Raised bile acid concentrations in SIDS lungs at necropsy

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Abstract

Previous studies of the abnormal physical properties of lung surfactant derived from infants experiencing prolonged expiratory apnoea, or who have died of sudden infant death syndrome (SIDS), have led to a search for the agent responsible. Bronchoalveolar lavage (BAL) has been performed upon 12 infants under 12 months at necropsy and the rinsings analysed for up to 26 bile acids using high performance liquid chromatography, which requires nanomolar quantities. They were also analysed for simultaneously retrieved phospholipid and proteolipid—a minor component of lung surfactant—as markers of lavage efficiency.

Total bile acid (TBA) was found to be higher in six SIDS cases, reaching a mean (SE) 8.54 (2.24) $\mu\text{mol/l}$ in the BAL fluid compared with 4.66 (1.47) $\mu\text{mol/l}$ in the six controls of similar age. When related to the concomitant surfactant yield, the TBA/proteolipid and TBA/phospholipid ratios both showed highly significant differences between index lungs and controls, providing another postmortem marker of SIDS with potential for development as a test of risk. Since the bile:phospholipid ratio determines whether phospholipase A_2 synthesises or hydrolyses phospholipid, the raised TBA/phospholipid ratio could be highly significant, causing this enzyme to function more like its role in the gut than in the lung.

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There can be few disorders for which so many or such diverse mechanisms have been proposed as the sudden infant death syndrome (SIDS). Although a brainstem abnormality or maturational delay to neuroregulation of cardio-respiratory control would appear the most compelling hypotheses, a review of postmortem findings in SIDS concludes that any such disorder would need to be 'very subtle'.¹ Moreover, as a result of studying infants experiencing recurrent cyanotic episodes, Southall *et al* conclude that these infants have essentially normal brainstems, arguing that the cause is more likely to be a 'mechanical defect', probably one influencing afferent neural feedback from the lungs to the brainstem.²

At resting volumes lung mechanics are dominated by the liquid-air interface—whether

this is continuous³ or not⁴—as witnessed by the reduction in lung recoil by 67–85% when this interface is eliminated by liquid filling.^{5–6} Since the mechanical effects of the interface are controlled by surfactant, there has been much interest in the surface active phospholipid harvested from the lungs of SIDS cases at necropsy. Both qualitative and quantitative deficiencies have been demonstrated,^{7–9} although it is difficult to ascertain whether these could actually lead to death.

One unexpected finding, which we reported,¹⁰ was the inversion of the relationship between surface tension (γ) and surface area (A) when surfactant harvested by bronchoalveolar lavage (BAL) was studied as a monolayer on a standard Langmuir trough widely used in pulmonary physiology.^{4–11} Instead of the $\gamma:A$ loops displaying normal hysteresis by cycling clockwise, those from infants who had experienced acute life threatening events (ALTEs) or sleep apnoea displayed either total inversion by cycling anticlockwise, or partial inversion to describe a 'figure of eight'. These findings were confirmed in a much larger study of 23 index cases and 26 controls which included two infants who later died of SIDS.¹²

In a subsequent double blind study of 55 infants under 24 months of age at necropsy,¹³ comprising 34 SIDS cases and 21 controls, hysteresis inversion of lavage samples on the Langmuir trough proved a highly significant ($p=10^{-4}$) postmortem marker. The sensitivity of this test (97%) far exceeded that for other markers reported, for example hypoplasia of the arcuate nucleus.¹⁴

Clearly there must be some agent that we are coextracting with the normal lipid components of lung surfactant, namely, phospholipid and proteolipid, which is responsible for hysteresis inversion in the index cases. Our efforts to identify the agent responsible were initially unsuccessful. Recently, however, we were evaluating the surface properties of a lavage sample from a neonate diagnosed with meconium aspiration in whom surfactant rescue with Exosurf had been instigated successfully when, to our surprise, this fluid displayed inversion of the $\gamma:A$ cycles similar to that seen in infants with ALTE or SIDS. Reasoning that one of the major components of meconium is bile salt, we added some deoxycholic acid to the aqueous hypophase of the Langmuir trough and were able to induce inversion of a monolayer of pure dipalmitoyl phosphatidylcholine which normally produces a large (fat) clockwise hysteresis loop. It was therefore decided to undertake a study to

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Table 1 Cause of death for controls

	Age (in weeks)*	Cause of death
1	40	Congenital heart disease
2	52	Bronchopneumonia
3	5	Congenital heart disease
4	2	Congenital heart disease
5	0.14	Congenital abnormality
6	7	Motor vehicle accident

* Mean age: 15.2 weeks.

determine whether there was a significant difference in bile salts between SIDS lungs and controls at necropsy.

Materials and methods

NECROPSIES

BAL was performed during necropsy on 12 infants (eight boys and four girls) of average age 3.1 months by inserting a tube directly through the larynx and instilling 5 ml of saline into a bronchus and retrieving as much as possible, usually 1.5–2.5 ml. This procedure was repeated once to obtain a second sample. Six of these infants (four boys and two girls) were determined by the coroner to have died from SIDS while six were controls. The causes of death in the six controls are listed in table 1.

Although great care was taken to follow the same procedure in all BAL procedures, the efficiency of harvesting alveolar contents varies between necropsies. Hence the BAL samples were analysed for lung surfactant, a known agent present on the alveolar surface in appreciable quantities,^{3,4,11} which could act as a marker to standardise the retrieval of bile salt. Another reason was the significance¹⁵ of the phospholipid to total bile acid (TBA) ratio in determining the action of the enzyme phospholipase A₂ present in type II alveolar cells.

ANALYSIS FOR SURFACTANT COMPONENTS

The two lavage samples obtained at necropsy were combined and elutriation performed with Folch solution (2:1 chloroform:methanol) to remove phospholipid, surfactant proteolipid, and most of the bile salt. After evaporation to dryness the phospholipid was determined as the quantity of phosphorus present by the standard method of Rouser *et al.*¹⁶ while proteolipid—as a minor component of pulmonary surfactant¹⁷—was determined by the standard method of Bohlen *et al.*¹⁸ The latter method was selected over many other means of estimating proteins because we found, in preliminary runs, that it gave results independent of the phospholipid content.

Phospholipid was determined by converting all phosphorus in chloroform soluble phospholipid to phosphate by oxidation with perchlorate. Phosphate was estimated colorimetrically with a phosphomolybdate indicator using a spectrophotometer (Shimadzu model UV-1201).

Surfactant proteins 'B' and 'C' are proteolipids¹⁷ whose combined content (proteolipid) can be determined as a second quantitative index of alveolar surfactant. A known weight of the Folch extract described above was dispersed in sodium phosphate at pH 8, adding fluorescamine (Sigma #F-9015) in acetone as

the indicator. The dispersion was measured on a fluorescence spectrophotometer (Shimadzu model RF-1501) at an emission wavelength of 475 nm with excitation at 365 nm. Calibration was performed using bovine serum albumin standards.

BILE SALT ANALYSIS

Samples from the residues of the Folch extraction were also sent to the National Institute of Occupational Health and Safety at Sydney University for analysis by high performance liquid chromatography (HPLC) according to the standard method described in detail by Wang and Stacey.¹⁹ Their method employs the Waters HPLC system with a Baseline 810 chromatography workstation (Waters Chromatography, Milford, MA, USA) set up with dual Model 501 pumps, a U6K injector, and a Blue Chip personal computer in conjunction with a fluorescence monitor (Shimadzu RF-535). This system is capable of identifying up to 26 bile acids (both free and conjugated) in the nanomolar range.

Results

BILE ACIDS

In all samples 12 bile acids were clearly identified in significant quantities, these including both conjugated and non-conjugated bile acids. The TBA is recorded in table 2, showing that TBA reached a mean (SE) of 8.54 (2.24) $\mu\text{mol/l}$ in fluid retrieved from SIDS lungs compared with 4.66 (1.47) $\mu\text{mol/l}$ for controls.

ANALYSIS FOR SURFACTANT COMPONENTS

Appreciable quantities of surfactant were recorded in all samples, the results of analysis for phospholipid and proteolipid being summarised in table 2. Also quoted for comparison purposes are the ratios of TBA to phospholipid and proteolipid respectively. It can be seen that the TBA/phospholipid ratio and the TBA/proteolipid ratio are significantly higher in the SIDS cases than in the controls.

STATISTICAL ANALYSIS

Using Student's *t* test, the higher TBA content of SIDS lungs relative to controls reached a moderate degree of significance and the same applies using non-parametric, two tailed statistics which are probably more appropriate to the type of population distribution under consideration here.

The ratios of TBA to surfactant, however, show a much more significant separation of index cases from controls. Using non-parametric statistics in the form of a ranking test (the U test²⁰) this gives a highly significant ($p < 0.02$) separation of TBA/proteolipid ratios for index cases compared with controls for the two sided test. The separation for the TBA/phospholipid ratio is also significant by this test with $p < 0.03$ for the two sided U test. These differences are depicted graphically in fig 1. In establishing the TBA/phospholipid ratio as a test of risk it would be necessary to determine and compare the range of each of these parameters.

Table 2 Phospholipid and proteolipid in BAL samples and their ratios to TBA

Units	TBA		Phospholipid (PL) $\mu\text{g/ml}$ (BAL)	Proteolipid (Pr) $\mu\text{g/ml}$ (BAL)	TBA/PL	TBA/Pr
	$\mu\text{mol/l}$ (BAL)	$\mu\text{g/ml}$ (BAL)				
Controls						
1	2.33	0.96	1336.0	322.0	0.0007	0.0030
2	2.27	0.95	1128.0	352.0	0.0008	0.0027
3	3.01	1.25	810.0	157.0	0.0015	0.0080
4	2.49	1.03	1018.0	179.0	0.0010	0.0058
5	11.12	4.62	406.0	63.0	0.0114	0.0733
6	6.73	2.79	262.0	82.0	0.0106	0.0340
Mean	4.66	1.93	826.7	192.5	0.0043	0.0211
SE	1.47	0.61	171.5	49.2	0.0021	0.0115
SIDS						
1	6.72	2.79	95.0	16.0	0.0294	0.1740
2	6.33	2.66	44.0	21.0	0.0605	0.1270
3	16.02	6.62	171.0	42.0	0.0387	0.1580
4	7.89	3.26	742.0	271.0	0.0044	0.0120
5	13.67	5.66	50.0	7.0	0.1130	0.8090
6	0.68	0.29	30.0	6.0	0.0097	0.0483
Mean	8.54	3.55	188.7	60.5	0.0426	0.2210
SE	2.24	0.93	112.6	42.4	0.0163	0.1200

Discussion

The quantities of phospholipid and proteolipid harvested by BAL in this study (table 2) are consistent with the yields reported in previous studies,¹² while the phospholipid/proteolipid ratios are also within the normal physiological ranges reported.²¹ Both components of surfactant (phospholipid and proteolipid) were harvested in lesser quantities in SIDS lungs than controls (see table 2) consistent with the findings of other groups for phospholipid,⁷⁻⁹ although our results do not reach statistical significance, perhaps attributable to the smaller numbers ($n=12$) involved in this study.

Against these consistencies in our harvest of surfactant by BAL, the appreciably higher yield of bile acids in SIDS cases, as demonstrated by both the TBA/proteolipid and TBA/phospholipid ratios in fig 1, becomes particularly interesting. However, before considering why bile salts might be directly or indirectly involved in SIDS, it is necessary to consider the source. Neither the pathologist performing the necropsy nor ourselves could find any overt evidence of aspiration in any of the lungs. However it could be argued that very little bile would need to reach the upper respiratory tract for it to elicit an obstructive reflex, just as bile is so effective in inducing pain and erosion in the oesophagus.²² Aspiration is an unlikely source of bile salt for the further reason that, if related to SIDS, it is more likely to occur in infants sleeping in the supine position,²³ thus

Figure 1 Depicts the differences in TBA recovered by BAL of lungs, at necropsy, of infants who have died of SIDS compared with controls of a similar age. Results are expressed as ratios to the major component of alveolar surfactant (phospholipid) and a minor component (proteolipid) to allow for any variation in efficiency of harvesting alveolar material by BAL. * Denotes a difference significant to 97% and ** a significance of 98%.

conflicting with experience that the incidence of SIDS is higher for the prone position.²⁴

A more likely source of bile salts would seem to be the circulation where serum bile acid concentrations are typically higher in infants at about 20–30 $\mu\text{mol/l}$ ²⁵ than in adults, especially over the age range at which most deaths occur from SIDS. Serum bile salt concentrations are raised by fasting and can rise several fold in infants, exceeding 100 $\mu\text{mol/l}$ in certain disease states.²⁶ Since unconjugated bilirubin increases the permeability of gall bladder epithelium, facilitating the uptake of bile by the circulation, it could be very pertinent that jaundice has been identified in 40% of SIDS victims at birth.²⁷

The association of bile salt with surfactant and its physical properties that led to this study is interesting for two reasons, the first being the chemical affinity of some bile acids for phosphatidylcholines which is the major component of phospholipid.²⁸ The second is the important role of phospholipase A₂ in the synthesis of surfactant in the lung.²⁹ In the gut, however, this enzyme hydrolyses long chain phosphatidylcholines (PC) to their lysophosphatidylcholines (LPC) as the initial step in breaking down ingested membranous PC(30), that is, it catalyses the reaction which is the reverse of that which it promotes in the lung. The critical parameter that determines the direction in which the reaction proceeds is the bile:phospholipid ratio,¹⁵ bile being described as an 'absolute requirement' for hydrolysis.³⁰ Hence the presence of abnormally high bile concentrations in the lung, as indicated by the high TBA/phospholipid ratios found in SIDS lungs (fig 1), could reverse the action of phospholipase A₂ in the lung, causing it to degrade PC to LPC. Thus one would predict a higher proportion of LPC in SIDS lungs—which is precisely what Morley *et al* have reported from their lung lavage studies.⁷ However interesting these associations may be, they do not lead to an obvious cause of death in SIDS.

One possible mechanism for the action of bile salts in the lung is the inversion of surface tension: surface area ($\gamma:A$) hysteresis imparted by LPC as found in the preliminary studies mentioned above. It may be fortuitous but this normal clockwise hysteresis in surface tension coincides with a similar clockwise hysteresis when plotted against surface area in the firing of inspiratory mechanoreceptors as monitored in the vagi.³¹ It is a moot point whether reversal of the $\gamma:A$ loop in the lung would lead to inversion of neural activity during the respiratory cycle and one which we are pursuing in animal models. If it does, then one could expect the brainstem to receive a distorted feedback from the lungs that might be so confusing as to stop respiration or lead to the events described by Southall *et al* in infants experiencing prolonged expiratory apnoea where, essentially, they fail to 'switch' from expiration to inspiration at the appropriate time.²

An alternative possibility relates to the putative loss of hypoxic drive derived from the carotid body. Erosion of the surface active phospholipid barrier believed to retain hydro-

gen ions (H^+) within the glomus cells³² could be effected by bile salts just as they are known to erode a similar barrier³³ excluding H^+ from the stomach wall in causing gastric ulcer.³⁴

Whatever the mechanism leading to death, bile acid concentrations would seem to offer another postmortem marker and an interesting avenue to pursue in elucidating the mechanism of SIDS, while it might provide a potential test of risk if reflected in TBA concentrations in blood. We are currently undertaking a study of TBA in blood obtained routinely by heel tap at birth to determine any correlation with the incidence of SIDS. A positive result could offer the basis for developing a prospective test of risk.

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