RESEARCH COMMUNICATION

Reduction of the surface-tension-lowering ability of surfactant after exposure to hypochlorous acid

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The reactive species hypochlorous acid (HOCl/OCl^-) is a major product of the respiratory burst in activated neutrophils. We studied the effects of HOCl/OCl^- on human surfactant and upon surfactants Survanta, KL_4 and Exosurf, utilizing a pulsating surfactometer for measuring surface tension. HOCl/OCl^- induced a marked dose-dependent decrease in the surface-tension-lowering activity of human surfactant. The surfactant containing surfactant proteins B and C (Survanta) was less sensitive; however, synthetic surfactants with or without peptides were not

affected by HOCl/OCl^- (KL_4 , Exosurf). Ascorbic acid and GSH protected human surfactant against inactivation by HOCl/OCl^- . We suggest that HOCl/OCl^- produced by activated phagocytes in the alveolar compartment of the lung could damage endogenous surfactant and affect the function of exogenously administered natural or other surfactants, especially if ascorbic acid and GSH levels in the lung lining fluids are subnormal, as is known to be the case in some inflammatory lung diseases.

INTRODUCTION

Surfactant is critical for maintaining low surface tensions in the alveoli, and the surfactant proteins A, B and C (SP-A, SP-B and SP-C) (especially the last two) play a critical role in this function [1,2]. Some reactive oxygen species, in addition to serum proteins, have been shown to diminish the surface-tension-lowering ability of surfactant [3,4], and this inhibition of function contributes to the severity of respiratory distress syndrome (RDS) in preterm infants [5]. Surfactant replacement therapy has yielded significant improvements in morbidity and mortality in premature infants with RDS [6] and, from preliminary studies, in full-term infants with severe respiratory failure [7]. More recently, surfactant treatment has been tested in patients with adult respiratory distress syndrome (ARDS) [8,9]. Surfactant replacement therapy has been performed with surfactant (containing SP-A, SP-B and SP-C) obtained from human amniotic fluid, with modified lipid-extracted surfactants of bovine or porcine origin containing SP-B and SP-C, and with totally synthetic preparations containing either synthetic peptides (such as KL_4 surfactant [10]) or without proteins at all (such as Exosurf), as described in Table 1.

In ARDS, increased numbers of phagocytes are recruited to the alveolar spaces [11], and there is evidence that they are activated to produce increased quantities of both proteases and reactive oxygen species, such as superoxide radical ($\text{O}_2^{\cdot-}$) and H_2O_2 [12,13]. In the presence of iron, these can be converted into more reactive species such as the hydroxyl radical, $\cdot\text{OH}$ [14]. It has already been shown that iron-dependent radical-generating systems damage surfactant surface-tension-lowering functions [3,4]. However, activated neutrophils produce large amounts of hypochlorous acid (HOCl/OCl^-), a reactive oxidizing species which can damage proteins by attacking thiol groups, methionine, tryptophan, and amino groups [15–17]. HOCl/OCl^- is generated by H_2O_2 -dependent oxidation of Cl^- ions by the neutrophil enzyme myeloperoxidase [18].

In this study, we show that HOCl/OCl^- damages the surface-tension-lowering ability of human surfactant. We have also examined the effect of HOCl/OCl^- upon a surfactant containing SP-B and SP-C, and upon synthetic surfactant preparations being used clinically (or undergoing clinical trials), as it is interesting to know if these various preparations are also susceptible to HOCl/OCl^- -induced oxidative damage.

MATERIALS AND METHODS

Sodium hypochlorite (NaOCl), 5,5'-dithiobis-(2-nitrobenzoic acid), reduced glutathione (GSH) and ascorbic acid were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Inorganic salts were from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Surfactant of human amniotic fluid origin (IND 26308) was prepared as previously reported [5]. Surfactant KL_4 was kindly supplied by Dr. Charles Cochrane at the Scripps Research Institute (La Jolla, CA, U.S.A.). Survanta and Exosurf were purchased from Ross Laboratories (Columbus, OH, U.S.A.) and Burroughs–Wellcome (Research Triangle Park, NC, U.S.A.) respectively.

Exposures to HOCl/OCl^-

The concentration of NaOCl was determined using the molar absorption coefficient at 290 nm of $350 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at pH 12. Serial dilutions of a stock solution of 40 mM NaOCl were made in normal saline and the required concentrations dispensed into the suspension of surfactant.

Two concentrations of each surfactant were used for HOCl/OCl^- treatment, 2.5 and 5.0 mg of phospholipid/ml dispersed in a final volume of 0.5 ml, concentrations approximating to those found in the alveolar lining fluids. The control vials contained only normal saline with phosphate buffer. The following order of mixing was used: 50 μl of surfactant, 350 μl of normal saline, 50 μl of 0.5 M $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ at pH 7.4, 50 μl of HOCl/OCl^- (diluted previously in normal saline), to

Abbreviations used: SP-A, -B and -C, surfactant proteins A, B and C; RDS, respiratory distress syndrome; ARDS, adult RDS.

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Table 1 Compositions of surfactants used in the study

Surfactant type	Composition
Natural human surfactant (from amniotic fluid)	Lipid + SP-A, SP-B and SP-C
Survanta	Lipid + bovine SP-B and SP-C
KL ₄ (synthetic)	Lipid + leucine/lysine peptide model of SP-B
Exosurf	Lipid only

produce the required concentration of each component of the mixture in the final volume of 500 μ l. After each treatment, the pH of the mixture was measured to ensure that it remained at 7.4.

Surface tension measurements

Treated and control samples were incubated from 0.25 to 2 h at 37 °C before surface tension measurements were taken using a pulsating-bubble surfactometer (Electronics Corporation, Amherst, NY, U.S.A.) as previously described [19]. Minimum surface tension in mN/m was recorded at 1, 2 and 3 min of inflation and deflation of a bubble at 20 cycles/min. The adsorption rate of surfactant to an air-liquid interface was measured through plots of surface tension versus time in seconds during formation of the bubble at maximum radius.

Effect of antioxidants

The effects of ascorbic acid and GSH were examined by mixing them, at the final concentrations stated, with surfactants before incubation with HOCl/OCl⁻.

RESULTS

At pH 7.4, HOCl is approx. 50 % ionized to hypochlorite anion, OCl⁻ [20]. Since we do not know whether HOCl, OCl⁻, or both of these species cause the damage described below, we have used the term HOCl/OCl⁻ throughout this paper.

Effects of surface adsorption

As shown in Figure 1, the surface adsorption of human surfactant was minimally affected by HOCl/OCl⁻, except at 10 mM concentrations of HOCl/OCl⁻. There was a similar minimal effect on surface adsorption with the other surfactants (results not shown).

Effects on surface tension minimum (mN/m) of surfactants

Table 2 shows, in common with other observations [21,22], that human surfactant, at concentrations of 2.5 and 5.0 mg/ml, lowered surface tensions to 0.9 and 1.4 mN/m respectively, while KL₄ surfactant achieved values of 3.2 and 3.0 mN/m, Survanta 6.0 and 4.0 mN/m, and Exosurf 27 nM/m at both concentrations. Treatment with HOCl/OCl⁻ affected these preparations differently. As we have shown that the biophysical activity of these preparations is affected by pH [22,23], it was essential to ensure that the pH remained at 7.4 by careful buffering. The effect of HOCl/OCl⁻ was complete after incubation for 0.25 h, in keeping with the high reactivity of these species [24].

Human surfactant was strongly affected by HOCl/OCl⁻. Even a 125 μ M concentration significantly ($P < 0.01$) decreased its

Figure 1 Surface adsorption measurements made after the formation of the bubble to maximum bubble diameter with human surfactant (2.5 mg/ml) with various concentrations of HOCl/OCl⁻

Surface adsorption at 10 s after bubble formation shows minimal change except at the highest concentration (10 mM) of HOCl/OCl⁻.

Table 2 Minimum surface tension (mN/m) of surfactants at concentrations of 2.5 mg/ml (A) and 5.0 mg/ml (B) after exposure to HOCl/OCl⁻

Inhibitory effects of HOCl/OCl⁻ on various surfactants at 2.5 mg/ml (A) and 5.0 mg/ml (B). Human surfactant shows the lowest surface tension, but is markedly affected by HOCl/OCl⁻. Exosurf containing no surfactant protein shows the highest baseline surface tension, but is unaffected by 2 mM HOCl/OCl⁻. Survanta and KL₄ have low surface tension as controls, but are also affected by HOCl/OCl⁻; however, KL₄ is affected only at a concentration of 2.5 mg/ml by 2.0 mM HOCl/OCl⁻. * $P < 0.0001$, † $P < 0.001$, †† $P < 0.01$ compared with the control value. Values are expressed as mean (S.E.M.) for $n = 6$.

HOCl/OCl ⁻ concn. (mM)	Minimum surface tension (mN/m)			
	Human surfactant	Survanta	KL ₄	Exosurf
(A) 2.5 mg/ml surfactant				
Control (0)	0.9 (0.9)	6.0 (0.5)	3.2 (0.4)	27 (0.3)
0.125	5.0 (0.2)††	7.0 (1.0)	3.5 (0.2)	28 (0.2)
0.250	8.0 (0.2)†	8.0 (1.0)	3.5 (0.3)	28 (0.1)
0.500	8.8 (0.7)†	9.0 (0.3)†	4.2 (0.2)	27 (0.5)
1.000	14.8 (1.1)*	17.7 (0.7)*	4.0 (0.2)	28 (0.2)
2.000	20.5 (0.2)*	20.0 (1.0)*	7.0 (0.2)††	27 (0.2)
(B) 5.0 mg/ml surfactant				
Control (0)	1.4 (0.4)	4.0 (0.4)	3.0 (0.4)	27 (0.25)
0.125	5.0 (0.8)††	7.4 (0.5)	3.8 (0.1)	27 (0.1)
0.250	7.9 (0.5)†	7.5 (0.6)†	3.6 (0.1)	27 (0.2)
0.500	14.0 (2.0)*	10.0 (0.3)*	3.3 (0.1)	27 (0.2)
1.000	18.0 (0.4)*	15.0 (0.7)*	3.5 (0.4)	28 (0.1)
2.000	19.0 (0.3)*	19.3 (0.4)*	5.8 (0.8)††	28 (0.1)

surface-tension-lowering ability (Table 2). By contrast, Survanta was only affected by HOCl/OCl⁻ at concentrations ≥ 0.5 mM and KL₄ surfactant only at concentrations ≥ 2 mM. Exosurf was not affected by even 2 mM HOCl/OCl⁻.

Table 3 Protective effect of GSH on HOCl/OCl⁻-induced inhibition of surface-tension-lowering ability of human surfactant

At 0.5 mM HOCl/OCl⁻, 0.2 and 0.5 mM GSH has a significant protective effect on HOCl/OCl⁻-induced inhibition of surface-tension-lowering capability, whereas at 1.0 mM HOCl/OCl⁻ 1.0 mM GSH was required to prevent HOCl/OCl⁻ inhibition. **P* < 0.0001 compared with the control value, (ANOVA). Values are expressed as means (S.E.M.), *n* = 6.

HOCl/OCl ⁻ concn. (mM)	Surface tension (mN/m)			
	— GSH	+ 0.2 mM GSH	+ 0.5 mM GSH	+ 1 mM GSH
Control	3.4 (0.4)	4.0 (0.2)	3.4 (0.4)	3.5 (0.1)
0.5	14.0 (0.2)*	6.2 (0.7)	5.8 (0.8)	—
1.0	18.0 (0.4)*	11.5 (1.4)*	6.7 (0.7)	5.8 (0.8)

Table 4 Protective effect of ascorbic acid (Asc) on HOCl/OCl⁻-induced inhibition of surface-tension-lowering ability of human surfactant

At 1.0 mM HOCl/OCl⁻ 0.5 mM ascorbic acid and 0.2 mM ascorbic acid has significant concentration-dependent effects on the HOCl/OCl⁻-induced inhibition of surface-tension-lowering capability. **P* < 0.0001 compared with the control value, (ANOVA). Values are expressed as means (S.E.M.), *n* = 6.

HOCl/OCl ⁻ concn. (mM)	Surface tension (mN/m)			
	— Asc	+ 0.2 mM Asc	+ 0.5 mM Asc	+ 2 mM Asc
Control	3.5 (0.4)	4.3 (0.2)	—	4.3 (0.2)
1.0	18.0 (0.4)*	10.0 (0.7)*	7.3 (0.7)	5.7 (0.8)

Effects of antioxidants

GSH and ascorbic acid are important antioxidants in broncho-alveolar lavage fluids [25–27], and their ability to scavenge HOCl/OCl⁻ has been reported [24,28]. Table 3 shows that GSH was able to protect human surfactant against damage by HOCl/OCl⁻. For example, 0.5 mM GSH protected significantly against the effects of 1 mM HOCl/OCl⁻. Table 4 shows that ascorbic acid was similarly protective at concentrations of 0.5 mM.

DISCUSSION

Surfactant is known to be susceptible to oxidative damage by activated neutrophils, but previous studies have concentrated upon iron-ion-dependent reactions [3,4]. However, in the present study we show that HOCl/OCl⁻, a major product generated by activated neutrophils, can rapidly destroy the surface-tension-lowering ability of surfactant. Inhibition was complete after 0.25 h (the shortest time in which measurements could be made), but given the reactivity of HOCl/OCl⁻, it is probable that damage was much quicker than this [24]. It has been reported that 5×10^6 activated neutrophils generate 88.3 ± 23.6 nmol of HOCl/OCl⁻ per ml in 2 h, i.e. about 100 μ M [29]. Although HOCl/OCl⁻ is very unlikely to accumulate 'free' *in vivo*, its effective concentration at localized sites of formation in the alveolar compartment may be much greater than this. Hence, the observation that 125 μ M HOCl/OCl⁻ damages the human surfactant surface-tension-lowering ability is not without physiological relevance, especially as large numbers of neutrophils are found in the airway fluid of neonates with RDS [30] and adults with ARDS [11].

Survanta was less susceptible to inhibition by HOCl/OCl⁻,

and KL₄ and Exosurf were nearly unaffected. This perhaps suggests that any effects of HOCl/OCl⁻ on the lipid component of surfactant are not the major factor(s) responsible for its surface-tension-lowering ability. However, different lipid compositions and conformations of proteins (where present) in each preparation could affect such tentative conclusions [22]. Our results suggest that surfactant preparations such as KL₄ surfactant or Exosurf may have the advantage of being less susceptible to oxidative damage, although the surface-tension-lowering capacity of Exosurf on the pulsating bubble and in animal models of RDS may be inferior to natural surfactants [31].

GSH is reported to be present in alveolar lining fluids at concentrations approaching 200 μ M and ascorbate at even higher concentrations [26,27]. Hence, they could be protective against HOCl/OCl⁻ generated *in vivo*. In several models of inflammatory lung diseases, it has been shown that GSH levels are diminished [32], so that damage to surfactant by HOCl/OCl⁻ is feasible. Such damage would be additional, and probably much faster, than that produced by proteases and by other reactive oxygen species, such as H₂O₂ and OH[•].

While our studies show that exposure of human surfactant or Survanta to HOCl/OCl⁻ *in vitro* results in inhibition of surface tension, the precise molecular mechanisms involved have not been established. HOCl/OCl⁻ has been shown to produce chlorohydrins from unsaturated fatty acids [33], and three (human, Survanta and KL₄) of the four surfactants utilized in the current studies contain unsaturated fatty acids. Exosurf, containing no unsaturated fatty acid, was least affected by HOCl/OCl⁻. However, it seems more likely that critical essential amino acid residues which are important for interaction with lipid constituents (possibly thiol and amino groups) on SP-A, SP-B and SP-C are being damaged by HOCl/OCl⁻. This could contribute to the particular sensitivity of human surfactant surface activity to be inhibited by HOCl/OCl⁻, for it represents the only surfactant studied to contain the major surfactant protein, SP-A. This speculation is buttressed by findings that SP-A is a particular target of attack by ozone [34] and by nitrogen dioxide [35], two other strong oxidants.

In summary, our results demonstrate a significant loss of surface-tension-lowering ability of human lung surfactant after exposure to an oxidizing agent known to be produced in large amounts by activated phagocytes *in vivo* [18]. In developing strategies for surfactant administration in clinical conditions of surfactant deficiency or surfactant dysfunction [7,10] and where alveolar inflammation can be expected to exist, it would appear useful to consider including selected antioxidants (such as GSH and ascorbic acid) in the administered therapeutic surfactant mixtures.

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