The anatomy, physics, and physiology of gas exchange surfaces: is there a universal function for pulmonary surfactant in animal respiratory structures?

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Synopsis (Orgeig and Daniels) This surfactant symposium reflects the integrative and multidisciplinary aims of the 1st ICRB, by encompassing *in vitro* and *in vivo* research, studies of vertebrates and invertebrates, and research across multiple disciplines. We explore the physical and structural challenges that face gas exchange surfaces in vertebrates and insects, by focusing on the role of the surfactant system. Pulmonary surfactant is a complex mixture of lipids and proteins that lines the air–liquid interface of the lungs of all air-breathing vertebrates, where it functions to vary surface tension with changing lung volume. We begin with a discussion of the extraordinary conservation of the blood–gas barrier among vertebrate respiratory organs, which has evolved to be extremely thin, thereby maximizing gas exchange, but simultaneously strong enough to withstand significant distension forces. The principal components of pulmonary surfactant are highly conserved, with a mixed phospholipid and neutral lipid interfacial film that is established, maintained and dynamically regulated by surfactant proteins (SP). A wide variation in the concentrations of individual components exists, however, and highlights lipidomic as well as proteomic adaptations to different physiological needs. As SP-B deficiency in mammals is lethal, oxidative stress to SP-B is detrimental to the biophysical function of pulmonary surfactant and SP-B is evolutionarily conserved across the vertebrates. It is likely that SP-B was essential for the evolutionary origin of pulmonary surfactant. We discuss three specific issues of the surfactant system to illustrate the diversity of function in animal respiratory structures. (1) Temperature: *In vitro* analyses of the behavior of different model surfactant films under dynamic conditions of surface tension and temperature suggest that, contrary to previous beliefs, the alveolar film may not have to be substantially enriched in the disaturated phospholipid, dipalmitoylphosphatidylcholine (DPPC), but that similar properties of rate of film formation can be achieved with more fluid films. Using an *in vivo* model of temperature change, a mammal that enters torpor, we show that film structure and function varies between surfactants isolated from torpid and active animals. (2) Spheres versus tubes: Surfactant is essential for lung stabilization in vertebrates, but its function is not restricted to the spherical alveolus. Instead, surfactant is also important in narrow tubular respiratory structures such as the terminal airways of mammals and the air capillaries of birds. (3). Insect tracheoles: We investigate the structure and function of the insect tracheal system and ask whether pulmonary surfactant also has a role in stabilizing these minute tubules. Our theoretical analysis suggests that a surfactant system may be required, in order to cope with surface tension during processes, such as molting, when the tracheae

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collapse and fill with water. Hence, despite observations by Wigglesworth in the 1930s of fluid-filled tracheoles, the challenge persists into the 21st century to determine whether this fluid is associated with a pulmonary-type surfactant system. Finally, we summarize the current status of the field and provide ideas for future research.

Introduction (Orgeig)

The aims of the First International Congress of Respiratory Biology were to foster integrative and multidisciplinary research. With these aims in mind we structured a symposium on pulmonary surfactant to encompass *in vitro* and *in vivo* research, studies into vertebrates and invertebrates, as well as research across multiple disciplines, including anatomy, biophysics, protein and lipid biochemistry, and physiology that spanned both basic and biomedical science. In particular, we have explored the physical and structural challenges that face gas-exchange surfaces in vertebrates and insects, by focusing on the role of the surfactant system.

Pulmonary surfactant is a complex mixture of lipids and proteins that lines the inner surface of the lungs of all air breathing vertebrates. In the animals studied to date, surfactant functions in varying the surface tension at the air–liquid interface during changes in lung volume, thereby reducing the work of breathing, maximizing lung compliance, and optimizing the available surface area for gas exchange. Among the lipid components of surfactants, phospholipids form the major constituent with phosphatidylcholine the major phospholipid class (Possmayer 2004). Among the protein components, there are four surfactant-associated proteins, surfactant protein (SP) A, B, C, and D. Of these, SP-B and SP-C are directly involved in the regulation of surface tension at the air–liquid interface, whereas SP-A and SP-D are largely involved in pulmonary immune functions and alveolar surfactant homeostasis (Haagsman and Diemel 2001). A lack of surfactant (which occurs in serious medical conditions including the immature lungs of preterm infants) or the inhibition of surfactant (which can occur during or after a pulmonary infection, inflammation, or the inhalation of a toxic substance), can lead to pulmonary failure. The consequences of surfactant failure include a greatly increased effort to breathe, decreased lung compliance and increased elastic recoil, pulmonary edema, and severe hypoxemia (Orgeig and Daniels 2004).

In this review, we set the scene by discussing the common structural constraints in the development and evolution of the blood–gas barrier of the vertebrates, of which pulmonary surfactant is a vital component. We then give brief overviews of the current knowledge concerning the functional biochemistry of the surfactant lipids and SP-B and -C. Thereafter, we discuss three particular issues/areas of study of the surfactant system so as to understand more completely the function of surfactant in animal respiratory structures: (1) How does surfactant behave under different conditions of temperature both *in vitro* and *in vivo* in animals that enter periods of torpor or hibernation? (2) What are the differences in structure and function between airway and alveolar surfactant, and are there any parallels that can be drawn from understanding the roles of surfactant in the tubular lungs of birds? (3) Is there a role for a pulmonary-type surfactant in the tracheae of insects? Finally, we summarize the current state of knowledge and provide ideas for future research.

Evolution of the structure and function of the blood–gas barrier in vertebrates (Maina)

The construction of the blood–gas (tissue) barrier (BGB) is a compromise design. On the one hand, the BGB must be strong in order to maintain structural integrity (under tension) from changing hemodynamic pressures on the blood capillary side, while at the same time tolerating surface tension and distension forces from the air side. On the other hand, the BGB must be thin enough to allow efficient flux of respiratory gases by passive diffusion. Presenting clear formidable bioengineering challenges, these conflicting structural requirements have been surmounted by remarkable trade-offs and compromises. From an evolutionary viewpoint, various designs would have undergone rigorous selection on the basis of relative costs and benefits, with the inferior ones being discarded and the optimal one conserved. Among the air-breathing vertebrates, generally, the thickness of the BGB is greatest in amphibians, intermediate in reptiles, and thinnest in mammalian and avian lungs (Maina and West 2005). The differences have been governed by (correlate with) many factors including phylogeny, body size, oxygen demands, lifestyle, and habitat. Comprised of a thin epithelial cell flange (covered by a surfactant film) that faces air, an extracellular matrix (EM) (=basement membrane), and an endothelial cell that fronts the blood capillary,

this ''three-ply'' (laminated) architecture of the BGB has been evolutionarily highly conserved (Maina and West 2005). For example, in the lungfishes (Dipnoi), the design has been in existence for over ~400 million years. Invariably present in the primitive lungs and their derived forms, surfactant also has been equally highly conserved (Orgeig and Daniels 1995; Sullivan et al. 1998; Daniels et al. 2004). In biology, designs that have changed little over long periods of time are termed *Bauplans* (=blue prints) or ''frozen cores''. In all likelihood, such designs are the only feasible or practical solutions for exacting functional requirements.

Recently, a systematic study of the morphogenesis of the BGB of the avian lung was carried out by Maina (2004). The development of the air capillaries (ACs) and the blood capillaries (BCs) in the gas-exchange tissue of the lung of the domestic fowl, *Gallus gallus* variant *domesticus* starts on day 15 of embryogenesis. Projecting copiously into the mesenchymal tissue of the formative bronchi, the ACs separate the BCs, thereby bringing air close to blood. The interconnection of the BCs and the thinning of the BGB occur through considerable mesenchymal cell displacement and disintegration. Commencing at day 17, the epithelial cells and endothelial cells contact and fuse. The deposition of the EM starts soon thereafter. At day 21, with a thin BGB formed, the lung is well-prepared for gas exchange at pipping and ultimately at hatching. Interestingly, in areas where the ACs lie adjacent to each other, extensions of the cytoplasm of type-I epithelial cells lie back-to-back and the EM is lacking. The EM is, however, always present in the BGB, i.e., at sites where the BCs and the ACs lie adjacent to each other. In the volume-constant (fixed) avian lung, the AC-to-AC areas are not subjected to tension, while the AC-to-BC ones are subjected to tension from intramural (capillary) blood pressure. This suggests, at least in the avian lung, that the tension-bearing component of the BGB is the EM. It is plausible that the presence of the type-IV collagen in the EM contributes to the strength of the BGB (Maina and West 2005), making it both adequately thin and sufficiently strong to equally promote gas exchange and withstand tension within its physiological ranges of operation. In addition to systematic disintegration of mesenchymal cells, confinement of the type-II cells to the atria and infundibulae and consignment of many of the perikarya of the type-I epithelial cells to the infundibulae allows for development of narrow ACs and thin BGB.

As a consequence of these and other structural and functional characteristics, the diffusing capacity (conductance) of the avian lung for oxygen is the most efficient of the vertebrates, enabling support for the highly aerobic lifestyle and great metabolic capacity characteristic of birds.

The role of pulmonary surfactant in regulating surface tension at the air–liquid interface of the lung (Veldhuizen and Possmayer)

Introduction

Both the discovery of surfactant and the finding that surfactant deficiency was a major contributor to lung dysfunction associated with prematurity, occurred in the 1950s and initiated research directed toward all aspects of the surfactant system. Our (Possmayer and Veldhuizen) research has focused on the relationship between the composition of mammalian surfactant and its biophysical function. Specifically, we have utilized reconstitution of isolated components from control and dysfunctional surfactants as a tool to understand the mechanisms by which mammalian surfactants reduce the surface tension *in vitro* and restore lung function *in vivo*.

In our studies, we have utilized oxidative damage to obtain dysfunctional surfactants. The rationale for this methodology is that oxidative stress is common in situations of acute lung injury and lung pollution. Furthermore, oxidative reactions are rapidly reproduced *in vitro* using either hypochlorous acid or the Fenton reaction. Initial studies with BLES, a clinical bovine lipid-extract surfactant containing the surfactant phospholipids and proteins SP-B and SP-C, demonstrated that oxidation impaired the ability of BLES to reduce tension and that this effect was reversed by adding exogenous, nonoxidized SP-A (Rodriguez-Capote et al. 2003). Subsequent studies addressed the following two questions: (1) does the impaired biophysical function *in vitro* correlate with a reduced ability to restore lung function *in vivo*, and (2) which components of surfactant are responsible for its impaired function?

Surfactant function *in vivo*

Although an impaired ability of oxidized surfactant to reduce surface tension *in vitro* indicates functional alterations to the surfactant, the *in vivo* situation is more complex than measurements of surface tension *in vitro*. For example, *in vitro* studies are not influenced by surfactant metabolism within the airspace. Therefore, experiments were performed in which control and oxidized surfactants were

administered to rats made surfactant deficient by lavage (rinsing the lung with saline), and lung function was monitored by blood oxygenation levels.

The results showed that surfactant-deficient rats, administered oxidized BLES at a dose similar to the endogenous pool of rats, had decreased lung function compared to those receiving nonoxidized BLES. Analysis of the lung lavage obtained 2 h after administration of the exogenous surfactant revealed no differences in the amounts of surfactant recovered (Bailey et al. 2004). Separate experiments revealed that the addition of SP-A to oxidized BLES restored the *in vivo* function of this material (Bailey et al. 2006). The overall conclusion of these experiments is that oxidation of surfactant impairs the *in vivo* function of this material and that the *in vitro* assessment correlated with the *in vivo* data. These studies provided the rationale for a more detailed assessment investigating which components of surfactant were responsible for the surfactant dysfunction due to oxidative damage.

Reconstitution studies of oxidized and nonoxidized surfactant components

A variety of chemical and compositional analyses demonstrated that SP and phospholipids were both modified by the oxidation procedure. In order to determine how either the phospholipid components or the proteins SP-B or SP-C contributed to the function/dysfunction of the surfactant, these components were isolated from both oxidized and nonoxidized BLES. Subsequently, the phospholipids and proteins were reconstituted and analyzed for ability to reduce surface tension, using a captive bubble surfactometer (Rodriguez-Capote et al. 2006).

The biophysical activity of reconstituted nonoxidized proteins and phospholipid mirrored the activity of BLES, whereas the reconstitution of oxidized materials resulted in material that behaved similarly to oxidized BLES. Cross-over experiments in which oxidized lipids were reconstituted with nonoxidized proteins resulted in biophysical activity close to that of samples with normal phospholipids, indicating that the oxidative modifications to the phospholipids by themselves were only partially responsible for the impaired activity due to oxidative damage. Reconstitution of oxidized SP-B and SP-C with nonoxidized phospholipids demonstrated similar impaired biophysical activity to that of oxidized BLES. These results suggested that modifications to the surfactant proteins are largely responsible for the impaired activity. Further, reconstitution studies demonstrated that the effects of oxidation on SP-B were more marked than were those on SP-C (Rodriguez-Capote et al. 2006). Moreover, nonoxidized SP-B could rescue samples with oxidized SP-C, but not vice versa.

In conclusion, these studies demonstrated that oxidative damage to surfactant can significantly impair surfactant function due to oxidative modifications to SP-B. The occurrence of these modifications *in vivo*, due to lung injury or pollution, needs to be investigated further. The specific amino acids of SP-B undergoing oxidative modifications and their effect on SP-B properties may provide insight into the function of this protein.

Structure–activity relations of surfactant proteins B and C in surfactant phospholipid interfaces (Perez-Gil)

It is established that the hydrophobic pulmonary SP-B and SP-C are required for the air-breathing mode of life of mammals (Nogee 2004). SP-B appears to be crucial in establishing the stable and functional air–liquid interface at the respiratory surface necessary for gas exchange. SP-B is also required for the proper maturation of SP-C, although the molecular mechanisms behind this coordinated processing are not understood. The presence of SP-B-related polypeptides has been reported in surface active material taken from fish, amphibian, reptilian, and avian lungs (Bernhard et al. 2001a; Johnston et al. 2001; Miller et al. 2001; Daniels et al. 2004), suggesting that SP-B activity may have been essential for the evolutionary origin of pulmonary surfactant.

Unfortunately, a model of the 3D structure of SP-B is lacking, precluding an understanding of its molecular mechanism of action. The structural similarity of SP-B to the saposin-like family of proteins suggests that SP-B may associate with phospholipid surfaces in an analogous manner, gaining access into deeper regions of the membranes and facilitating movement of lipid species out from the bilayers towards the interface. The amphipathic character of certain helical segments of SP-B as well as the particularly high affinity of some clusters of aromatic residues in the protein for association with lipid interfaces, seem critical features for the biophysical activity of the protein (Serrano et al. 2006). Molecular characterization of SP-B-like proteins from nonmammalian sources should establish whether these features are part of an evolutionarily conserved SP-B action. Current models propose that SP-B is required to ensure the insertion of surface-active molecules into relatively compressed films.

Reduction of interfacial affinity is associated with a strong decay of the ability of the protein to promote phospholipid interfacial adsorption against pressures higher than 35 mN/m. SP-B-promoted efficient adsorption to re-establish equilibrium pressures around 45 mN/m is required for (1) promoting rapid association of the newly secreted surfactant with the interface and (2) respreading of the interfacial lipid/protein complexes during the expansion moiety of the breathing cycle (Serrano and Perez-Gil 2006).

In contrast to SP-B, the presence of surfactant protein SP-C has only been reported in mammals to date, where its expression is tightly coupled to the differentiation of lung tissue. However, with the exception of one study on two species of bird (Bernhard et al. 2001a), in which SP-C was reported absent, to our knowledge no systematic, wide-ranging study has been undertaken to search for SP-C in nonmammals. Notably, a very recent study reports the SP-C gene sequence and expression of the SP-C gene in developing lungs of the frog *Xenopus laevis* (Hyatt et al. 2007), opening the question of the role of this lipopeptide in the evolution of the surfactant system. Whether SP-C is also present in reptiles and birds should be extensively explored. SP-C is the only surfactant protein that is solely synthesized by surfactant-producing type-II pneumocytes, which would suggest that SP-C may have appeared coincident with the evolution of the lung, and seems particularly well-suited to stabilize the specific architecture of the mammalian bronchoalveolar lung. Genetic models suggest that SP-C may play subtle roles in the long-term stabilization of the alveoli. Absence of SP-C is associated with chronic inflammatory responses. The 3D structure of SP-C, as determined in organic solvents, is perfectly suited for a transmembrane orientation in surfactant membranes, with a relatively dynamic 12-residue N-terminal segment probably defining interactions with neighboring protein and/or lipid molecules in the same or different lipid layers. Palmitoylation of cysteines in the N-terminal segment of SP-C stabilizes associations of the protein with ordered phases, such as those existing in highly compressed surfactant films at the end of exhalation. Biophysical models, therefore, propose that SP-C could be responsible for maintaining the association of surfactant complexes with the interface at the extremely low surface tensions (high surface pressures) required to stabilize mammalian alveoli (Serrano and Perez-Gil 2006). However, this proposed model still requires verification *in vivo*.

Hydrophobic proteins SP-B and SP-C have also shown activities potentially involved in immune defence actions in the alveolar spaces. Several members of the saposin-like family, to which SP-B belongs, have natural antibiotic properties, and peptides designed from the sequence of certain segments of SP-B demonstrate effective microbicidal activity against respiratory pathogens (Ryan et al. 2006). SP-C binds endotoxin (lipopolysaccharide, LPS) with some affinity and has been proposed to participate in removal of LPS from the airways without activation of pro-inflammatory pathways (Chaby et al. 2005). These activities suggest a coordinated action of pulmonary surfactant with respect to both physical stabilization and protection of the respiratory epithelium against environmental elements. This central coordinating role of surfactant also suggests that pulmonary surfactant might have evolved from primitive innate defence systems preexisting at organism–environment interfaces (Sullivan et al. 1998; Daniels et al. 2004).

The surfactant film under thermal stress

Introduction (Orgeig and Hall)

Experimentation to understand how animals maintain a functional pulmonary surfactant film involves many challenges. The film that lowers surface tension is located deep within the lungs where experimental access is limited. The alveolar films are at most a few molecules thick, and they form noncovalent structures that do not provide a simple basis for isolation. Studies of interfacial films are fraught with difficulties, and replication *in vitro* of alveolar conditions can be particularly difficult. In large part because of these problems, the most fundamental questions concerning how pulmonary surfactant functions, remain unanswered.

Perhaps the most productive investigative approach has used physiological studies to establish landmark characteristics of how the surfactant films behave *in situ*, and then to determine what properties must be present in order to replicate that behavior *in vitro*. Physiological studies, for instance, have established that pulmonary surfactant forms the alveolar films rapidly (Enhorning and Robertson 1972; Lachmann et al. 1979; Nicholas et al. 1982), and that when compressed by the decreasing alveolar surface area during exhalation, surfactant films achieve and sustain remarkably low surface tensions for prolonged periods (Horie and Hildebrandt 1971; Valberg and Brain 1977; Wilson 1981; Schürch 1982; Smith and Stamenovic 1986). Testing models of how surfactant may achieve these characteristics, however,

has been difficult because alveolar conditions are hard to perturb.

Thermal stress represents one perturbation that can be applied both *in vitro* and *in vivo*. Although the variation of temperature among most of the commonly studied animals is limited, heterothermic mammals provide a much larger range. This section presents studies of how simple films respond *in vitro* to different temperatures, and of how alveolar surfactant changes in heterothermic mammals in response to changes in body temperature.

In vitro behavior of surfactant films at different temperatures (Hall, Biswas, and Yan)

Temperature, along with composition, determines the dynamic characteristics of lipid structures. Like other compounds, phospholipids form distinct structural phases at different temperatures. Individual phospholipids, for instance, can form the highly ordered structure, with fully extended acyl chains and regular molecular packing, of gel (L_β) phase bilayers and tilted-condensed (TC) monolayers. When heated, these structures melt at specific temperatures characteristic of the individual phospholipid to the disordered liquid–crystalline (L_α) bilayers, and the liquid-expanded (LE) monolayers, characterized in both cases by kinked chains and disordered packing. Added cholesterol modulates the structure of the highly ordered phase, resulting in the extended chains but disordered packing of liquid-ordered (L_o) bilayers and monolayers (McIntosh 1978; Yeagle 1985; McMullen and McElhaney 1996). As expected from their structures, the different forms have different dynamic properties, with gel-phase bilayers and TC monolayers having the greatest rigidity, and liquid–crystalline bilayers and LE monolayers the greatest fluidity (Gitler 1972; Chapman 1975; Melchior and Steim 1976; Lewis and Engelman 1983).

The different dynamic behavior of these structures may determine how pulmonary surfactant satisfies the different requirements for its proper function. Rapid formation of an interfacial film requires a fluid structure, and vesicles adsorb much more slowly in the gel phase than in the liquid–crystalline phase (Phillips and Hauser 1974). The low alveolar surface tensions, however, indicate films that are rigid. Compressed monolayers, if they are fluid, will collapse from the surface to form 3D structures when their density exceeds the maximum equilibrium value. Surface tension for these fluid films normally falls during compression only to the level achieved during adsorption. To function effectively, pulmonary surfactant not only must have the initial fluidity necessary to form films rapidly, but also the rigidity required to sustain the low surface tensions observed in the lungs (Clements 1977).

The composition of pulmonary surfactant suggests a basis whereby these divergent requirements might be satisfied. In contrast to most biological phospholipids, pulmonary surfactant of most mammals contains large amounts of a compound, dipalmitoylphosphatidylcholine (DPPC, or PC-16:0/16:0) that in single-component bilayers melts from the gel phase to the liquid–crystal phase at 41°C, above temperatures relevant for the physiology of most mammals. At 37°C, DPPC can form TC monolayers, which replicate the resistance to collapse observed *in situ*. DPPC, however, represents at most half of the surfactant phospholipids and in some cases less than a quarter (Lang et al. 2005). The other lipids individually produce more fluid structures. Modulating the composition of the different structures formed by pulmonary surfactant might achieve the required different dynamics.

More recent evidence, however, has suggested that a difference in composition between adsorbing vesicles and the functional film may be unnecessary. If compressed fast enough to reach low surface tensions, fluid films transform to rigid structures that avoid collapse (Crane and Hall 2001; Smith et al. 2003). These supercompressed fluid films behave in several respects like 3D supercooled liquids, which, if cooled fast enough to sufficiently low temperatures, form amorphous solids, or glass (Debenedetti 1996). They retain the disordered structure of the initial fluid but attain the rigidity of a solid. The TC and supercompressed fluid monolayers replicate equally well the resistance of alveolar films to collapse.

Thermal stress represents a perturbation that might help distinguish which film is present in the lungs. Melting of the rigid films allows them to collapse, after which surface tension should promptly rise. The pulmonary mechanics of excised lungs should change accordingly, with the interfacial component of recoil forces increasing at the melting temperatures. TC and supercompressed fluid monolayers were therefore held at surface tensions below equilibrium values to determine the temperatures at which rates of collapse increase in vitro (Yan et al. 2007).

The experiments used films formed and manipulated on the surface of captive bubbles (Schürch et al. 1989; Putz et al. 1994). TC films contained DPPC spread at the interface and then compressed to the desired surface tension at 26°C. The supercompressed films instead contained extracted calf

surfactant (calf lung surfactant extract, CLSE). Both films melted at distinct temperatures that depended on the surface tension at which they were heated. At all surface tensions below 25 mN/m, TC melted at temperatures ~10°C higher than for the supercompressed CLSE. The increase in rates of collapse occurred at 47–55°C for TC DPPC, and at 37–41°C for CLSE.

Only the melting of the supercompressed fluid film corresponded well to the changes observed previously over the range of 35–42°C in the pulmonary mechanics of rat lungs (Clements 1967, 1977). Those results were interpreted initially as supporting an alveolar film that is substantially enriched in DPPC because the mechanics changed at the melting temperature of DPPC in bilayers (Clements 1977). The dependence on surface tension, however, shows that an alveolar film of TC DPPC would melt at substantially higher temperatures, and that the change in pulmonary mechanics of rat lungs corresponds instead to the melting of the supercompressed fluid films.

Other physiological measurements, however, have obtained different results. With cat (Horie et al. 1974) and rabbit (Inoue et al. 1981, 1982) lungs, the increases in interfacial recoil forces were smaller than in rat lungs, and they occurred at higher temperatures. Although the two kinds of films melt *in vitro* at distinct temperatures, the conflicting physiological data currently prevent any conclusions concerning which film is more likely to be present in the alveolus.

The effect of torpor on surfactant film structure (Orgeig, Daniels, Lang, Panda, and Possmayer)

Although most common experimental animals maintain a constant temperature close to 37°C, heterothermic mammals regulate body temperature over a wide range depending on ambient temperature, food availability, or circadian rhythms. During periods of torpor or hibernation, metabolism decreases and body temperature is maintained at a lower level than during activity. In general, torpor encompasses short periods of the order of hours, whereas hibernation is a deep and prolonged period of torpor lasting weeks (Geiser 1998). These mammals provide useful systems for testing how the surfactant system responds to changes in temperature *in vivo*.

Pulmonary surfactant demonstrates significant changes in composition and function during both torpor and hibernation in the three species that have been investigated to date; the marsupial fat-tailed dunnart, *Sminthopsis crassicaudata*, the microchiropteran bat, *Chalinolobus gouldii* and the golden-mantled ground squirrel, *Spermophilus lateralis*. Although the phospholipid composition remains largely unaltered during torpor or hibernation (Lang et al. 2005), cholesterol increases (Langman et al. 1996; Codd et al. 2002; Lang et al. 2005). The biophysical activity also changes, with surfactant from animals in torpor or during arousal achieving the lowest surface tension during compression *in vitro* at that temperature which most closely matches the *in vivo* temperature of the animal at the time of surfactant isolation (Lopatko et al. 1999; Codd et al. 2002, 2003). We (Daniels, Lang, Orgeig, Panda, and Possmayer) extend these initial findings here to determine, for torpid and active dunnarts, what structural changes in surfactant films result from compositional differences and cause functional alterations.

Brief methodology

Dunnarts were killed and lavaged when they were in either the warm-active state ($T_b = 35 \pm 0.21$°C, mean ± SE) or after they had remained in a torpid state for 8 h ($T_b = 13.14 \pm 0.2$°C, mean ± SE). Lavaged material was purified and centrifuged to isolate the large aggregate (LA) fraction of surfactant, as described previously (Lang et al. 2005). LA fractions were analyzed biochemically using enzymatic colorimetric kits for total cholesterol and for phosphatidylcholine, and for protein using the Lowry assay (Panda et al. 2004). Surface activity on solvent-spread films was determined on a Langmuir–Wilhelmy balance (Kibron Inc., Finland) at 23 ± 1°C. Adsorbed films containing 1mol% NBD-PC (1-palmitoyl-2-[12-{(7-nitro-1, 3-benzooxadiazol-4-yl) amino}dodecanoyl]-sn-glycero-3-phosphocholine) were compressed to either 5, 10, 15, 20, 25, or 30 mN/m and imaged with a Karl Zeiss epifluorescence microscope (Panda et al. 2004). Langmuir–Blodgett films were prepared on freshly cleaved mica at a constant surface pressure (π) of 30 mN/m with a transfer ratio of 1:1. Monolayer structures were imaged by atomic force microscopy (AFM), using a Nanoscope IIIa (Santa Barbara, CA, USA) in contact mode with a silicon nitride tip with a force constant of 0.6 mN/m (Panda et al. 2004).

Biochemical characterization

We found no difference in total phosphatidylcholine (PC; warm-active: 441 ± 121 μg; torpid: 433 ± 155 μg), total cholesterol (Chol; warm-active: 40 ± 14 μg; torpid: 36 ± 25 μg), or total protein

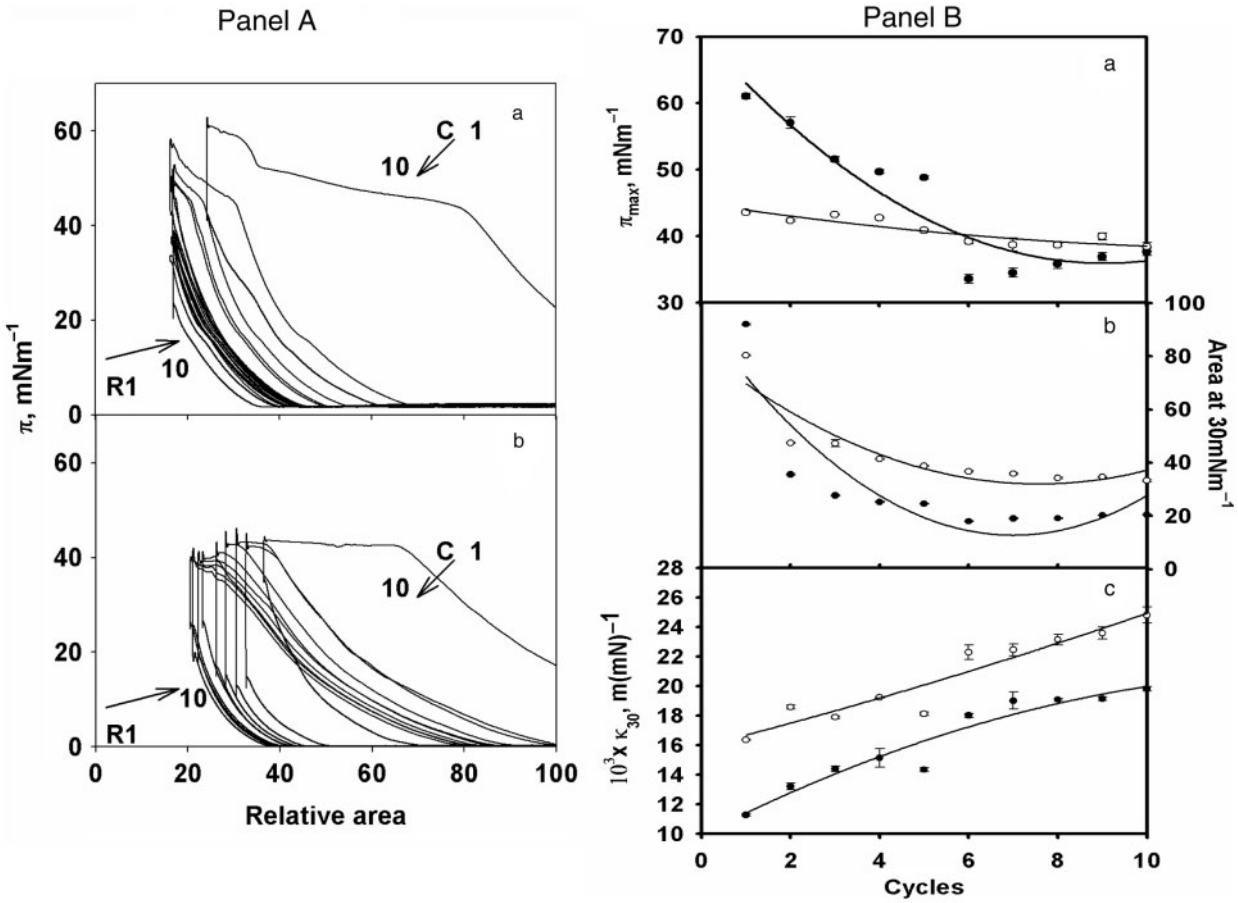

Fig. 1 (Panel A) Surface pressure–area compression–relaxation curves for solvent-spread surfactant films from warm-active dunnarts (a) and torpid dunnarts (b) at 23°C. C1 → 10 and R1 → 10 indicate the positions and directions of the compression and relaxation cycles, respectively. Subphase: 0.15 M NaCl, 1.5 mM $CaCl_2$, 1.0 mM Tris–HCl buffer at pH = 7.0. Film compression rate: 0.85 nm^2/mol/min with an initial area of 1.20 nm^2/mol. **(Panel B)** Quantitative representation of film parameters of the compression–relaxation cycles of solvent-spread warm-active (solid symbols) and torpid (open symbols) films at 23°C from Panel A. (a) Maximum attainable surface pressure, π_{max}; (b) Area at which a π of 30 mN/m was first attained and (c) Compressibility at 30 mN/m.

(warm-active: 920 ± 174 μg; torpid: 1136 ± 143) between LA fractions of surfactant isolated from warm-active and torpid dunnarts. Hence, the ratios of these components also did not change between warm-active and torpid dunnarts: protein/PC (μg/μg) was 2.09 for warm-active and 2.62 for torpid dunnarts; Chol/PC (μg/μg) was 0.09 for warm-active and 0.08 for torpid dunnarts. This result is unlike our previous findings with a range of species in which we documented consistently that cholesterol relative to phospholipid increases during torpor (Langman et al. 1996; Codd et al. 2002; Lang et al. 2005). In these previous studies, however, biochemical characterization was performed on whole-lavage material rather than on LA fractions. The different results may suggest that the increase in cholesterol observed in whole lavage is caused by increases in cholesterol in the small aggregate, non-surface-active component of lavage fluid, which is eliminated in the isolation of LAs.

Measurements of surface activity

Analysis of π-area isotherms indicated that in two out of three surface-activity parameters, surfactant isolated from torpid dunnarts (denoted T) demonstrated greater activity than did surfactant isolated from warm-active dunnarts (denoted W), when measured at 23°C on the Wilhelmy–Langmuir balance. When analyzing isothermal compressibility at 30 mN/m (k_A), which is determined from the equation $k_A = (-1/A_{30})\ (dA/d\pi)_{30}$ and measures the change in compression relative to the change in π, T-surfactant demonstrated higher compressibility (Fig. 1 Panel B, b), (i.e., a higher compression relative to π), which indicates a poorer surface activity than that of W-surfactant. However,

while on the first four cycles of dynamic surface pressure—area measurements, W-surfactant attained higher π on maximum compression than did T-surfactant, the W-surfactant film was unstable, with π decreasing on the 5th cycle to below that of T-surfactant (Fig. 1 Panel A, a and Panel B, a). Therefore, for cycles 5–10, T-surfactant attained higher π than did W-surfactant. In terms of the area compression that is required to achieve max π, T-surfactant demonstrated a lower area compression, i.e., the area at which 30 mN/m was reached was higher in T-surfactant for all cycles except the first (Fig. 1 Panel B, b). Hence, in terms of max π and surface area compression, T-surfactant was more stable and more surface active at higher cycles. Hence, at 23°C, which is a temperature that more closely matches the body temperature of torpid animals, T-surfactant demonstrated greater surface activity.

Measurements of fluorescence microscopy

The fluorescent probe NBD-PC, having a bulky fluorescently labeled tail, cannot partition into organized regions, known as domains. It is excluded from the ordered (or liquid–condensed) phase and thus generates a brilliant contrast between the fluid (or disordered) and the condensed (or ordered) domains. These latter domains appear black when visualized under the epifluorescence microscope. As can be seen from Fig. 2 (Panel A), phase partitioning (i.e., coexistence of ordered and disordered phase) was present initially in W-surfactant but only up to a π of 15 mN/m. Thereafter, the probe-excluded domains disappeared and were replaced by microdomains 1–2 μm in diameter, which were virtually beyond the resolution of the imaging analysis. Hence, above a π of 15 mN/m there was a perturbation to the packing of the surfactant film, indicating that the W-surfactant film was unstable under the conditions of measurement. On the other hand, T-surfactant demonstrated phase coexistence throughout film compression up to 30 mN/m (Fig. 2 Panel B), indicating stable packing into liquid–condensed regions. Moreover, the area of coverage of probe-excluded liquid–condensed regions in T-surfactant increased throughout compression and was greater than that of T-surfactant (Fig. 2 Panel C, a). Hence, in terms of film structure and behavior during compression, T-surfactant was a more effective surfactant at 23°C. As these measurements were performed on solvent-extracted surfactant, a process that eliminates any potential contaminating nonsurfactant proteins, it is possible to exclude contaminating serum proteins as a cause for the packing perturbation seen in W-surfactant. At this stage, we cannot confirm the cause of this phenomenon.

Atomic force microscopy

Atomic force microscopy confirmed the presence of large circular liquid–condensed domains in T-surfactant with mean diameter of 4.76 ± 0.17 μm and mean height profile of 0.91 ± 0.07 nm (Fig. 2 Panel D, c and d). On the other hand, W-surfactant demonstrated much smaller circular domains with mean diameter of 1.43 ± 0.08 μm, but with a very similar mean height profile of 0.90 ± 0.02 nm (Fig. 2 Panel D, a and b). These height profiles were very similar to those that would be obtained with a pure DPPC film (Panda et al. 2004), thereby supporting the fluorescence data by concluding that these were liquid–condensed, DPPC-enriched domains. An unusual structural feature, present only in T-surfactant, was the existence of microdomains (LC2) within the larger liquid–condensed domains (LC1) with mean diameter of 1.70 ± 0.8 μm and a mean height profile of 1.56 ± 0.37 nm, i.e., extending a further 0.64 nm above LC1 (Fig. 2 Panel D, c and d). This height profile was greater than any domains usually observed with natural or artificial surfactant mixtures, and hence we are unsure as to the composition of these microdomains. One possibility is that they represent higher order condensed DPPC-cholesterol regions, in which the cholesterol intercalates between the acyl chains inducing further rigidity, thereby, further reducing the tilt of the acyl chains relative to the plane of the liquid-expanded film and in turn increasing the height profile. Alternatively, addition of cholesterol may cause other structural anomalies such as formation of stacked layers, as recently reported in a study where cholesterol was added to BLES, an exogenous clinical surfactant formulation (Nag et al. 2007).

Summary

In summary, surfactants isolated from dunnarts exposed to different physiological conditions behaved very differently under the same *in vitro* conditions. Moreover, these differences in biophysical behavior were matched by significant differences in film structure as assessed by fluorescence and AFM. In general, the function and structure of T-surfactant films were more similar to those expected of natural surfactant isolated from traditional mammalian models that are classed as ''good'' surfactants. These studies were performed at 23°C, a temperature more similar to the body temperature of torpid dunnarts, which may explain the improved function

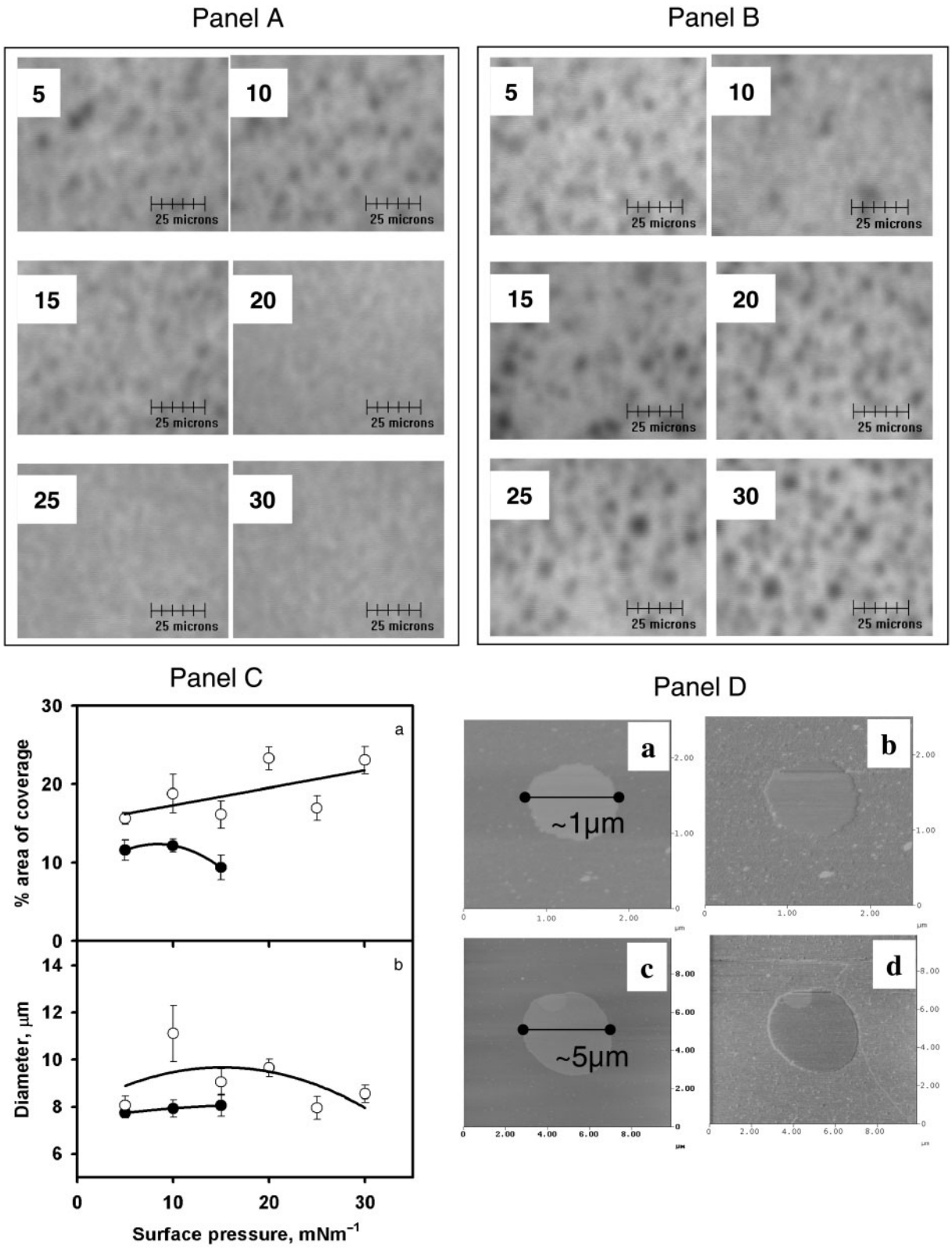

Fig. 2 Fluorescence images of solvent-spread surfactant film from warm-active (**Panel A**) and torpid (**Panel B**) dunnarts measured at 23°C at 5, 10, 15, 20, 25, 30 mN/m surface pressure (indicated inside the images). Subphase: 0.15 M NaCl, 1.5 mM $CaCl_2$, 1.0 mM Tris–HCl buffer at pH = 7.0. (**Panel C)** Quantitative fluorescence image analysis of solvent-spread surfactant films of warm-active (solid symbols) and torpid (open symbols) dunnarts at different surface pressures. (a) Percentage of area of coverage of the probe-excluded regions; (b) Diameter (μm) of the probe-excluded regions. (**Panel D)** Atomic force microscopy images of solvent-spread surfactant films from warm-active (a, b) and torpid (c, d) dunnarts at 30 mN/m surface pressure. The films were transferred onto freshly cleaved mica by the Langmuir–Blodgett transfer technique. Area of scan a, b: $2.5 \times 2.5\ \mu m^2$; c, d: $10 \times 10\ \mu m^2$. Images a and c were taken in height mode, while b and d were taken in phase mode. LE = liquid expanded phase; LC = liquid condensed phase.

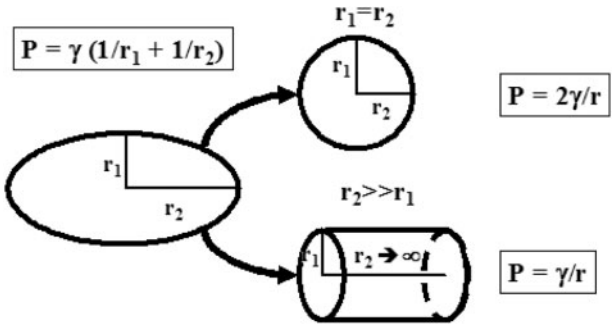

Fig. 3 Application of the Young–Laplace equation (left) to differently structured air–liquid interfaces, i.e., spheres like alveoli (right top) and tubules like mammalian bronchioli and avian air capillaries (right bottom). P = (opening) pressure; γ = surface tension; r = radius.

of T-surfactant over W-surfactant. However, future studies performed at *in vitro* temperatures that match the *in vivo* temperatures are required to confirm this hypothesis. Moreover, studies have to be repeated with natural surfactants as well as surfactants in which various components are selectively removed or added, such as cholesterol, the hydrophobic surfactant proteins SP-B and SP-C and/or individual phospholipid molecular species.

Functional and molecular characteristics of surfactant in different vertebrate respiratory structures (Bernhard)

Introduction

The contractile forces of lungs depend in large part on the surface tension of their inner surfaces and the radii of their terminal gas-exchange units. Hence, a surface active agent is essential to oppose surface tension and to prevent lung collapse. Due to the incidence of neonatal respiratory distress syndrome in premature neonates with a surfactant insufficiency, the focus of ''surfactant research'' was initially restricted to alveolar function (Adams et al. 1970). However, based on the Young–Laplace equation, the need to reduce surface tension is defined by the radii of (air–liquid) interfaces, and therefore applies to all narrow lung structures, whether spherical, saccular, or tubular (Enhorning et al. 1995) (Fig. 3). Alveoli can demonstrate a radius (r), where $r_1 = r_2 = 20–125\,\mu m$, as opposed to that of tubules, where values can be as low as 8–10 μm for r_1 and approaching infinity (∞) for r_2 (e.g., the terminal bronchioles of the mammalian lung or the air capillaries in the bird lung). This would suggest that a reduction of surface tension is essential for saccular lungs (preterm human infants beyond 24 weeks gestational age; term newborn rat and mouse) and lungs from very preterm human infants during the tubular phase (below 24 weeks gestational age). So, why should surfactant be unnecessary for stabilizing mammalian airways and tubular bird lungs, where it is evidently present (Bernhard et al. 1997, 2001a). Indeed comparative and developmental studies refute the concept that the ''function of surfactant is restricted to keep open the alveolus,'' and they do support that surfactant is essential for bronchiolar function (Mander et al. 2002), as it is for tubular lungs of birds. Moreover, recent data have demonstrated a significant difference in surfactant molecular composition for airway surfactant between alveolar and avian lungs, suggesting that there is a strong relationship between biochemistry, structure, and function.

Bronchiolar versus alveolar surfactant of mammals

It is clear from auto-radiography, radio-labeling experiments and analysis of proteins, and gene expression that secretion of the complete lipidomic and proteomic set of surfactant components in mammalian lungs is restricted to the type-II pneumocytes (PNII), while other cells may well contribute to the secretion of mature surfactant proteins, SP-A and SP-D. However, airway epithelia neither comprise lamellar bodies nor do they contain or secrete significant amounts of those phospholipid molecular species characteristic for lung surfactant, particularly DPPC (PC16:0/16:0), palmitoyl-myristoyl-PC (PC16:0/14:0) and palmitoyl-palmitoleoyl-PC (PC16:0/16:1). Hence, airway surfactant, which demonstrates a phospholipid composition identical to that of the alveolar compartment and not of the underlying airway epithelium, must be regarded as an alveolar overflow (Bernhard et al. 1997). In addition to any theoretical reasoning (see above), biochemical and functional analysis shows that such airway surfactant is functionally important, since it reduces surface tension in airways, improves mucociliary transport, and essentially contributes to airway patency under clinical conditions (Heeley et al. 2000).

Bronchiolar surfactant of mammals versus air capillary surfactant of birds

The tubular, rather than spherical, structure of bronchioles in mammals and in air capillaries in bird lungs allows us to address questions relating to the structure, function, and biochemistry of the alveoli. Both similarities and differences exist, and we are only beginning to understand their meaning and

to learn from these biological differences between models.

Similarities between mammalian airway and bird surfactants

Both airway and bird surfactant spread from distant locations. Bronchiolar surfactant is derived from alveolar overflow, whereas in birds, surfactant in the gas-exchanging ACs is derived from PNII that are located in the atria (Bernhard et al. 1997, 2001a). Similarly, there are essential differences in the structure of airway and tubular surfactant relative to alveolar surfactant. Neither bird surfactant (Daniels and Orgeig 2001) nor airway surfactant appear to form tubular myelin structures or demonstrate a surface-associated reservoir (Bernhard et al. 2001a), which are common characteristics of alveolar surfactant, suggesting fundamental compositional differences. Indeed, concentrations of those components supporting the formation of tubular myelin or surface-associated reservoirs, i.e., SP-A and SP-C are either reduced (airway surfactant) or may be absent or present in altered form (bird surfactant) compared to alveolar surfactant (Bernhard et al. 1997, 2001a; Sullivan et al. 1998). However, SP-B concentration is identical in bird and alveolar surfactant, supporting its role in lamellar body formation and in affecting surface tension (Bernhard et al. 2001a).

A further similarity between airway and bird surfactant is that neither of them easily reach minimal surface tension near zero mN/m in response to cyclic compression (Bernhard et al. 1997, 2001a). This ''low'' surface activity is not a functional problem because, unlike alveoli, neither airways nor the rigid tubular bird lung undergo cyclic changes in air–liquid interface area during respiration. While the surface tension within bird lung ACs is unknown, a surface-associated reservoir does not appear to exist and, using gel filtration chromatography, a SP-C molecule of size or hydrophobicity comparable to that of mammals has not been detected in chicken and duck surfactant material (Bernhard et al. 2001a). Whether it is entirely absent or present in altered form has to be confirmed. These differences suggest that any surface-enrichment in the major component PC16:0/16:0 must be based on different principles from those that apply in mammalian alveoli. While bronchioli may narrow during expiration, the minimal surface tension of airway surfactant upon cyclic area compression only reaches values of about 20 mN/m, which is equilibrium surface tension of phospholipids, and is similar to surface tensions measured in airways *in vivo* (Bernhard et al. 1997; Im Hof et al. 1997).

Unique composition of bird surfactant

There are also characteristics of bird surfactant that differentiate it from both alveolar and airway surfactant of mammals and provide insights into the possible roles of individual components. While the currently assumed absence (Bernhard et al. 2001a) or possibly altered form of SP-C in bird lungs fits with the absence of a surface-associated reservoir, reservoirs are essential for replenishing material losses in mammals, e.g., through the bronchioles, and for competing with cyclic squeeze-out from, and replenishment to, the alveolar interface during respiration. Neither of these situations occurs in birds, as there are no narrow tubules (bronchioli) apart from the ACs themselves and there is essentially no change in air–liquid interface area during the respiratory cycle, since bird lungs function as a rigid flow-through system rather than as bellows.

Moreover, phospholipid composition of bird (chicken and duck) surfactant is essentially different from that of mammalian surfactants. Principally, the major components of surfactant, i.e., dipalmitoyl-PC (PC16:0/16:0), palmitoylmyristoyl-PC (PC16:0/14:0), and palmitoyl-palmitoleoyl-PC (PC16:0/16:1) and in some species their alkyl-acyl-analogues, are sorted in PNII according to fatty acyl chain length (Bernhard et al. 2001b). While their fractions differ during prenatal and postnatal development, as well as across mammals, they add up to about 75% of PC in mammalian (Lang et al. 2005) and bird surfactant. Importantly, bird surfactant nearly exclusively comprises PC16:0/16:0 with the fluidic components, disaturated PC16:0/14:0 and PC16:0/16:1, being virtually absent or reduced (Bernhard et al. 2001b). In contrast, mammalian alveolar and airway surfactants contain much more PC16:0/14:0 and PC16:0/16:1 at the expense of PC16:0/16:0. Such differences are probably related to surfactant function under dynamic conditions rather than to curvature, since curvature of gas-exchanging surfaces is highest in bird lungs comprising little or none of these fluidic PC species (Bernhard et al. 2001b). Additionally, the absence of both PC16:0/14:0 and cells analogous to alveolar macrophages (alvMΦ) in bird lungs (Bernhard et al. 2001a, 2001b), together with specific effects of this PC component on macrophage differentiation and inhibition of T-cell proliferation (Gille et al. 2007), highlights the possibility that short-chain PC components of surfactant are important contributors to parenchymal homoeostasis in mammals.

In summary, surfactant is essential for stabilization of the lung, but its function is not restricted to the alveolus. Instead, it is, according to the Young–Laplace equation, essential for air–liquid interfaces of all ''narrow'' lung structures including mammalian bronchioles and avian air capillaries, two vertebrate systems which have been extensively investigated. Molecular differences are principally attributable to functional characteristics, which include differences in interface dynamics, the need for surfactant reservoirs and, possibly, the principles of parenchymal immune homoeostasis.

Is there a role for a pulmonary-type surfactant in the tracheae of insects? (Hetz)

Introduction: structure and function of the insect tracheal system

The insect tracheal system is unique in the animal kingdom. The tracheal system can be considered as an invagination of the cuticle building a network of small air-filled tubes branching inside the insect body and reaching from the body surface to the organs. The hypodermis secretes the two main layers of the tracheal intima, the endocuticle and the exocuticle (Whitten 1972). As a respiratory surface, however, the tracheal intima is much thinner than the outer cuticle, thereby allowing gas exchange by diffusion. The exocuticular part is restricted to larger tracheae, where it builds up a thin lining that stabilizes the structure of the trachea, and also to air sacs that are equipped with special cuticular structures (Wasserthal 1997) and may act as bellows-like structures during respiration (Wobschall and Hetz 2004). As the tracheal system diverges and branches into smaller and thinner tracheae, the surface may increase with decreasing diameter until it ends with thin tracheoles in close contact with the tissues (Schmitz and Perry 1999).

External gas exchange is controlled by closable spiracles. One pair is originally present in every thoracic and abdominal segment (Nikam and Khole 1989). Within the insect body, the tracheae form a network that allows the respiratory gases to diffuse through the tracheal system. Diffusive transport of respiratory gases in air rather than convective transport of oxygen and carbon dioxide in body fluids, allows an energetically ''cheap'' supply of respiratory gases. External gas exchange was originally considered to be purely driven by diffusion (Krogh 1920), but recent data suggest that under some circumstances, some portion of active or passive ventilation also contributes to gas exchange (Westneat et al. 2003).

Characteristics that may suggest the need for a pulmonary type surfactant in the insect tracheal system

Molting

The tracheal system has certain characteristics that beg the question of whether a pulmonary-type surfactant system is required to maintain proper functioning of the tracheal system. An important issue in insect life is the need to molt as the animals grow. Molting of the first-, second- and third-order tracheae may be complicated. An exuvial fluid is secreted by hypodermis cells during the molting cycle. It contains enzymes like chitinase and proteases (Passonneau and Williams 1953). Unfortunately, most of the characterization of insect molting fluid has been done for reasons other than understanding potential surfactant effects (Jungreis 1974). The enzymes within the exuvial fluid partly digest the old cuticle, while the new cuticle is produced by the hypodermis. During the molting process, the old cuticle is pulled out of the tracheal system. The remainder of the exuvial fluid may, therefore, serve to reduce capillary forces between old and new tracheal walls thereby preventing disruption of the old cuticle and exhibiting surfactant-like properties.

Ventilation leading to collapse

If diffusion does not suffice to meet metabolic demands, the tracheal system has to be ventilated actively. Ventilation of the tracheal system with rhythmical collapsing and expanding of the tracheal lumina was visualized using synchrotron radiation (Westneat et al. 2003). Tracheal air sacs with complicated intima structures may work as bellows if large volumes of air are exchanged through convection. Wasserthal (1981, 1997) also observed tracheal ventilation in wings of moths during wing inflation after molting and contributed ideas about the effect of hemolymph pressure in tracheal ventilation. The internal tracheal structures are believed to collapse and expand during these convection movements. If they do, in fact, collapse there may be a need for a surfactant that facilitates subsequent expansion.

Fluid-filled tracheoles

Wigglesworth (1935) used the transparent flea, *Xenopsylla*, to study respiration under changing ambient oxygen levels. The flea closes its spiracles

from time to time but seems to adapt spiracular conductance to metabolic demand and to availability of ambient oxygen. In previous papers, Wigglesworth (1930, 1931) discovered that the tracheoles contain water and the water column changes its length according to ambient oxygen concentration. According to Wigglesworth, the osmolarity of the fluid around the tracheoles changes due to the accumulation of intermediate metabolites in response to the endotracheal oxygen partial pressure leading to water uptake and release from the tracheoles. His schematic drawing (Fig. 12 in his paper) has now been used in many physiology textbooks. Kestler (1985) expanded Wigglesworth's model and discussed it as possibly serving to ventilate the tracheoles during a respiratory cycle.

The question arises, how a thin fluid-column can be drawn back and forth by simple changes in osmotic pressure? Discussions concerning why and how the water column within the tracheoles changes its dimension are based on some physical constraints and two questions. Firstly, does the content of the tracheoles consist solely of water and second, is the mechanism driven passively and only by osmosis?

Wigglesworth's theory relies mainly on the ability of the surrounding cells to absorb fluid from the tracheal system as is the case in initial tracheal filling in mosquito larvae that had been kept submerged for the first days after hatching (Wigglesworth 1938). Upon being given access to the surface, mosquito larvae begin filling the trachea with air, whereas they remain fluid-filled without surface access. Filling was completed after 20 min in young larvae but took longer in older larvae. Passonneau and Williams (1953) analyzed the composition of the molting fluid in moth pupae. Jungreis (1979) characterized the osmotic pressure of molting fluid repeatedly and found that it was isoosmotic or slightly hyperosmotic to the surrounding hemolymph. Secretion and absorption of molting fluids was thought to be based on active transport of ions with water following passively. It seems possible that a similar mechanism is utilized for the ventilation of the tracheoles with changes in the osmotic pressure, and thus the length of the fluid column, being driven by active transport of K^+ and HCO_3^-. This would mean, however, that changes in the water column would be energetically costly and would not be due solely to changes in oxygen partial pressure.

To our knowledge, no one has measured the magnitude of changes in osmotic pressure simultaneously with the extension of a water column in living fleas, which is important for the tracheal filling theory. Wigglesworth himself used the interpretation of Bodine (1928) to discuss the significance of the external oxygen partial pressure for the formation of inorganic acids. If this interpretation is correct, we can ask how great a change in osmolarity is required to drive the tracheole fluid back and forth? A glass capillary with a diameter of 10 μm would suck in pure water against a pressure of about 12 kPa due to capillary forces. This pressure corresponds to a change in osmolarity of about 4.9 mOsmol/l, a value that does not appear unreasonable at a first glance since the normal osmolarity in insects can be as high as 400 mOsmol/l, or even higher. If we assume a tracheole diameter of a single micrometer, however, the corresponding change in osmolarity would be about 49 mOsmol/l, which represents an increase in osmolarity of ~12%, a huge change for intracellular osmolarity.

So, the question remains open. Is the change in osmolarity around the tracheoles so great such that there is no need for surfactant-like material within the tracheoles to draw the fluid back and forth? Or are the osmotic forces around the tracheoles relatively small, such that there is likely to be a need for a surfactant facilitating movement of the tracheolar fluid?

Conclusions and future directions (Orgeig and Daniels)

Despite the extraordinary variation in respiratory structures among the vertebrates, the structure of the BGB is highly conserved. Driven by evolutionary pressures to optimize diffusion of gases across the respiratory epithelium, the BGB is extremely thin, but on the other hand very strong to withstand the numerous distension forces acting upon it. Among the vertebrates, the bird lung is recognized as the most complex and efficient, possessing the thinnest of BGB. To date, however, we have very little understanding of the biomechanics of the water/BGB interface, nor do we understand the evolution of the molecular regulation of the structure of the BGB that optimizes the conflicting requirements of thinness and strength.

The pulmonary surfactant system, a mixture of lipids and proteins that forms a film at the air–liquid interface and hence forms part of the BGB on the air side of the lung, is also extraordinarily conserved. The two hydrophobic surfactant proteins, SP-B and SP-C are intimately involved in regulating the formation, maintenance, and dynamic function of the surfactant phospholipid film. The fact that oxidative stress to SP-B is detrimental to the biophysical function of pulmonary surfactant, that

SP-B deficiency is lethal and that it appears to be evolutionarily conserved, suggests that SP-B is likely to have been essential for the evolutionary origin of pulmonary surfactant. To date, the precise molecular mechanism of action of SP-B within lipid layers has not been established as the 3D structure has yet to be elucidated. However, an analysis of the specific amino acids of SP-B undergoing oxidative modifications and their effect on SP-B properties, as well as an evolutionary analysis of SP-B amino-acid sequence may provide insight into the function of this protein. On the other hand, the specific role of SP-C in the bronchoalveolar lung of mammals is open to question. Although the 3D structure of mammalian SP-C is known, proposed models for its mechanism of action have yet to be confirmed *in vivo*. Moreover, exploration of the presence and role of SP-C in surfactant materials from other animal groups is required.

In order to investigate fundamental aspects of the composition and function of the surfactant film, a powerful approach has been to use physiological studies to establish characteristics of how the surfactant films behave *in situ*, and then to ascertain which properties must be present, in order to replicate that behavior *in vitro*. As the surfactant film consists predominantly of lipids, one useful physiological perturbation involves changes in temperature. *In vitro* studies of tilted-condensed DPPC films versus supercompressed fluid monolayers of CLSE have established that at all surface tensions below 25 mN/m, DPPC films melted at temperatures $\sim$10°C higher than did supercompressed CLSE films. The temperature range for the latter was 37–41°C, corresponding most closely to changes in pulmonary mechanics in rat lungs over a similar range of temperatures. This suggests that, contrary to previous beliefs, the alveolar film does not have to be substantially enriched in DPPC, but that similar properties of rate of film formation can be achieved with more fluid films. Furthermore, this fits well with recent evidence that there are many species of mammals, including heterothermic mammals that do not possess the traditional 40–50% of DPPC (Lang et al. 2005). However, mechanical studies of the lung in other mammalian species (e.g., cat and rabbit) do not currently support this hypothesis. Hence, future studies need to concentrate on matched *in vivo* and *in vitro* studies over identical temperature ranges and with matched species-specific extracts of surfactants to establish the likely composition of the actual alveolar film *in vivo*. Moreover, these studies need to be complemented with structural studies at temperatures matching the *in vivo* temperature on both whole and selectively depleted surfactants so as to establish likely film structures *in vivo* under different physiological conditions and the components primarily responsible for these.

Comparative physiology has proven a powerful approach to highlight or elucidate the importance of individual components and/or certain functional aspects of the pulmonary surfactant system. Hence, it is clear that surfactant is essential for lung stabilization in vertebrates but its function is not restricted to the spherical alveolus. Instead, surfactant is also important in narrow tubular respiratory structures such as the terminal airways of mammals and the ACs of birds. This begs the question as to whether a surfactant-type material is also required in the minute tracheae and tracheoles that constitute the respiratory system of most insects.

Although surfactant material has been detected in the gas mantle of the pulmonate snail, a molluscan invertebrate (Daniels et al. 1999), to the authors' knowledge, to date no studies have detected pulmonary-type surfactant material in the tracheal respiratory system of insects, members of the largest invertebrate phylum, the arthropods. Upon theoretical analysis, however, it appears likely that such a system would be required, in order to cope with surface-tension forces in minute tubules during processes such as molting, when the tracheae collapse and when they fill with water. Hence, despite the observation by Wigglesworth in the 1930s of fluid within the tracheae, the challenge persists into the 21st century to determine whether this fluid is associated with a pulmonary-type surfactant system.

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