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Surface properties of sulfur- and ether-linked phosphonolipids with and without purified hydrophobic lung surfactant proteins

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Abstract

Two novel C16:0 sulfur-linked phosphonolipids (S-lipid and SO_2-lipid) and two ether-linked phosphonolipids (C16:0 DEPN-8 and C16:1 UnDEPN-8) were studied for surface behavior alone and in mixtures with purified bovine lung surfactant proteins (SP)-B and/or SP-C. Synthetic C16:0 phosphonolipids all had improved adsorption and film respreading compared to dipalmitoyl phosphatidylcholine, and SO_2-lipid and DEPN-8 reached maximum surface pressures of 72 mN/m (minimum surface tensions of <1 mN/m) in compressed films on the Wilhelmy balance (23 °C). Dispersions of DEPN-8 (0.5 mg/ml) and SO_2-lipid (2.5 mg/ml) also reached minimum surface tensions of <1 mN/m on a pulsating bubble surfactometer (37 °C, 20 cycles/min, 50% area compression). Synthetic lung surfactants containing DEPN-8 or SO_2-lipid + 0.75% SP-B + 0.75% SP-C had dynamic surface activity on the bubble equal to that of calf lung surfactant extract (CLSE). Surfactants containing DEPN-8 or SO_2-lipid plus 1.5% SP-B also had very high surface activity, but less than when both apoproteins were present together. Adding 10 wt.% of UnDEPN-8 to synthetic lung surfactants did not improve dynamic surface activity. Surfactants containing DEPN-8 or SO_2-lipid plus 0.75% SP-B/0.75% SP-C were chemically and biophysically resistant to phospholipase A_2 (PLA_2), while CLSE was severely inhibited by PLA_2. The high activity and inhibition resistance of synthetic surfactants containing DEPN-8 or SO_2-lipid plus SP-B/SP-C are promising for future applications in treating surfactant dysfunction in inflammatory lung injury.

Keywords: Lung surfactants; Surfactant therapy; Phosphonolipids; Lipid analogs; DEPN-8

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1. Introduction

Lung surfactant is a complex mixture of surface-active phospholipids and proteins that promotes normal respiration by lowering and varying surface tension at the liquid–air alveolar interface (Notter, 2000). A deficiency in lung surfactant in premature infants causes the neonatal respiratory distress syndrome (RDS), and surfactant dysfunction is an important contributor to clinical acute lung injury (ALI) and the acute respiratory distress syndrome (ARDS) (Notter, 2000; Wang et al., 2005). When endogenous surfactant is deficient or dysfunctional, it can be replaced by active exogenous substitutes. Therapy with active exogenous surfactants is life-saving in premature infants, and is currently being extended to pediatric and adult patients with ALI/ARDS (Chess et al., 2005; Notter, 2000; Willson et al., 2005). Optimizing surfactant therapy for ALI/ARDS is challenging, and requires exogenous surfactants with maximal surface activity plus the ability to resist inhibition from endogenous substances in injured, inflamed lungs. The most widely used current clinical exogenous surfactants are animal-derived rather than synthetic in nature (Chess et al., 2005; Notter, 2000). However, synthetic exogenous surfactants containing novel chemical components with specific structural and activity benefits are of significant interest.

In prior work, we have reported the synthesis of several phospholipase-resistant phosphonolipids that are structurally analogous to dipalmitoyl phosphatidylcholine (DPPC), the most prevalent glycerophospholipid in native lung surfactant (Liu et al., 1994a, 1994b, 1995; Turcotte et al., 1991, 1977; Wang et al., 2003). These studies have shown that DEPN-8, a synthetic diether analog, has better adsorption and spreading properties than DPPC while maintaining an ability to reduce surface tension to <1 mN/m in dynamically compressed interfacial films (Liu et al., 1994a; Turcotte et al., 1991; Wang et al., 2003). In addition, a synthetic exogenous surfactant containing DEPN-8 plus 1.5 wt.% of mixed (unseparated) bovine lung surfactant proteins (SP)-B/C was shown to have overall surface activity equivalent to calf lung surfactant extract (CLSE, the substance of the highly active clinical exogenous surfactant Infasurf) (Wang et al., 2003). This synthetic surfactant was resistant to inhibition by phospholipase A_2 (PLA2), while glycerophospholipids in CLSE were susceptible to cleavage by this enzyme (Wang et al., 2003).

In addition to carrying out further studies with DEPN-8, the current paper investigates the surface-active properties of new synthetic phosphonolipids alone and in combination with purified bovine hydrophobic SP-B and/or SP-C. New compounds include two disaturated C16:0 sulfur-linked phosphonolipids (S-lipid and SO_2-lipid), as well as a C16:1 unsaturated diether phosphonolipid (UnDEPN-8), all having the same choline N-headgroup as DEPN-8 and DPPC. Synthetic surfactants containing phosphonolipids plus pure SP-B, pure SP-C, or an equal weight of SP-B + SP-C are examined to assess synergy between the hydrophobic apoproteins. Biophysical measurements include surface pressure–area (π–A) isotherms and respreading in interfacial films on the Wilhelmy balance, adsorption from a stirred subphase, and overall dynamic surface activity on the physiologically relevant pulsating bubble surfactometer (37 °C, 20 cycles/min, 50% area compression). The ability of synthetic lung surfactants containing DEPN-8 or SO_2-lipid + 0.75% SP-B + 0.75% SP-C to resist inhibition from PLA_2 is also specifically examined.

2. Materials and methods

2.1. Synthetic lipids

DPPC (1,2-dipalmitoyl-*sn*-3-phosphocholine, >99% pure) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL). The four synthetic phosphonolipids studied (DEPN-8, S-lipid, SO_2-lipid, and UnDEPN-8) are shown schematically in Fig. 1. DEPN-8 [(±)-trimethyl(3-phosphonopropyl)ammonium, mono(2,3-bis(hexadecyloxy)propyl) ester] was made and purified as detailed previously (Wang et al., 2003). Details on the methods used in preparing S-lipid, SO_2-lipid, and UnDEPN-8 are given in Appendix A. In brief, 1-thioglycerol for the synthesis of S-lipid and SO_2-lipid was converted to the 1-*S*-hexadecyl-*rac*-thioglycerol by alkylation with hexadecyl bromide in alcoholic KOH (95%) (Chang et al., 2004). The primary hydroxyl group was selectively tritylated to produce 1-*S*-hexadecyl-3-*O*-trityl-*rac*-thioglycerol (93% yield), and the remaining hydroxyl group was alkylated with hexadecyl bromide to give 1-*S*-hexadecyl-2-*O*-

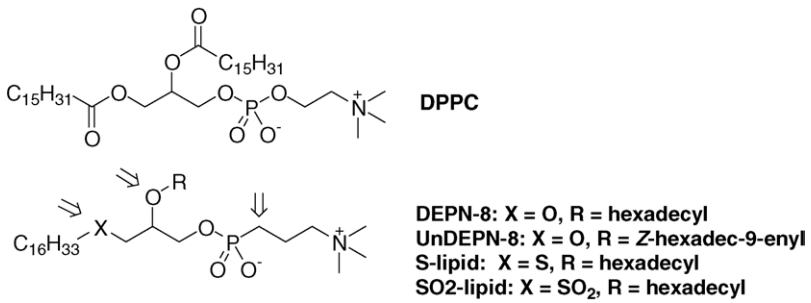

Fig. 1. Schematic diagram of the synthetic phosphonolipid compounds studied. Shown schematically are DEPN-8, S-lipid, SO_2-lipid, and UnDEPN-8. Arrows (⇒) indicate the position of functional group changes in relation to DPPC. The molecular changes at these locations impart structural resistance to phospholipases A_1, A_2, and D (Lin et al., 1997; Wang et al., 2003). In addition, DEPN-8 has been shown to be partially resistant to cleavage by phospholipase C (Lin et al., 1997).

hexadecyl-3-*O*-trityl-*rac*-thioglycerol (94% yield). The trityl group was cleaved in 95% yield with aqueous methanol to produce 1-*S*-hexadecyl-2-*O*-hexadecyl-*rac*-thioglycerol (Nali et al., 1986), which was oxidized to the corresponding sulfone 1-SO_2-hexadecyl-2-*O*-hexadecyl-*rac*-sulfonylglycerol prior to installation of the phosphono headgroup (Chang et al., 2004). Final isolation and recrystallization afforded S-lipid [(±)-trimethyl(3-phosphonopropyl)ammonium, mono(2-hexadecyloxy-3-hexadecylsulfanylpropyl)ester, 48% yield] and SO_2-lipid [(±)-trimethyl(3-phosphonopropyl)ammonium, mono(2-hexadecyloxy-3-hexadecylsulfonylpropyl)ester, 59% yield]. UnDEPN-8 [(±)-trimethyl(3-phosphonopropyl) ammonium, mono(2-hexadec-9-enyloxy-3-hexadecyloxypropyl)ester] was prepared by alkylation of 1-*O*-hexadecyl-3-*O*-trityl-*rac*-glycerol with commercially available *Z*-hexadec-9-enyl mesylate under KOH/DMSO conditions (Johnstone and Rose, 1979) as used in preparing DEPN-8 (Wang et al., 2003). Double bond geometry remained intact over all manipulations based on ^{1}H and ^{13}C NMR spectroscopy, and the final waxy UnDEPN-8 compound was obtained at a yield of 51%. All compounds had single spots on thin layer chromatography with a solvent system of 30:9:25:7:25 (v/v) chloroform:methanol:2-propanol:water:triethylamine (Touchstone et al., 1980).

2.2. CLSE and hydrophobic lung surfactant proteins (SP-B, SP-C)

CLSE (a gift from ONY Inc., Amherst, NY) was prepared by chloroform:methanol extraction of large aggregate surfactant lavaged from intact calf lungs as reported previously by Notter and co-workers (e.g., Hall et al., 1992b, 1994; Wang et al., 1995). SP-B and SP-C were purified from CLSE by isocratic normal-phase liquid chromatography (LC) on Silica C8 (60 μm mesh, J.T. Baker, Phillipsburg, NJ) (Baatz et al., 2001). All columns, tubing, and fittings were constructed of organic-resistant materials to avoid degradation of plastics (Spectrum Medical Industries Inc., Los Angeles, CA). Approximately 700 mg of CLSE in 7:1 MeOH:chloroform + 5% 0.1N HCl was concentrated by evaporation under nitrogen to 4 ml volume and applied to a 450 ml LC column pre-equilibrated with the same solvent. Added CLSE was allowed to completely absorb to the column prior to elution with additional 7:1 MeOH:chloroform + 5% 0.1N HCl at a flow rate of 0.4 ml/min. Eluted components were assessed by a UV detector (Pharmacia Biotech, Uppsala, Sweden) at 254 nm. The phospholipid content of eluted fractions was determined by the methodology of Shin et al. (Shin, 1962) with the aid of a Fiske-Subbarow phosphate analysis kit (Sigma Chemical Co., St. Louis, MO). Chloroform and methanol (HPCL grade) and HCl (ACS grade) were from Baxter Scientific (Chicago, IL). Final isolates of pure bovine SP-B and SP-C had no detectable contaminating proteins based on SDS polyacrylamide gel electrophoresis (SDS-PAGE) and N-terminal amino acid analysis (Baatz et al., 2001).

2.3. Phospholipase A2 (PLA2) studies

PLA_2 (Sigma Chemical, St. Louis, MO) was suspended in 5 mM Tris(hydroxymethylaminomethane)

buffer containing 5 mM $CaCl_2$ (pH 7.4), and was incubated with surfactants dispersed in the same solvent for 30 min at 37 °C (final enzyme concentration was 0.1 units/ml) (Enhorning et al., 1992; Holm et al., 1991). After incubation, surfactant–PLA_2 mixtures were examined for surface activity on the pulsating bubble (see below). In addition, aliquots of PLA_2-incubated surfactants containing S-lipid and SO_2-lipid were also examined for chemical degradation by thin layer chromatography (Touchstone et al., 1980). Chemical degradation was assessed by scraping and analyzing chromatographic bands using the phosphate assay of Ames (1966).

2.4. Wilhelmy balance studies on spread interfacial films

Surface pressure–area (π–A) isotherms were measured on a custom-designed Wilhelmy surface balance with a Teflon trough and continuous ribbon barrier to minimize film leakage as described previously (Tabak and Notter, 1977). Surfactant mixtures were dissolved in 9:1 (v/v) hexane–ethanol and spread dropwise from a syringe at the air–water interface of a saline subphase (1.5 mM $CaCl_2$, 150 mM NaCl). Surface pressure (the magnitude by which surface tension was lowered relative to the pure subphase) was determined from the force on a hanging sandblasted platinum slide dipped into the interface, and film compression was initiated after a 10 min pause for solvent evaporation. Two types of films were investigated: (1) monolayers ("lift-off" films) spread to a dilute initial surface concentration of 120–150 $Å^2$/molecule and cycled at 10 min/cycle; (2) "surface excess" films spread to 15 $Å^2$/molecule and cycled at a rate of 5 min/cycle. Studies of surface excess films are of particular interest for lung surfactants because they emphasize respreading behavior during continuous cycling in the collapse regime where the highest surface pressures (lowest surface tensions) are reached. The compression ratio of the balance was 4.35:1, and temperature was 23 ± 1 °C. Dynamic respreading in surface excess films was defined from calculated π–A isotherm areas between compressions 2/1 and 7/1 as reported previously (Notter, 2000; Wang et al., 1995). These isotherm areas (arbitrary units) provide a measure of surfactant material ejected from the interface during compression that re-incorporates effectively back into the film during expansion (an area of zero indicates complete respreading between the designated cycles, and larger areas indicate poorer respreading) (Notter, 2000; Wang et al., 1995).

2.5. Adsorption apparatus

Adsorption studies were done at 37 ± 0.5 °C in a Teflon dish containing a 35 ml subphase of 150 mM NaCl + 1.5 mM $CaCl_2$ (Notter et al., 1982, 1983). A Teflon-coated magnetic bar was used to gently stir the subphase to minimize diffusion resistance. Adsorption experiments were initiated at time zero by injecting 2.5 mg of phosphonolipid dispersed in 5 ml of subphase by probe sonication on ice into the 35 ml stirred subphase (final surfactant concentration was 0.0625 mg/ml in 40 ml of total subphase). Adsorption surface pressure was followed as a function of time from the force on a partially submerged sandblasted platinum Wilhelmy slide (Notter et al., 1982, 1983).

2.6. Pulsating bubble surfactometer

The dynamic surface tension lowering ability of surfactant dispersions was measured at a cycling rate of 20 cycles/min at 37 ± 0.5 °C on a pulsating bubble surfactometer (General Transco, Largo, FL). This instrument, based on the original design of Enhorning (1977), gives a physiologically relevant assessment of overall surface activity that includes a combination of dynamic film behavior and adsorption at physical conditions similar to those in the pulmonary alveoli. An air bubble, communicating with ambient air, was formed in 40 μl of dispersed surfactant held in a sample chamber mounted on a precision pulsator unit. The bubble was pulsated between maximum and minimum radii of 0.55 and 0.4 mm (50% area compression), and surface tension at minimum bubble radius (minimum surface tension) was calculated as a function of time of pulsation from the pressure drop across the bubble interface using the Laplace equation (Enhorning, 1977; Hall et al., 1993; Notter, 2000). Surfactants were dispersed in 1.5 mM $CaCl_2$ + 150 mM NaCl by probe sonication on ice (three 15 s bursts at 40 W power) at concentrations of 0.5 or 2.5 mg phosphonolipid/ml.

3. Results

Representative π–A isotherms for solvent-spread films of S-lipid, SO_2-lipid, DEPN-8, UnDEPN-8, and

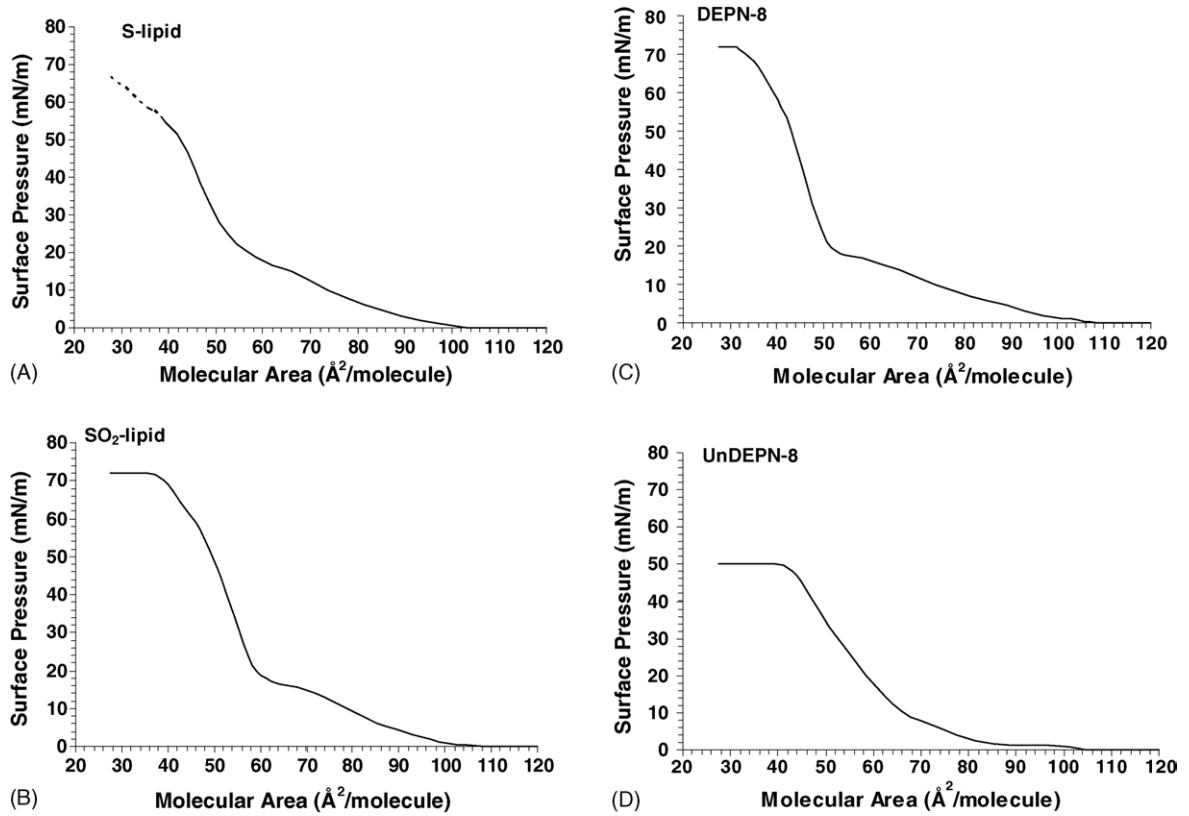

Fig. 2. Surface pressure-area isotherms for spread monolayers of phosphonolipids in a Wilhelmy balance. (Panel A) S-lipid; (Panel B) SO_2-lipid; (Panel C) DEPN-8; (Panel D) UnDEPN-8. Monolayers were spread in hexane–ethanol (9:1, v/v) to 120–150 $Å^2$/molecule and compressed at 10 min/cycle at 23 °C. Isotherms shown are representative first compressions from four to five closely reproduced experiments for each compound. The isotherm for S-lipid is dotted above a surface pressure of 51 mN/m, where a change to lower compressibility occurred. Lift-off areas (mean ± S.E.M.) for the complete isotherm groups for each compound are in Table 1.

DPPC are shown in Fig. 2 (monolayers) and Fig. 3 (surface excess films). Average monolayer lift-off areas (the point where non-zero surface pressures were initially apparent during compression), as well as maximum surface pressures and respreading ratios from surface excess films, are given in Table 1. Monolayer lift-off areas were similar for all phosphonolipids (Table 1), and monolayers and surface excess films of SO_2-lipid, DEPN-8, and DPPC reached maximum surface pressures of 72 mN/m (minimum surface tensions <1 mN/m) during compression (Figs. 2 and 3). Monolayers of S-lipid had lower maximum pressures that continued to increase after compression past collapse based on the limiting area of the hydrocarbon chains (36 $Å^2$/molecule) (Fig. 2A). Surface excess films of S-lipid reached maximum surface pressures of 72 mN/m on cycles 4–7, but had lower maximum pressures of 51–56 mN/m on cycles 1–3 (Fig. 3, Table 1). Monolayer and surface excess films of UnDEPN-8 had the lowest maximum surface pressures (∼50 mN/m), consistent with the fluid unsaturated C16:1 chain present in this compound. S-lipid had the best respreading of any compound studied, and all phosphonolipids had greatly improved respreading compared to DPPC (Fig. 3, Table 1). Both sulfur-containing phosphonolipids also had greater respreading than DEPN-8 (Table 1).

In addition to studying solvent-spread interfacial films in the Wilhelmy balance, the adsorption and dynamic surface activity of phosphonolipids were also investigated following dispersal in the aqueous phase.

As pure compounds, all phosphonolipids had improved adsorption compared to DPPC, with overall adsorption facility ordered as S-lipid ~ SO_2-lipid > UnDEPN-8 > DEPN-8 > DPPC (Fig. 4). In binary mixtures, 9:1 DEPN-8:UnDEPN-8 had increased adsorption compared to DEPN-8 alone, while 9:1 SO_2-lipid:UnDEPN-8 had reduced adsorption compared to SO_2-lipid alone (Fig. 5). In pulsating bubble experiments, DEPN-8

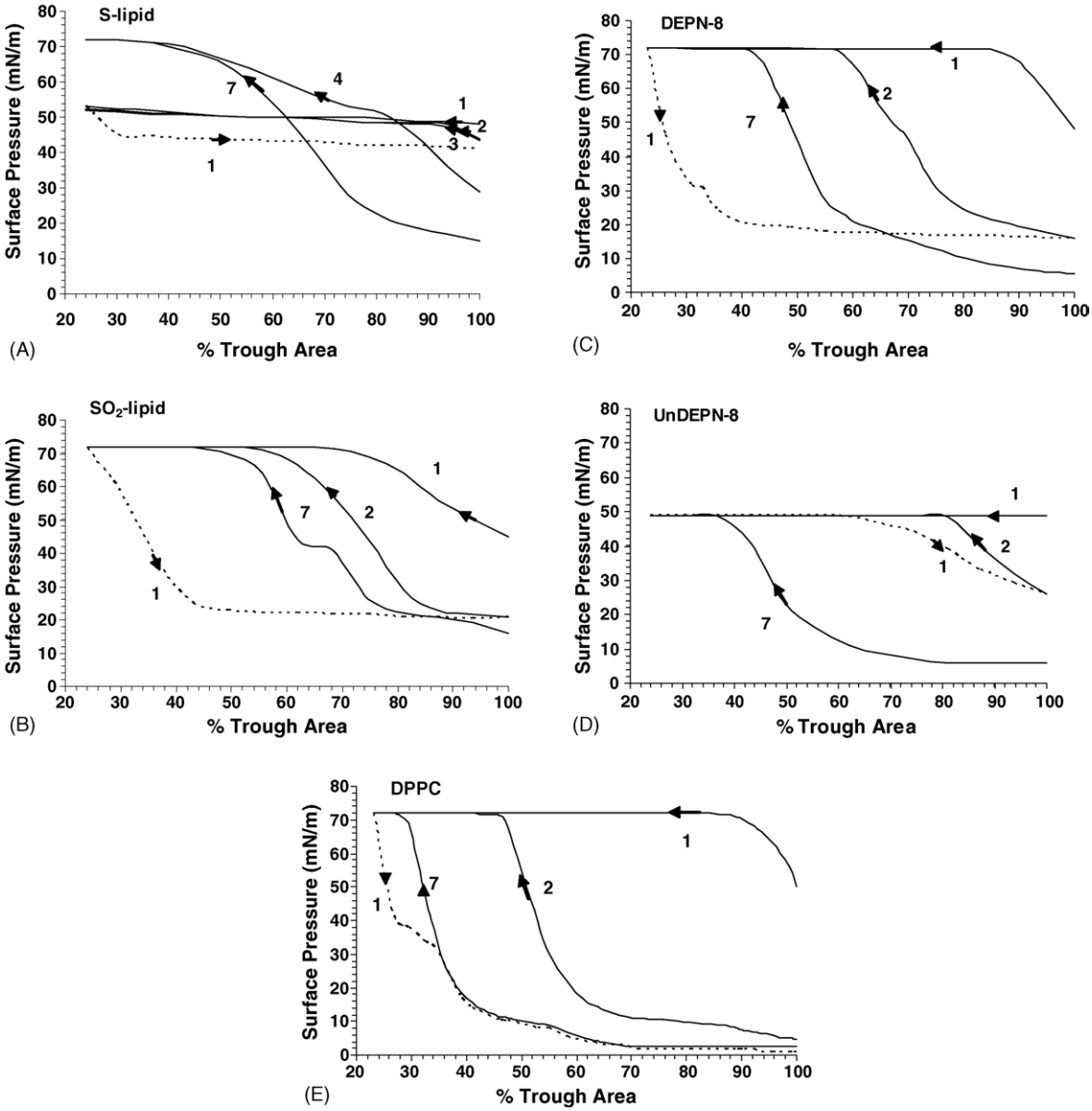

Fig. 3. Surface excess films of phosphonolipids compared to DPPC. (Panel A) S-lipid; (Panel B) SO_2-lipid; (Panel C) DEPN-8; (Panel D) UnDEPN-8; (Panel E) DPPC. Surface excess films were spread to 15 $Å^2$/molecule and compressed at 5 min/cycle at 23 °C. Isotherms shown are representative of three to five closely reproduced experiments per compound, and include compression curves 1, 2, and 7 plus expansion curve 1. In addition, compressions 3 and 4 are shown for S-lipid, which did not reach 72 mN/m until cycle 4. Table 1 gives maximum surface pressures and respreading areas (mean ± S.E.M.) for the complete sets of isotherms obtained for each compound.

Table 1
Respreading areas and maximum surface pressures for spread interfacial films of synthetic phosphonolipids compared to DPPC on a Wilhelmy balance

Compound	Dynamic respreading based on isotherm areas between compressions		Maximum surface pressure (mN/m)	Lift-off area (Å^2/molecule)
	Cycle 2/1	Cycle 7/1		
DPPC	28.3 ± 0.3	48 ± 0.3	72	104 ± 1
DEPN-8	19.5 ± 1.0	33.1 ± 1.5	72	105 ± 1
SO_2-lipid	14.3 ± 0.7	21.6 ± 0.4	72	102 ± 1
S-lipid	0.2 ± 0	–	72 (≥cycle 4)	101 ± 1
UnDEPN-8	3.1 ± 0.3	26.6 ± 0.4	50	103 ± 2

Data are mean ± S.E.M. for $n = 4$–6. Lift-off areas are for monolayers initially spread to 120–150 Å^2/molecule and compressed at 10 min/cycle at 23 °C. Respreading areas and maximum surface pressures are for surface excess films spread to 15 Å^2/molecule (5 min/cycle, 4:35 to 1 compression, 23 °C). Larger respreading areas (arbitrary units) indicate less respreading. Maximum surface pressures for S-lipid were significantly less than 72 mN/m for cycles 1–3 (51–56 mN/m). Data for DPPC are from Wang et al. (2002, 2003).

had the greatest overall dynamic surface activity of the phosphonolipids studied, reaching minimum surface tensions <1 mN/m at both 0.5 and 2.5 mg/ml (Tables 2 and 3). Dispersions of SO_2-lipid also reached minimum surface tensions of <1 mN/m on the bubble at 2.5 mg/ml (Table 3), but had minimum surface tension values of only 14.2 ± 0.3 mN/m after 20 min of pulsation at 0.5 mg/ml (Table 2). S-lipid reached minimum surface tensions of only 25.4 ± 0.2 mN/m (0.5 mg/ml) and 13.3 ± 0.3 mN/m (2.5 mg/ml) after 20 min of pulsation on the bubble apparatus, and UnDEPN-8 had the worst dynamic surface tension lowering ability as a pure component (Tables 2 and 3).

In added studies related to synthetic exogenous surfactant development, phosphonolipids (DEPN-8, SO_2-lipid, and S-lipid) were examined for activity on the bubble apparatus when combined with purified bovine SP-B and/or SP-C. Synthetic surfactants containing DEPN-8 or SO_2-lipid plus either 1.5% SP-B or 0.75% SP-B + 0.75% SP-C had very high activity, rapidly reducing surface tension to <1 mN/m on the pulsating bubble (Tables 2 and 3). SP-C had significantly less activity than SP-B when present individually with

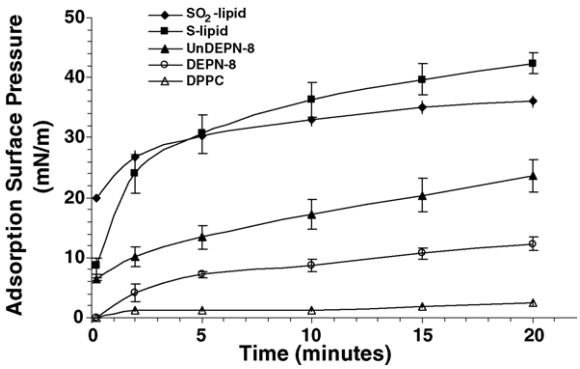

Fig. 4. Surface pressure-time adsorption isotherms for aqueous dispersions of synthetic phosphonolipids. Phosphonolipids in 150 mM NaCl + 1.5 mM $CaCl_2$ were injected at time 0 into a stirred subphase of the same solvent (final phosphonolipid concentration was 0.0625 mg/ml), and surface pressure was measured as a function of time with a hanging Wilhelmy slide at 37 °C (Section 2). Adsorption curve for DPPC is from Wang et al. (2002, 2003). Data are mean ± S.E.M. for $n = 3$–5.

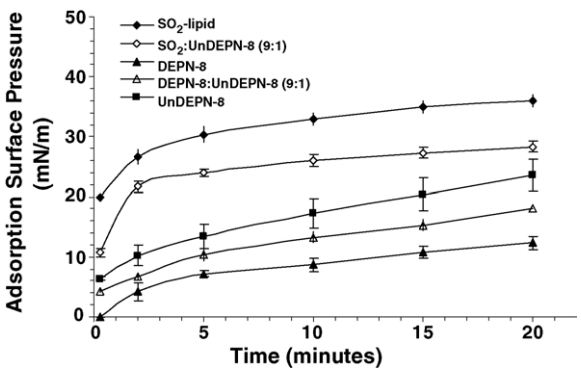

Fig. 5. Adsorption of binary mixtures of phosphonolipids to the air–water interface. Surfactants were injected at time 0 into a stirred subphase as in the legend to Fig. 4 (final lipid concentration was 0.0625 mg/ml). Binary phosphonolipid mixtures contained 90 wt.% DEPN-8 or SO_2-lipid plus 10 wt.% UnDEPN-8. Data are mean ± S.E.M. for $n = 3$–5.

Table 2
Dynamic surface tension lowering of synthetic phosphonolipids with and without hydrophobic surfactant proteins at low concentration (0.5 mg lipid/ml)

Surfactant mixture	Dynamic surface tension (mN/m) at minimum bubble radius at							
	0.25 min	0.5 min	1 min	2 min	5 min	10 min	15 min	20 min
UnDEPN-8	57.8 ± 2.8	50.9 ± 1.9	27.5 ± 0.4	25.8 ± 0.4	25.3 ± 0.1	25.5 ± 0.2	25.4 ± 0.4	25.1 ± 0.1
S-lipid	48.2 ± 1.1	44.5 ± 1.2	41.7 ± 1.1	35.8 ± 0.6	30.6 ± 0.5	28.1 ± 0.3	25.7 ± 0.3	25.4 ± 0.2
SO_2-lipid	20.0 ± 0.3	19.7 ± 0.4	19.4 ± 0.5	18.7 ± 0.6	16.6 ± 0.5	15.3 ± 0.3	14.2 ± 0.3	14.2 ± 0.3
DEPN-8	38.0 ± 1.2	26.7 ± 3.4	22.2 ± 2.5	17.7 ± 1.8	12.0 ± 1.8	8.6 ± 1.0	4.7 ± 1.0	< 1
CLSE	17.6 ± 0.4	14.8 ± 1.4	10.9 ± 2.2	9.2 ± 2.1	6.8 ± 2.0	< 1		
S-lipid + 1.5% SP-B	36 ± 1.1	33.6 ± 1.2	30.1 ± 1.1	25.8 ± 2.1	22.9 ± 1.0	20.7 ± 0.4	18.6 ± 0.4	17.6 ± 0.2
S-lipid + 1.5% SP-C	43.8 ± 1.6	40.5 ± 2.0	36.6 ± 2.1	31.8 ± 1.7	26.9 ± 0.7	24.1 ± 0.2	23.5 ± 0.1	23.3 ± 0.0
S-lipid + 0.75% SP-B + 0.75% SP-C	22.8 ± 1.5	20.7 ± 1.3	18.9 ± 0.9	16.7 ± 0.9	15.1 ± 0.5	13.6 ± 0.4	11.4 ± 0.3	10.3 ± 0.4
SO_2-lipid + 1.5% SP-B	19.1 ± 1.0	18.4 ± 1.2	16 ± 0.3	11.6 ± 1.6	9.0 ± 1.0	6.9 ± 0.7	< 1	
SO_2-lipid + 1.5% SP-C	18.5 ± 0.7	17 ± 0.7	15.7 ± 0.5	14.8 ± 0.2	14.1 ± 0.3	13.1 ± 0.3	10.6 ± 0.4	8.8 ± 0.2
SO_2-lipid + 0.75% SP-B + 0.75% SP-C	7.8 ± 2.6	4.1 ± 2.1	1.8 ± 1.5	< 1				
DEPN-8 + 1.5% SP-B	19.6 ± 1.0	17.9 ± 0.9	14.7 ± 0.3	10.7 ± 0.8	4.9 ± 0.2	< 1		
DEPN-8 + 1.5% SP-C	25.6 ± 1.5	23.9 ± 1.1	22.4 ± 1.2	17.3 ± 0.6	11.5 ± 0.3	6.1 ± 0.6	1.3 ± 0.5	< 1
DEPN-8 + 0.75% SP-B + 0.75% SP-C	10.9 ± 0.8	8.3 ± 1.2	5.7 ± 2.0	3.1 ± 1.6	1.2 ± 1.2	< 1		

Data are mean ± S.E.M. for $n = 4$–6. Surface tension at minimum radius (minimum surface tension) is on a bubble surfactometer (37 °C, 20 cycles/min, 50% area compression, 0.5 mg phospholipid/ml in 150 mM NaCl + 1.5 mM $CaCl_2$).

Table 3
Dynamic surface tension lowering of synthetic phosphonolipids with and without hydrophobic surfactant proteins at high concentration (2.5 mg lipid/ml)

Surfactant mixture	Dynamic surface tension (mN/m) at minimum bubble radius at							
	0.25 min	0.5 min	1 min	2 min	5 min	10 min	15 min	20 min
UnDEPN-8	35.2 ± 5.1	25.5 ± 0.4	24.9 ± 0.5	24.7 ± 0.5	24.1 ± 0.3	23.8 ± 0.1	24.0 ± 0.4	23.8 ± 0.2
S-lipid	41.7 ± 1.2	37.8 ± 1.0	32.8 ± 1.4	24.6 ± 1.6	19.6 ± 0.9	16.9 ± 0.5	14.5 ± 0.2	13.3 ± 0.3
SO_2-lipid	21.0 ± 0.4	20.0 ± 0.6	17.6 ± 0.9	15.3 ± 1.3	11.3 ± 1.4	6.3 ± 1.4	2.8 ± 1.4	< 1
DEPN-8	31.7 ± 1.6	27.4 ± 1.9	21.2 ± 1.6	14.0 ± 1.5	5.6 ± 1.3	1.9 ± 1.1	< 1	
CLSE	4.5 ± 2.0	< 1						
S-lipid + 1.5% SP-B	22.6 ± 0.7	19.3 ± 0.1	17.0 ± 0.8	13.4 ± 1.0	12.4 ± 1.0	11.1 ± 0.8	10.9 ± 0.7	9.7 ± 0.2
S-lipid + 1.5% SP-C	36.2 ± 1.2	29.2 ± 0.6	22.7 ± 0.9	18.8 ± 0.9	16.1 ± 0.5	12.3 ± 0.4	11.8 ± 0.2	11.6 ± 0.2
S-lipid + 0.75% SP-B + 0.75% SP-C	21.8 ± 1.2	19.1 ± 0.8	17.5 ± 0.8	14.2 ± 0.9	11.7 ± 0.8	10.7 ± 0.2	10.2 ± 0.3	9.2 ± 0.1
SO_2-lipid + 1.5% SP-B	17.7 ± 1	13.4 ± 1.0	7.8 ± 0.5	4.2 ± 0.6	< 1			
SO_2-lipid + 1.5% SP-C	19.6 ± 0.4	17.7 ± 0.2	14.2 ± 0.5	7.2 ± 1.1	1.9 ± 0.8	< 1		
SO_2-lipid + 0.75% SP-B + 0.75% SP-C	4.4 ± 2.1	3.0 ± 1.4	1.9 ± 1.0	< 1				
DEPN-8 + 1.5% SP-B	18.5 ± 0.3	12.4 ± 1.5	6.8 ± 2.0	1.6 ± 1.0	< 1			
DEPN-8 + 1.5% SP-C	29.3 ± 4.0	23.0 ± 2.4	18.5 ± 1.6	13.4 ± 1.0	5.1 ± 0.5	< 1		
DEPN-8 + 0.75% SP-B + 0.75% SP-C	10.8 ± 1.5	7.8 ± 1.2	5.6 ± 1.0	1.8 ± 0.6	< 1			

Data are mean ± S.E.M. for $n = 4$–6. Surface tension at minimum radius (minimum surface tension) is on a bubble surfactometer (37 °C, 20 cycles/min, 50% area compression, 2.5 mg phospholipid/ml in 150 mM NaCl + 1.5 mM $CaCl_2$).

Table 4
Dynamic surface tension lowering of synthetic surfactants containing 9:1 DEPN-8:UnDEPN-8 or 9:1 SO_2-lipid:UnDEPN-8 plus hydrophobic surfactant apoproteins

Surfactant mixture	Surface tension (mN/m) at minimum bubble radius at							
	0.25 min	0.5 min	1 min	2 min	5 min	10 min	15 min	20 min
9:1 DEPN-8:UnDEPN-8 + 0.75% SP-B + 0.75% SP-C (0.5 mg/ml)	27.8 ± 1.3	23.0 ± 0.6	20.7 ± 0.5	18.4 ± 0.9	15.2 ± 1.4	9.3 ± 0.9	6.8 ± 1.2	3.1 ± 0.6
9:1 DEPN-8:UnDEPN-8 + 0.75% SP-B + 0.75% SP-C (2.5 mg/ml)	25.2 ± 3.4	18.0 ± 2.0	15.9 ± 2.0	10.5 ± 1.8	<1			
9:1 SO_2-lipid:UnDEPN-8 + 0.75% SP-B + 0.75% SP-C (0.5 mg/ml)	27.4 ± 1.4	21.1 ± 1.2	17.0 ± 0.9	13.9 ± 0.6	11.3 ± 0.6	6.6 ± 1.1	3.3 ± 0.6	<1
9:1 SO_2-lipid:UnDEPN-8 + 0.75% SP-B + 0.75% SP-C (2.5 mg/ml)	25.7 ± 0.5	20.9 ± 0.7	15.1 ± 0.4	7.9 ± 0.1	<1			

Data are mean ± S.E.M. for $n = 4$–6. Surface tension at minimum radius (minimum surface tension) is shown as a function of time of pulsation on a bubble surfactometer (37 °C, 20 cycles/min, 50% area compression, 0.5 or 2.5 mg phosphonolipid/ml). The activities of comparable surfactants containing DEPN-8 or SO_2-lipid without 10% UnDEPN-8 are given in Table 2 (0.5 mg/ml) and Table 3 (2.5 mg/ml).

DEPN-8 or SO_2-lipid in synthetic surfactants. However, overall dynamic surface activity was greatest for surfactants that contained 0.75% SP-B + 0.75% SP-C (Tables 2 and 3), indicating possible synergy between these hydrophobic apoproteins that was most pronounced at a low surfactant concentration of 0.5 mg/ml. Addition of UnDEPN-8 at 10 wt.% did not improve the surface activity of synthetic surfactants containing DEPN-8 or SO_2-lipid + 0.75% SP-B + 0.75% SP-C (Table 4 compared to Tables 2 and 3). In fact, addition of 10% UnDEPN-8 had a negative rather than positive effect on overall dynamic surface activity for the mixtures studied.

A final set of experiments examined the ability of synthetic surfactants to resist inhibition by PLA_2. When incubated with 0.1 units/ml of PLA_2 for 30 min, synthetic surfactants containing S-lipid or SO_2-lipid + 0.75% SP-B + 0.75% SP-C exhibited no chemical degradation based on phosphate analysis of thin layer chromatographic bands (Table 5). Surfactants containing DEPN-8 + 1.5% of mixed bovine SP-B/C have previously been shown to be resistant chemically to PLA_2 (Wang et al., 2003), while glycerophospholipids in CLSE are highly susceptible to PLA_2-induced degradation (Table 5). Consistent with the structural resistance of phosphonolipids to degradation by PLA_2, synthetic surfactants containing DEPN-8 or SO_2-lipid + 0.75% SP-B + 0.75% SP-C maintained high dynamic surface activity in the presence of this lytic enzyme (Fig. 6). However, the surface activity of CLSE on the bubble apparatus was severely impaired by the presence of PLA_2 (Fig. 6).

4. Discussion

This study has examined the surface-active biophysics of several novel phosphonolipids as pure compounds and in synthetic lung surfactants containing purified bovine hydrophobic SP-B and/or SP-C. All of the disaturated C16:0 phosphonolipids studied (SO_2-lipid, DEPN-8, and S-lipid) were synthesized to have structural analogy to DPPC, the most prevalent glycerophospholipid in mammalian lung surfactant (Fig. 1). However, modified structural linkages and groups (ether, sulfur, and phosphono) in analog compounds were by design resistant to cleavage by biological phospholipases (Lin et al., 1997; Wang et al., 2003). These

Table 5
Lack of effect of phospholipase A_2 (PLA_2) in chemically degrading synthetic phosphonolipids in exogenous surfactants compared to glycerophospholipids in calf lung surfactant extract (CLSE)

Phospholipid (phosphonolipid) class	CLSE	CLSE + PLA_2	DEPN-8 + 1.5% SP-B/C	DEPN-8 + 1.5% SP-B/C + PLA_2	SO_2-lipid + 0.75% SP-B + 0.75% SP-C	SO_2-lipid + 0.75% SP-B + 0.75% SP-C + PLA_2	S-lipid + 0.75% SP-B + 0.75% SP-C	S-lipid + 0.75% SP-B + 0.75% SP-C + PLA_2
Lysophosphatidylcholine	0.4 ± 0.2	29.5 ± 2.4						
Sphingomyelin	1.0 ± 0.2	1.2 ± 0.5						
Phosphatidylcholine	84.4 ± 0.4	55.1 ± 3.2	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Phosphatidylinositol	4.0 ± 0.6	3.8 ± 0.7						
Phosphatidylethanolamine	3.7 ± 0.7	3.8 ± 1.0						
Phosphatidylglycerol	4.7 ± 0.3	4.1 ± 0.6						
Residue	1.8 ± 0.2	2.5 ± 0.2						

Data are mean ± S.E.M. for $n = 3$. Tabulated data for CLSE and DEPN-8 are from a previous study by Wang et al. (2003). Surfactants were incubated with PLA_2 (0.1 units/ml) for 30 min at 37 °C, and degradation was assessed by measuring lipid classes in weight percent based on phosphate analysis of thin layer chromatographic bands.

structural modifications also influence molecular packing and interactions relative to glycerophospholipids, and thus affect surface tension lowering, adsorption, and film spreading behavior. All of the C16:0 phosphonolipid analogs studied here had surface properties that differed significantly from those of DPPC. In addition, SO_2-lipid and S-lipid had substantial differences in adsorption, spreading, and dynamic surface tension lowering compared to the diether phosphonolipid DEPN-8 (Figs. 2–4, Tables 1–3). As a pure compound, DEPN-8 had the greatest overall dynamic surface activity of any phosphonolipid investigated, followed by SO_2-lipid (Tables 2 and 3). DEPN-8 and SO_2-lipid had even greater activity when combined with purified bovine SP-B or SP-B/SP-C in synthetic lung surfactants (Tables 2 and 3, Fig. 6).

Our prior research has investigated the behavior of DEPN-8 in surface films and aqueous dispersions (Liu et al., 1994a, 1994b, 1995; Skita et al., 1995; Turcotte et al., 1991, 1977; Wang et al., 2003). DEPN-8 has a gel to liquid–crystal transition temperature (T_c) of 45 °C compared to 41 °C for DPPC (Liu et al., 1995; Skita et al., 1995), and forms tightly packed interfacial films able to reduce surface tension to <1 mN/m dur-

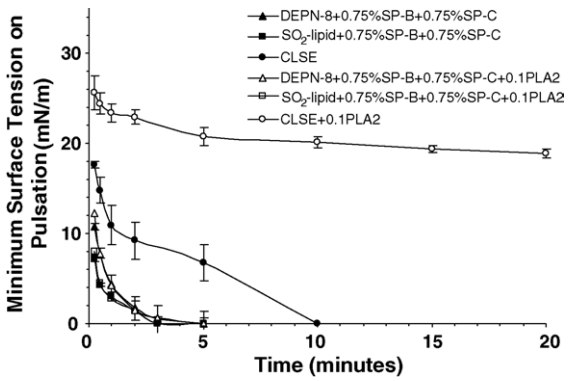

Fig. 6. Effects of PLA_2 on the dynamic surface activity of synthetic surfactants containing SO_2-lipid or DEPN-8 + 0.75% SP-B + 0.75% SP-C. Surface tension at minimum bubble radius was measured as a function of time of pulsation on a bubble surfactometer (37 °C, 20 cycles/min, 50% area compression, 0.5 mg phosphonolipid/ml) for dispersions of SO_2-lipid or DEPN-8 combined with 1.5 wt.% of purified bovine hydrophobic apoproteins (0.75% SP-B + 0.75% SP-C) with and without PLA_2 (0.1 units/ml). Also shown for comparison is the activity of calf lung surfactant extract (CLSE) in the presence and absence of PLA_2. Data are mean ± S.E.M. for $n = 6$–8.

ing dynamic compression (Liu et al., 1994a, 1994b; Turcotte et al., 1991). DEPN-8 has also been shown to have significantly greater adsorption and film respreading than DPPC (Liu et al., 1994a, 1994b; Turcotte et al., 1991). Ether linkages between the fatty chains and glycerol backbone in DEPN-8 have greater flexibility than ester linkages, and contribute to the ability of this compound to form interdigitated bilayers that may enhance respreading and adsorption (Skita et al., 1995). The relative hydrophobicity and size of chain linkage groups in phosphonolipid molecules also potentially affect surface behavior. The thioether linkage in S-lipid was the most hydrophobic of those studied, while the chain linkage of SO_2-lipid was the largest and most hydrophilic. Sulfur linkages in S-lipid and SO_2-lipid were associated with improved adsorption and film respreading compared to ether linkages in DEPN-8 (Table 1, Fig. 4). S-lipid had the best respreading and adsorption of the C16:0 compounds studied, suggesting that the thioether linkage was particularly beneficial for these surface properties. However, the SO_2-linkage was more beneficial to dynamic surface tension lowering in SO_2-lipid compared to the thioether linkage in S-lipid (Tables 1–3). Moreover, despite having reduced adsorption compared to sulfur-linked phosphonolipids, the diether analog DEPN-8 had the greatest overall dynamic surface activity on the pulsating bubble (Tables 2 and 3). Specific molecular biophysical mechanisms for the surface property differences found between sulfur-linked and ether-linked analogs were not addressed in our experiments, and need to be elucidated in more detail in future research.

An important emphasis in the present study was on the overall dynamic surface activity of synthetic lung surfactants containing phosphonolipids combined with hydrophobic apoproteins purified from lavaged bovine lung surfactant. Surface activity assessments were done on surfactants that contained pure SP-B, pure SP-C, and equal weights of the two together to address potential apoprotein synergy. We have previously reported that a surfactant containing DEPN-8 + 1.5 wt.% of mixed (unpurified) bovine SP-B/C had very high surface activity that equaled or exceeded that of CLSE (Wang et al., 2003). However, this prior work did not examine DEPN-8 combined with pure isolates of SP-B and SP-C as done here. Synthetic surfactants containing DEPN-8 or SO_2-lipid plus 1.5% SP-B were highly active, but dynamic surface tension lowering was found to be maximal for preparations that contained equal amounts by weight of both hydrophobic proteins (0.75% SP-B + 0.75% SP-C) (Tables 2 and 3). Evidence of apoprotein synergy was particularly apparent in the activity of surfactants containing SO_2-lipid + 1.5% SP-B versus 0.75% SP-B + 0.75% SP-C at a concentration of 0.5 mg/ml (Table 2). Previous studies by Wang et al. (1996) did not report appreciable synergy between bovine SP-B and SP-C in mixtures with biological glycerophospholipids. The small but noticeable synergy observed here may reflect differences in interactions of these apoproteins with synthetic phosphonolipids as opposed to glycerophospholipids, as well as newer preparative methodology used in purifying bovine SP-B and SP-C (Baatz et al., 2001).

In addition to studying surfactant apoproteins combined with C16:0 disaturated phosphonolipids in synthetic surfactants, the C16:1 compound UnDEPN-8 was examined to assess the effects of a fluid secondary lipid on surface activity. Endogenous lung surfactant is known to contain a mix of rigid disaturated phospholipids together with fluid unsaturated phospholipids that have increased respreading ability (Notter, 2000). Addition of 10 wt.% of UnDEPN-8 improved the adsorption of DEPN-8, but not that of SO_2-lipid (Fig. 5). Moreover, addition of 10% UnDEPN-8 did not improve (and in fact was detrimental to) dynamic surface tension lowering in synthetic surfactants containing DEPN-8 or SO_2-lipid plus hydrophobic apoproteins (Table 4). Unsaturated lipids like UnDEPN-8 have decreased molecular packing ability relative to disaturated lipids, and reach lower maximum surface pressures (higher minimum surface tensions) during dynamic compression. Although the addition of fluid lipids can potentially increase adsorption and film spreading in synthetic lung surfactants, phosphonolipids like DEPN-8 and SO_2-lipid already have significant improvements in these surface behaviors compared to glycerophospholipids like DPPC. This may lessen the need for secondary unsaturated zwitterionic components in synthetic surfactants that contain these active disaturated phosphonolipids.

The results of the present study are positive for the general approach of using phospholipase-resistant phosphonolipids like DEPN-8 or SO_2-lipid in synthetic surfactants for treating surfactant dysfunction

during inflammatory lung injury. Phospholipases are known to be released in the lungs during states of inflammatory injury in vivo (Holm et al., 1991; Touqui and Arbibe, 1999; Vadas and Pruzanski, 1986). Lytic enzymes including PLA_2 are also found in meconium (Schrama et al., 2001), which can be aspirated to cause lung injury in newborns. A number of studies have shown that phospholipases are directly inhibitory to surfactant activity (Duncan et al., 1996; Enhorning et al., 1992; Holm et al., 1991; Schrama et al., 2001; Wang et al., 2003). Phospholipases not only degrade active surfactant glycerophospholipids, but also produce reaction products (lysophosphatidylcholine, free fatty acids) that can further inhibit surfactant function (Hall et al., 1992a; Holm et al., 1999; Wang and Notter, 1998) and injure the alveolocapillary membrane (Hall et al., 1990; Niewoehner et al., 1987). Ether-, sulfur-, and phosphono-linkages in phosphonolipids are, on a structural basis, not susceptible to cleavage by phospholipases A_1, A_2, and D. The resistance of DEPN-8 to chemical degradation by phospholipases has been directly demonstrated in previous studies (Lin et al., 1997; Wang et al., 2003). Results here documented that synthetic surfactants containing SO_2-lipid or DEPN-8 + 0.75% SP-B + 0.75% SP-C were not impaired in surface activity by PLA_2 (Fig. 6), and that sulfur-containing phosphonolipids were chemically resistant to degradation by this enzyme (Table 5). In contrast, the active bovine lung surfactant extract CLSE was significantly impaired in surface tension lowering ability by PLA_2 (Fig. 6).

The most widely used current exogenous surfactants for treating RDS in premature infants are animal-derived. Laboratory research and clinical trials have documented that exogenous surfactant drugs derived from animals (Infasurf (CLSE), Survanta, Curosurf) have greater biophysical and physiological activity than first-generation synthetic surfactants like Exosurf and ALEC (Cummings et al., 1992; Egan et al., 1983; Hall et al., 1992b; Hudak et al., 1996, 1997; Mizuno et al., 1995; Notter, 2000; Seeger et al., 1993; Vermont–Oxford Neonatal Network, 1996). However, synthetic lung surfactants have significant potential advantages over animal-derived surfactants as pharmaceutical products. Synthetic surfactants manufactured in vitro have greater compositional reproducibility, and also can incorporate novel molecular components and properties such as structural resistance to phospholipases as emphasized here. The compositional complexity and batch-to-batch variability of animal surfactants increases the scope and expense of required quality control during manufacture. At least in principle, synthetic drugs become increasingly cost-effective over time once initial development costs are recovered. Synthetic surfactants are also free from concerns about prion-caused diseases like bovine spongioform encephalitis that potentially affect animal lung supplies. In addition to synthetic lipid components like DEPN-8 or SO_2-lipid as studied here, synthetic lung surfactants can also in principle be formulated to contain human-sequence peptide components, making them less subject to cultural and religious considerations compared to bovine or porcine preparations.

In summary, this paper has examined the surface activity of several new synthetic phosphonolipids including two C16:0 disaturated sulfur-containing compounds (SO_2-lipid and S-lipid) and a C16:1 unsaturated diether analog (UnDEPN-8). Also studied were DEPN-8, a highly active disaturated ether-linked phosphonolipid synthesized in our prior work, as well as the major lung surfactant glycerophospholipid DPPC and the clinically relevant bovine surfactant extract CLSE. Phosphonolipids were studied for adsorption, film behavior, and overall dynamic surface activity alone and in combination with purified SP-B and/or SP-C from bovine lung surfactant. Synthetic lung surfactants containing DEPN-8 or SO_2-lipid + 1.5% SP-B or 0.75% SP-B + 0.75% SP-C had surface activity approaching or equaling that of CLSE, with maximal activity found when both apoproteins were present together. Mixtures of DEPN-8 or SO_2-lipid + 0.75% SP-B + 0.75% SP-C maintained high surface activity when incubated with PLA_2, while CLSE was inhibited in activity by this lytic enzyme. Sulfur-containing phosphonolipids (SO_2-lipid and S-lipid) were also directly shown to be chemically resistant to PLA_2, as demonstrated previously for DEPN-8.

Acknowledgements

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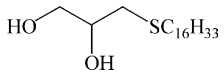
Appendix A. Details on lipid starting materials for preparing S-lipid, SO_2-lipid, and UnDEPN-8

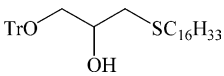
Detailed reaction intermediates and methods used in the synthesis and purification of DEPN-8 have been published previously by Notter, Turcotte and co-workers (e.g., Turcotte et al., 1991; Wang et al., 2003). In addition, some synthesis details for S-lipid and SO_2-lipid have also recently been reported (Chang et al., 2004). A summary of the starting lipid compounds used for preparing S-lipid, SO_2-lipid, and UnDEPN-8 is given below.

A.1. Synthesis of 1-S-hexadecyl-rac-thioglycerol



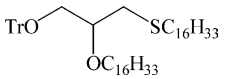
1-Thioglycerol (11.59 g, 105 mmol) and 1-bromohexadecane (30.5 g, 100 mmol) were dissolved in 95% EtOH (180 ml). Ethanolic KOH solution (1 M, 105 ml) was added dropwise over a period of 60 min with stirring under N_2. The reaction mixture was then stirred overnight (18 h), diluted with 1 l of water, and cooled. The white crystalline sulfide was collected, washed with cooled water (10 °C) and recrystallized from methanol to obtain 1-*S*-hexadecyl-*rac*-thioglycerol, 95% yield; mp: 65–66 °C (literature 69–70 °C, Morris-Natschke et al., 1986).

A.2. Synthesis of 1-S-hexadecyl-3-O-trityl-rac-thioglycerol



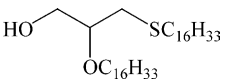
1-*S*-hexadecyl-*rac*-thioglycerol(15.0 g,45.1 mmol), triphenylmethyl chloride (15.1 g, 54.1 mmol) and Et_3N (5.47 g, 54.1 mmol) were dissolved in dry THF (150 ml) and CH_3CN (48 ml) at room temperature and the mixture was refluxed for 8 h and cooled. The resulting salt was filtered and washed with EtOAc (100 ml). The combined organic phases were washed with water (2 × 40 ml), 1% HCl (2 × 40 ml), saturated $NaHCO_3$ (100 ml), water and brine. After drying over $MgSO_4$, the mixture was filtered, evaporated, and purified by column chromatography using 15:1 EtOAc:hexane as the elution solvent to give 1-*S*-hexadecyl-3-*O*-trityl-*rac*-thioglycerol, 93% yield, mp: 56–58 °C (literature 60.5–61.5 °C, Morris-Natschke et al., 1986).

A.3. Synthesis of 1-S-hexadecyl-2-O-hexadecyl-3-O-trityl-rac-thioglycerol



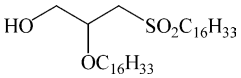
DMSO (20 ml) and powered KOH (2.62 g, 46.7 mmol) were mixed and stirred for 10 min, followed by addition of 1-*S*-hexadecyl-3-*O*-trityl-*rac*-thioglycerol (6.72 g, 11.7 mmol) and 1-bromohexadecane (7.13 g, 23.4 mmol) and stirring for 8 h at 55 °C. Water (40 ml) was added, and the mixture was then extracted with EtOAc (3 × 50 ml). The combined organic layers were washed with water, dried over $MgSO_4$, filtered, evaporated and purified by column chromatography (Al_2O_3, hexane) to provide 1-*S*-hexadecyl-2-*O*-hexadecyl-3-*O*-trityl-*rac*-thioglycerol, 94% yield; mp: 34–35 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): 7.20–7.45 (m, 15H), 3.46–3.54 (m, 3H), 3.21 (dd, J = 1.6, 4.8 Hz, 2H), 2.69 (ABX, J = 5.2, 6.4, 13.6 Hz, 2H), 2.48 (t, J = 7.4 Hz, 2H), 1.49–1.58 (m, 4H), 1.26–1.32 (m, 52H), 0.88 (t, J = 6.6 Hz, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz): 144.1, 128.7, 127.7, 126.9, 86.6, 79.3, 70.7, 64.8, 34.2, 33.1, 31.9, 30.1, 29.8, 29.7, 29.6, 29.4, 29.3, 28.9, 26.2, 22.7, 14.1; IR (CH_2Cl_2): 3059, 3033, 2924, 1449, 1264 cm^{-1}. Analysis, calculated for $C_{54}H_{86}O_2S$: C, 81.10; H, 10.76; found: C, 81.28; H, 10.62.

A.4. Synthesis of 1-S-hexadecyl-2-O-hexadecyl-rac-thioglycerol



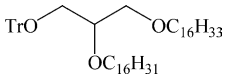
1-*S*-hexadecyl-2-*O*-hexadecyl-3-*O*-trityl-*rac*-thioglycerol (7.70 g, 9.63 mmol) and *p*-TsOH (414 mg, 2.4 mmol) were dissolved in 95% methanol (140 ml) and refluxed for 14 h. After cooling, the mixture was extracted with 10:1 hexane:EtOAc, washed with water, dried over $MgSO_4$, filtered, evaporated, and purified by flash chromatography (silica gel, 15:1 hexane:EtOAc) to provide known (Nali et al., 1986) 1-*S*-hexadecyl-2-*O*-hexadecyl-*rac*-thioglycerol in 85% yield, mp: 35–36 °C.

A.5. Synthesis of 1-SO_2-hexadecyl-2-O-hexadecyl-rac-sulfonylglycerol



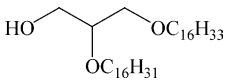
1-SO_2-hexadecyl-2-*O*-hexadecyl-*rac*-sulfonylglycerol was prepared as described in Chang et al. (2004).

A.6. 1-O-hexadecyl-2-O-Z-hexadec-9-enyl-3-O-trityl-rac-glycerol



1-*O*-hexadecyl-3-*O*-trityl-*rac*-glycerol (Stewart and Kates, 1989) (810 mg, 1.45 mmol) and powdered KOH (337 mg, 6 mmol) were dissolved in dry DMSO (6 ml) at room temperature. $C_{16}H_{31}OMs$ (500 mg, 1.54 mmol) in DMSO (3 ml) was added, and the reaction mixture stirred at room temperature overnight followed by heating to 60 °C for 2.5 h. After cooling to room temperature, 5% NH_4Cl (aqueous, 10 ml) was added and the mixture was extracted with EtOAc, washed with brine, dried over $MgSO_4$ and purified by flash chromatography (silica gel, 1:30 EtOAc:hexane) to provide 1-*O*-hexadecyl-2-*O*-*Z*-hexadec-9-enyl-3-*O*-trityl-*rac*-glycerol in 95% yield. ^{1}H NMR ($CDCl_3$, 400 MHz): 7.45–7.40 (m, 5H), 7.29–7.17 (m, 10H), 5.38–5.31 (m, 2H), 3.52 (m, 4H), 3.39 (t, $J = 6.8$ Hz, 2H), 3.17 (m, 2H), 2.01 (m, 4H), 1.61–1.49 (m, 4H), 1.26 (m, 45H), 0.88 (t, $J = 6.8$ Hz, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz): 144.1, 129.8, 129.0, 128.8, 128.7, 128.6, 127.7, 127.6, 126.8, 126.7, 86.5, 78.3, 71.6, 71.1, 70.6, 63.6, 31.9, 31.7, 30.1, 29.8, 29.7, 29.5, 29.4, 29.3, 28.9, 27.2, 26.1, 22.7, 22.6, 14.1; IR (neat, cm^{-1}): 3086, 3059, 3032, 3003, 2925, 2853.

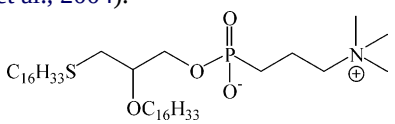
A.7. 1-O-hexadecyl-2-O-Z-hexadec-9-enyl-rac-glycerol



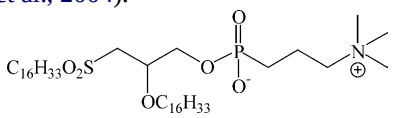
1-*O*-hexadecyl-2-*O*-*Z*-hexadec-9-enyl-3-*O*-trityl-*rac*-glycerol (3.97 g, 5.08 mmol) and *p*-TsOH (218 mg, 1.27 mmol) were dissolved in 95% methanol (140 ml) and refluxed for 15 h. After cooling, the mixture was extracted with 10:1 hexane:EtOAc, washed with water, dried over $MgSO_4$, filtered, evaporated, purified by flash chromatography (silica gel, eluent 15:1 hexane: EtOAc) to give 1-*O*-hexadecyl-2-*O*-*Z*-hexadec-9-enyl-*rac*-glycerol in 85% yield, mp: 35–36 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): 5.29 (m, 2H), 3.66 (m, 1H), 3.55 (m, 2H), 3.47 (m, 4H), 3.39 (dt, $J = 6.8$ and 0.9 Hz, 2H), 2.34 (d, $J = 5.8$ Hz, 1H), 1.97 (m, 4H), 1.51 (m, 4H), 1.25 (m, 44H), 0.84 (m, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz): 129.85, 127.74, 78.28, 71.77, 70.82, 70.33, 62.96, 31.89, 31.75, 30.03, 29.69, 29.66, 29.58, 29.46, 29.43, 29.41, 29.21, 28.94, 27.17, 27.14, 26.05, 22.64, 22.61, 14.0; IR (neat, cm^{-1}): 3449, 3059, 3004, 2926, 2854, 1465, 1117; EIMS, *m/z* (%): 538 (M^+, 15), 317 (92), 260 (53), 222 (78), 183 (100), 105 (64), 83 (82); HRMS, m/*z*: calculated for $C_{35}H_{70}O_3$ $[M]^+$: 538.5328; Found: 538.5338.

Synthesis procedures for final phosphonolipids used 700–1100 mg of doubly functionalized glycerols and generally followed previously published methods (Chang et al., 2004; Wang et al., 2003) with the exception that crystallization was from 2:1 $CHCl_3$/acetone after flash chromatography. Final phosphonolipid compounds were as follows.

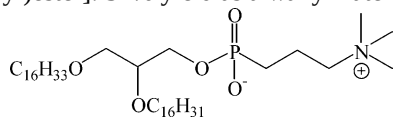
S-lipid [(±)-trimethyl(3-phosphonopropyl)ammonium, mono(2-hexadecyloxy-3-hexadecylsulfanyl-propyl)ester]: 48% yield, mp: 203–204 °C (decomposition). Spectral data have been reported previously (Chang et al., 2004).



SO_2-lipid [(±)-trimethyl(3-phosphonopropyl)ammonium, mono(2-hexadecyloxy-3-hexadecylsulfonyl-propyl)ester]: 59% yield, mp: 234–235 °C (decomposition). Spectral data have been reported previously (Chang et al., 2004).



UnDEPN-8 [(±)-trimethyl(3-phosphonopropyl) ammonium, mono(2-hexadec-9-enyloxy-3-hexadecyl-oxypropyl)ester]: 51% yield as a waxy material.



^{1}H NMR ($CDCl_3$:CD_3OD = 1:1, 400 MHz) (ppm): 5.25 (m, 2H), 3.80 (m, 2H), 3.62–3.48 (m, 4H), 3.38 (m, 5H), 3.06 (s, 9H), 1.92 (m, 6H), 1.48 (m, 6H), 1.18 (m, 44H), 0.79 (*t*, J = 6.8 Hz, 6H); ^{13}C NMR ($CDCl_3$:CD_3OD = 1:1, 100 MHz): 129.2, 129.1, 77.6 (d, J = 6.8 Hz), 71.0, 70.0 (d, J = 10.7 Hz), 66.3 (d, J = 12.3 Hz), 62.7, 52.1, 31.1, 31.3, 29.4, 29.1, 29.0, 28.9, 28.8, 28.7, 28.6, 28.3, 26.5, 25.5, 25.4, 21.9, 16.9, 13.1; ^{31}P NMR ($CDCl_3$:CD_3OD = 1:1, 162 MHz): δ, 23.2; IR (CH_2Cl_2): 3051, 2926, 2854, 1467, 1264, 1052, 741 cm^{-1}; LRMS, TOF MS CI (+ve ion), m/z (%): 703 (($M+1$)$^+$, 24), 397 (35), 381 (100); HRMS: calculated for $C_{41}H_{85}NO_5P$ ([M + H]$^+$): 702.6152; found: 702.6165.

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