactant, which will facilitate their aquatic behaviour. This manuscript describes the differences in composition and function between a California sea lion newborn and adult.

2. Materials and methods

2.1. Animals

Lungs from California sea lions (*Zalophus californianus*) were obtained from wild-caught animals held at the Marine Mammal Centre, Sausalito, California. One male newborn (1 day old), mass 4.5 kg, and one female adult, mass 104 kg, were obtained. Both adult and pup died from non-respiratory-related illness (demoic acid poisoning). Lungs were removed and material was sampled immediately after the death of the animal.

2.2. Lavage protocol and surfactant extraction and analysis

The lungs were removed from the chest cavity, and one lobe was cannulated with polypropylene tubing and rinsed with ice-cold 0.9% NaCl. The lavage fluid was then centrifuged at 150 *g* for 5 min at 4 °C to remove any free cells and other cellular debris. The surfactant fraction was isolated from the supernatant using sodium bromide density gradient ultracentrifugation modified from Shelley et al. (1977). The surfactant fraction was reconstituted in a solution containing 154 mM NaCl and 1.5 mM $CaCl_2$, aliquoted into smaller volumes and either frozen or lyophilised for shipping.

For lipid analysis, total lipids were extracted in chloroform:methanol (1:2) (Bligh and Dyer, 1959). Cholesterol was measured with a high performance liquid chromatography system as previously described (Daniels et al., 2004) and phospholipid molecular species were analysed as described below.

2.3. Electrospray ionisation mass spectroscopy

Phospholipid molecular species were measured by electrospray ionisation mass spectroscopy (ESI-MS) on a triple–quadrupole tandem mass spectrometer (Quattra *Ultima*, Micromass, Manchester, England). Total phospholipid extracts were dissolved in 25 μl of methanol:chloroform:water:NH_4OH (7:2:0.8:0.2) for single stage MS, followed by tandem MS/MS analysis of phosphatidylcholine (PC), phosphatidylinositol (PI), and phosphatidylglycerol (PG) molecular species using nanospray ESI-MS. Dry heated nitrogen was used as both the cone and desolvation gas (70 and 450 L h^{-1} respectively), and dry argon was used as the collision gas (3.5×10^{-3} mbar). All data were recorded at mass resolution, as a signal average of 10–20 scans per collection, with a scan time of 2.5 to 12 s. Data were processed using MassLynx NT software (Waters, Milford, MA). Molecular species are denoted as *A*:*x*/*B*:*y*, where *A* and *B* are the number of carbon atoms in the fatty acids esterified at the *sn*-1 and *sn*-2 positions, respectively, and *x* and *y* are the number of double bonds in the fatty acids. The majority of molecules are in the diacyl form (fatty acids attached to the glycerol backbone via ester bonds), however, some are in the alkyl-acyl form (one fatty acid attached to the glycerol backbone via an ether bond), and are denoted by an 'a' after the double bonds (e.g., PC16:0a/18:1).

2.4. Enzyme-linked immunosorbent assay

Surfactant protein A and B (SP-A and SP-B) were quantified from the unextracted lavage using an enzyme-linked immunosorbent assay (ELISA) modified from McMahan et al. (1987). For SP-A, a 96-well ELISA plate (high binding; Greiner, Germany) was coated with 0.1 M Na_2HPO_4 containing a 1:100 dilution of goat anti-rabbit SP-A antibody and incubated overnight at 37 °C. The plate was then washed with 0.01 M Tris pH 8.0, 0.05% Tween 20, 0.01% thimerosal. Non-specific binding was blocked with 0.15 M NaCl, 0.01 M Tris pH 7.4, 5 mg mL^{-1} bovine serum albumin (BSA), 2.5% human albumin and 2.5% goat serum. A standard curve was then prepared using purified human SP-A (a generous gift from Prof. J.A. Whitsett, Children's Hospital Research Center, Cincinnati, OH, USA) in 0.05 M Na_2HPO_4/NaH_2PO_4 pH 7.4, 0.15 M NaCl, 0.5 mL Triton X100. Samples were added to the plate in duplicate, and incubated for 2 h at 37 °C.

Plates were then washed and incubated with primary antibody (1:1000 rabbit anti-human SP-A (Chemicon, USA) in blocking buffer) for 1 h at 37 °C. Plates were washed again and incubated with secondary antibody (1:1000 goat anti-rabbit IgG-HRP conjugate (Sigma-Aldrich, USA) in a buffer containing 0.05 M Na_2HPO_4/NaH_2PO_4 pH 7.4, 0.15 M NaCl, 0.05% Tween 20, 0.01% thimerosal, 2.5% human albumin and 2.5% goat serum) for 1 h at 37 °C. 60 mg of *o*-phenylenediamine dissolved in 0.065 M Na_2HPO_4/NaH_2PO_4 pH 6.3, 0.017 M citric acid with 0.01% thimerosal was added to the plate for the final colour reaction, which was stopped after approximately 10 min with 50% H_2SO_4. Absorbance was then recorded at 492 nm.

For SP-B, the ELISA plate was initially incubated with 0.1 M Na_2HPO_4 containing no antibodies. Where buffers