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Adaptations to hibernation in lung surfactant composition of 13-lined ground squirrels influence surfactant lipid phase segregation properties

Lakshmi N.M. Suri [a], Antonio Cruz [c], Ruud A.W. Veldhuizen [b], James F. Staples [d], Fred Possmayer [e], Sandra Orgeig [a,*], Jesus Perez-Gil [c,**]

[a] Sansom Institute for Health Research and School of Pharmacy & Medical Sciences, University of South Australia, Adelaide, SA 5000, Australia
[b] Lawson Health Research Institute, University of Western Ontario, London, Ontario, Canada
[c] Department of Biochemistry, Faculty of Biology, Complutense University, Madrid, Spain
[d] Department of Biology, University of Western Ontario, London, Ontario, Canada
[e] Departments of Obstetrics & Gynaecology and Biochemistry, University of Western Ontario, London, Ontario, Canada

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ABSTRACT

Pulmonary surfactant lines the entire alveolar surface, serving primarily to reduce the surface tension at the air–liquid interface. Surfactant films adsorb as a monolayer interspersed with multilayers with surfactant lipids segregating into different phases or domains. Temperature variation, which influences lipid physical properties, affects both the lipid phase segregation and the surface activity of surfactants. In hibernating animals, such as 13-lined ground squirrels, which vary their body temperature, surfactant must be functional over a wide range of temperatures. We hypothesised that surfactant from the 13-lined ground squirrel, *Ictidomys tridecemlineatus*, would undergo appropriate lipid structural re-arrangements at air–water interfaces to generate phase separation, sufficient to attain the low surface tensions required to remain stable at both low and high body temperatures. Here, we examined pressure–area isotherms at 10, 25 and 37 °C and found that surfactant films from both hibernating and summer-active squirrels reached their highest surface pressure on the Wilhelmy–Langmuir balance at 10 °C. Epifluorescence microscopy demonstrated that films of hibernating squirrel surfactant display different lipid micro-domain organisation characteristics than surfactant from summer-active squirrels. These differences were also reflected at the nanoscale as determined by atomic force microscopy. Such re-arrangement of lipid domains in the relatively more fluid surfactant films of hibernating squirrels may contribute to overcoming collapse pressures and support low surface tension during the normal breathing cycle at low body temperatures.

1. Introduction

Pulmonary surfactant lines the entire alveolar surface and is involved primarily in reducing the surface tension at the air–liquid-interface [1]. Upon secretion by alveolar type II epithelial cells, surfactant adsorbs as a monolayer interspersed with multilayers at the alveolar interface [2,3]. Temperature alterations, which have profound effects on lipid physical properties, can result in the phase co-existence in monolayers and in bilayer membranes of native surfactant [3–5]. The effect of temperature on surfactant composition and function in heterothermic mammals such as bats and dunnarts has previously been investigated [5–7]. However the effect of temperature on phase segregation properties of surfactant films has not been examined.

Recently, we showed that hibernation in the 13-lined ground squirrel, *Ictidomys tridecemlineatus*, induces an increase in unsaturated surfactant phospholipid (PL) species resulting in an increase in overall fluidity. Nevertheless, surfactant from hibernators (body temperature ~5 °C) and summer-active animals (body temperature 37 °C) remains functional over a wide range of temperatures [8]. Specifically, hibernating ground squirrels had higher amounts of monounsaturated phosphatidylcholine (PC) species, including PC16:0/16:1, PC16:0/18:1, and unsaturated phosphatidylglycerol (PG) species, but lower levels of disaturated PC and PG species, such as PC16:0/16:0 and PG16:0/16:0 compared with summer-active animals (see data summarized in Table 1). Such alterations in the surfactant composition of heterothermic mammals would presumably affect the phase segregation properties of surfactant films. But what is not clear is how such surfactant films with higher unsaturation avoid film collapse, a feature which might be necessary for attaining low surface tensions. All natural and lipid extract surfactants examined to date form ordered structures known as

* Corresponding author. Tel.: +61 8 83022649; fax: +61 8 83021087.
** Correspondence to: J. Perez-Gil, Department of Biochemistry, Faculty of Biology, Complutense University, 28040 Madrid, Spain. Tel.: +34 91 3944994; fax: +34 91 3944672.
E-mail addresses: Sandra.orgeig@unisa.edu.au (S. Orgeig), jpg@bbm1.ucm.es (J. Perez-Gil).

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Table 1
Proportion of disaturated species of phosphatidylcholine (PC) and phosphatidylglycerol (PG) in surfactant large aggregates from warm active and torpid squirrels.

	Disaturated PC species (mol%)	Disaturated PG species (mol%)
Summer-active	65.1 ± 6.6	36.2 ± 6.2
Hibernating	47.6 ± 5.0	22.5 ± 1.1

Data obtained from reference [8].

domains when their films are compressed to lower surface area [9–11]. We hypothesised that fluid surfactant films of hibernating 13-lined ground squirrels undergo lipid organisational re-arrangements at the interface and generate a compression-driven phase separation of lipid domains which permit the films to remain stable and avoid collapse before reaching high surface pressures at low temperatures.

In the current study, we studied the domain or phase organisation of films formed by surfactant from 13-lined ground squirrels with pressure–area isotherms, epifluorescence and atomic force microscopy in order to better understand how surfactant films with relatively high concentrations of unsaturated PL species are able to remain stable and functional at different body temperatures. An improved mechanistic understanding of the adaptive strategies employed by heterothermic mammals to maintain functional surfactant films at different body temperatures may help to identify essential structure-function determinants in surfactant. Such insights could facilitate the development of improved surfactant therapies for respiratory complications associated with conditions such as severe hypothermia or hypothermic surgery [12–14].

2. Materials and methods

2.1. Animals and lavage

All animal procedures were approved by the University of Western Ontario animal use subcommittee and were in accordance with the Canadian Council of Animal Care's guidelines. The 13-lined ground squirrels were wild-caught at the University of Manitoba's field research station in Carman MB, Canada. After transfer to the University of Western Ontario (London, ON, Canada) animal husbandry procedures described by Muleme et al. [15] were followed. Summer-active animals, with body temperatures near 37 °C, were euthanised by a sodium pentobarbital overdose (270 mg/ml, 0.2 ml/100 g). Hibernating animals, sampled in the winter (December to early March), had body temperatures near 5 °C. Hibernating animals undergo periodic increases in body temperature every 5–7 days known as arousal. Therefore these animals were euthanised by cervical dislocation to prevent inducing arousal. A repetitive lavage procedure using an ice-cold 0.15 M NaCl solution was performed in order to remove pulmonary surfactant as described previously [8]. The total lavage was centrifuged at 150 *g* for 10 min to yield a pellet containing cells and cellular debris which was discarded. The supernatant was re-centrifuged at 40,000 *g* for 15 min, to pellet the active large aggregates (LA) sub-fraction. The 40,000 *g* pellet was re-suspended in 2.0 ml sterile 0.15 M NaCl solution. PL concentration of the LA was determined according to the method of Bligh and Dyer followed by a modified Duck–Chong phosphorus assay [16,17]. Samples were lyophilised and shipped to Complutense University, Madrid, Spain where further experiments were conducted.

2.2. Pressure (π)–area (A) isotherms

A specially designed custom built Wilhelmy–Langmuir balance with a 190 cm^2 trough (Nima Technology, Inc., Coventry, UK) was used to generate pressure–area isotherms of spread films of surfactant samples. The trough was equipped with a leak-proof Teflon-ribbon barrier and connected to a thermostat controlled water bath (Microbeam, Barcelona, Spain). The trough was thoroughly cleaned with chloroform/methanol (3:1), distilled water and double distilled water, as described elsewhere [18]. After cleaning, the trough was filled with 0.15 M NaCl, 5 mM Tris buffer, pH 7 and a set of 5 complete isotherms was performed at a speed of 89–90 cm^2/min to clear any surface contamination until a baseline of 0 mN/m surface pressure was recorded. Surfactant samples (suspended in 0.15 M NaCl solution) were thoroughly vortexed before each experiment and were added drop-by-drop and allowed to spread for 10 min to form a surface film. After compressing first to a target pressure of 10–20 mN/m, the film was allowed to equilibrate for 10 min before finally being compressed at a rate of 65 cm^2/min until the films reached their maximum surface pressures [19,20]. Isotherms were generated at 10 °C (the minimum temperature at which this Wilhelmy–Langmuir balance can be controlled), 25 °C and at 35–37 °C for both hibernating (n = 3–4) and summer-active (n = 3) samples. Calculations for the amount of surfactant added, area changes and surface pressure were based on the molecular weight of DPPC. The values for percent compressed area of samples in the balance were plotted against surface pressure.

2.3. Epifluorescence and atomic force microscopy

Films to be analysed by epifluorescence and atomic force microscopy (AFM) were transferred simultaneously onto solid supports following the procedure previously described to prepare Langmuir–Blodgett surfactant films [21]. Surfactant samples in 0.15 M NaCl were labelled with 1 mol% in dimethylsulphoxide (DMSO) aliquoted NBD-PC (1-palmitoyl-2-{12-((7-nitro-2,1,3-benzoxadiazol-4-yl)amino)dodecanoyl} phosphatidylcholine) and vortexed at 1350 rpm (5 min vortex and 10 min interval) for 1 h at 45 °C. Two separate sets of experiments were conducted as described in the following sections.

2.3.1. Epifluorescence and AFM images of Langmuir–Blodgett films transferred at 25 °C and constant target pressure

All samples (hibernating and summer-active, n = 3) were transferred at 25 °C for both epifluorescence and AFM. Following the cleaning procedure described above [18], the balance trough was filled with 0.15 M NaCl and 5 mM Tris buffer and maintained at a constant temperature of 25 °C. Glass slides for epifluorescence were washed with chloroform/methanol (2:1) and sonicated for 5 min. After the slides were completely air-dried, they were mounted onto the balance. Freshly cleaved mica, cut to a size of 1 cm^2 area was mounted onto the balance alongside the glass slide at the far end of the trough facing the barrier. Sufficient amounts of labelled surfactant material were added dropwise onto the aqueous surface until the films reached a static surface pressure of 4–6 mN/m. At this stage surfactant samples were allowed to spread for 10 min. The barrier was compressed at 25 cm^2/min to reach a target pressure of 35 mN/m at which point the spread monolayer was allowed to equilibrate for 10 min. This compression speed was selected to match the speed used to transfer efficiently continuously compressed films (see below). Finally, the film was transferred to both the glass slide facing the PS film in the Wilhelmy–Langmuir balance and the mica surface at a transfer rate of 5 mm/min. In order to prevent pressure reduction resulting from the transfer of the PS film, automatic compensation of area was employed [21].

2.3.2. Epifluorescence images of Langmuir–Blodgett films transferred at 10 and ~37 °C continuously with isotherms

Constantly varying surface pressure (COVASP) films were prepared as previously described [22], by transferring the interfacial film onto glass supports continuously whilst reaching different pressures, starting from low to high surface pressures, until they reached a plateau. After loading the surface of the balance with labelled samples, and following the spreading and equilibration steps described above, films were transferred onto glass slides for epifluorescence microscopy