

ORIGINAL ARTICLE

Surfactant degradation activity in bronchoalveolar lavage fluid from guinea pigs challenged with antigen

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Background and objective: Surfactant dysfunction is a characteristic of bronchial asthma, but mechanisms of dysfunction following antigen exposure are not understood. The aim of this study was to examine whether bronchoalveolar lavage fluid (BALF) has surfactant degradation activity after antigen challenge, using an animal model of asthma.

Methods: BALF was collected 24 h after a challenge with aerosolized antigen solution in actively sensitized guinea pigs and from non-sensitized control guinea pigs. The surface tension of BALF was measured by pulsating bubble surfactometer. Surfactant activity was expressed as the minimum surface tension of BALF after 5 min of pulsation. BALF was separated into a cellular phospholipid fraction and supernatant, and reconstituted into 'pellet + supernatant' and 'pellet + saline' fractions.

Results: Surfactant activity of BALF from sensitized antigen-challenged animals was reduced after 4 h of incubation at 37°C but a decrease was not observed in BALF from non-sensitized control animals. The decrease of surfactant activity in BALF from challenged animals was prevented by incubation at 4°C. Disappearance of surfactant activity after incubation at 37°C was observed in the 'pellet + supernatant', but not in the 'pellet + saline' fraction. The decrease of surfactant activity in BALF was also partially suppressed by the secretory phospholipase A2 inhibitor, indoxam, and by a cocktail of protease inhibitors.

Conclusion: Surfactant-degrading activity was present in the supernatant of BALF from antigen-challenged guinea pigs. This activity may be attributed to secretory phospholipase A2 and to proteases present in the antigen-challenged airway.

Key words: asthma, guinea pig, phospholipase A2, surface tension, surfactant.

INTRODUCTION

Pulmonary surfactant is a mixture of phospholipids and surfactant-specific proteins that reduce alveolar surface tension during respiration.¹ Surfactant dysfunction is considered to be an important cause of infant and adult respiratory distress syndromes.^{2,3} Moreover, adequately functioning surfactant is necessary for the patency of narrow conducting airways and for low airway resistance.⁴ Bronchial asthma is a chronic inflammatory disease and recent studies have shown that one of the characteristics of bronchial

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asthma is surfactant dysfunction.^{5–10} As evidence of direct surfactant degradation, Ackerman *et al.* demonstrated surfactant hydrolysis by phospholipase A2 (PLA2) and eosinophil lysophospholipases.⁷

Surfactant degradation can hypothetically be attributed to two mechanisms. One mechanism is hydrolysis of surfactant phospholipids by PLA2.^{11,12} PLA2 comprises a diverse family of lipolytic enzymes that attack the sn-2 carbonyl of glycerophospholipids. Based on the primary structure, mammalian PLA2s can be classified into intracellular and secretory (sPLA2).^{13,14} Bronchoalveolar lavage fluid (BALF) from asthmatic patients challenged with endobronchial antigen instillation demonstrated increased amounts of sPLA2 activity and 1-palmitoyl-lysoPC6-1.¹⁵ Another mechanism of surfactant degradation is degradation of surfactant apoproteins. Surfactant contains four unique proteins, SP-A, SP-B, SP-C and SP-D. SP-A, SP-B and SP-C contribute to the biophysical functions of surfactant. There have been recent reports that in acute lung injury, leukocyte elastase degraded surfactant apoproteins and decreased surfactant activity.¹⁶

The aim of this study was to examine and confirm degradation of surfactant in BALF from animals challenged with inhaled antigen, and to examine putative mechanisms.

METHODS

Animals

Male Hartley albino guinea pigs (200–250 g) were obtained from Sankyou Laboratory Service (Toyama, Japan). All animal procedures in this study were in accordance with the standards specified in the institutional Guideline for the Care and Use of Laboratory Animals.

Animal sensitization

Guinea pigs were actively sensitized by a modification of the method reported by Andersson.¹⁷ Briefly, the animals were immunized with 2.0 mg of ovalbumin and 100 mg of aluminium hydroxide (Al(OH)₃). A booster injection of 10 µg of ovalbumin together with 100 mg of Al(OH)₃ was given 3 weeks after the primary immunization. Twelve animals were immunized and another 12 animals were not immunized for use as controls.

Antigen challenge

Three weeks after the booster injection, the guinea pigs were challenged with aerosolized ovalbumin solution. Procaterol (0.1 mg/kg) was administered intraperitoneally 30 min before antigen inhalation and diphenhydramine (30 mg/kg) 15 min before inhalation. Conscious guinea pigs were placed in a dual chamber plethysmograph for measurement of airway resistance (sRaw) by modification of the

method described by Pennock *et al.*¹⁸ Animals were challenged with 10 mg/mL ovalbumin solution for 60 s by a DeVilbiss 646 nebulizer (DeVilbiss Co., Somerset, PA, USA) using compressed air at 7.57 L/min (Minipon 54B-588, Origin Medical Industry Co., Ltd, Tokyo, Japan) and passed through the nasal chamber. Ovalbumin challenges were performed three times every other day and sRaw was measured prior to and for 20 min after the last challenge. BALF was obtained 24 h after the last inhalation. The 12 sensitized animals were challenged with ovalbumin solution and the 12 non-sensitized control animals were challenged with saline.

BAL

Twenty-four hours after the last challenge, each animal was anaesthetized by an intraperitoneal injection of 75 mg/kg of sodium pentobarbitone, and its trachea was cannulated with a polyethylene tube. Five millilitres of sterile saline was infused through a tracheal cannula and manually recovered by gentle aspiration with a disposable syringe. This procedure was repeated five times using the same 5 mL of saline. Saline was added to the obtained BALF to bring the total volume up to 5 mL. In order to obtain a measurable concentration of surfactant, BAL was performed on another guinea pig using the 5 mL obtained from the first animal. Therefore, the final BALF sample consisted of BALF from two animals. The total cell number was determined using a Turk solution and counted in duplicate on a Burkner haemocytometer. Differential cell counts were performed on cytopsin preparations (Cytospin 2, Shandon, UK) by counting 300 cells after staining using the May–Grunwald-Giemsa method. BALF was centrifuged at 80 g for 5 min to remove cells and the supernatant was stored at –80°C until measurement of surface tension and biochemical analysis.

Biochemical analysis of the BALF

Phospholipid was measured enzymatically with a commercially available kit (Wako Chemicals, Osaka, Japan).¹⁹ Protein content was determined by the pyrogallol red-molybdenum complex method.²⁰

Surface tension measurement

A pulsating bubble surfactometer (PBS, Electronics, Amherst, NY, USA) was used to measure the BALF dynamic surface properties.²¹ A bubble, which is a large model of an alveolus, is expanded in 20 µL of the sample liquid, and through a vertical capillary it communicates with ambient air. The bubble pulsates at a rate of 20 cycles per minute, whereby its radius alternates from a maximum of 0.55 mm to a minimum of 0.4 mm. Thus, the area of the surfactant film formed at the air–liquid interface of the bubble will be compressed to 50% of its maximum size. As the pressure difference across the bubble surface (dP) is

Table 1 Body weights of control and sensitized-challenged guinea pigs and laboratory data from BALF

	Control (<i>n</i> = 12)	Sensitized-challenged (<i>n</i> = 12)
Body weight (g)	655 ± 10	663 ± 12
Recovery (mL)	2.9 ± 0.1	3.1 ± 0.1
BAL analysis	(<i>n</i> = 6)	(<i>n</i> = 6)
Total cell count (×10 ⁵ cells/mL)	11.9 ± 1.7	24.7 ± 3.3*
Macrophages (×10 ⁵ cells/mL)	10.2 ± 1.7 (85.7 ± 3.6%)	14.1 ± 2.0* (63.6 ± 6.8*)
Neutrophils (×10 ⁵ cells/mL)	0.02 ± 0.01 (0.1 ± 0.07%)	0.5 ± 0.3* (2.4 ± 0.8%)
Lymphocytes (×10 ⁵ cells/mL)	0.8 ± 0.3 (7.3 ± 2.2%)	0.6 ± 0.1 (2.1 ± 0.5%)
Eosinophils (×10 ⁵ cells/mL)	0.8 ± 0.3 (6.8 ± 2.6%)	9.6 ± 1.8* (31.9 ± 6.9%)
Phospholipids (mg/L)	742 ± 61	493 ± 70*
Total protein (mg/L)	822 ± 85	871 ± 112
Proteins/phospholipids	1.11 ± 0.09	1.87 ± 0.27*

**P* < 0.05.

Values are mean ± SEM.

continuously recorded and the bubble radius (*r*) changes according to a fixed pattern, surface tension (ST) can be calculated according to the law of Laplace, $ST = 1/2dP(r)$. For this study, the value at the minimum bubble size (ST_{min}) during 5 min of pulsation was assessed. From the present study and the report of Liu *et al.*,²² ST_{min} is variable during the first 3 min with the variability becoming very small after 5 min.

Measurements of surfactant degradation activity

1 Changes in surfactant activity in BALF during incubation at 37°C.

Cell-free BALF was prepared and incubated at 37°C. ST_{min} was measured before and after incubation at 2 and 4 h. The time course of surfactant activity after incubation was compared between sensitized ovalbumin-challenged BALF (sensitized-challenged) and non-sensitized saline-challenged BALF (control).

2 Surface activity of the pellet of BALF and influence of the supernatant of BALF.

Differential centrifugation can separate alveolar surfactant into pellets.^{23–25} Aliquots of BALF from sensitized-challenged animals were centrifuged at 20 000 *g* for 60 min at 4°C to separate the supernatant from the pellets. The pellets, including surfactant aggregates, were resuspended in the same volume of saline or supernatant. The surfactant activity of the solutions was measured after incubation at 4°C or 37°C for 4 h.

3 Influence of sPLA2 inhibitor and protease inhibitors on changes in surfactant activity.

The cell-free BALF of sensitized-challenged animals was incubated at 37°C for 4 h with 6, 60 or 600 nM of the IIA-sPLA2 inhibitor, indoxam (Shionogi, Japan) or protease inhibitor cocktail (1 unit contains AEBSF 2.0 mM, EDTA 1.0 mM, bestatin 130 μM, E-64 1.4 μM, leupeptin 1.0 μM, aprotinin 0.3 μM; P 2714, Sigma, St Louis, MO, USA) at dilutions of 0.01, 0.1 and 1 unit, or vehicle. After incubations with indoxam or inhibitor cocktail ST_{min} was measured and compared with ST_{min} of BALF incubated at 4°C for 4 h.

Statistics

Two-tailed Student's *t*-test for unpaired samples was used for analysis of differences between the means of two variables. One-way analysis of variance (ANOVA) without replication was used as a test of significant differences between the means of more than two groups of data. A *P* < 0.05 was considered significant.

RESULTS

Airway response to antigen inhalation

Antigen inhalation increased airway resistance in sensitized animals. Baseline sRaw values were 1.0 ± 0.0 cmH₂O·s (mean ± SE) in control and 1.1 ± 0.1 cmH₂O·s in sensitized animals. The maximum increase in sRaw within 20 min of antigen inhalation was 8.7 ± 1.3 cmH₂O·s. Such a response was not seen in control animals after inhalation of saline (1.1 ± 0.0 cmH₂O·s).

Body weights and BALF biochemical and cellular analysis

Body weight was not significantly different between the two groups of animals (Table 1). The total cell number in the BALF was significantly increased in the antigen-challenged group compared with that of the control group. In the antigen-challenged group, eosinophils were significantly increased. The protein concentration in BALF was not significantly different, but total phospholipid was significantly lower in the antigen-challenged group than in controls. As a result, the ratio of proteins/phospholipids was significantly higher in the antigen-challenged group compared with that of the control group.

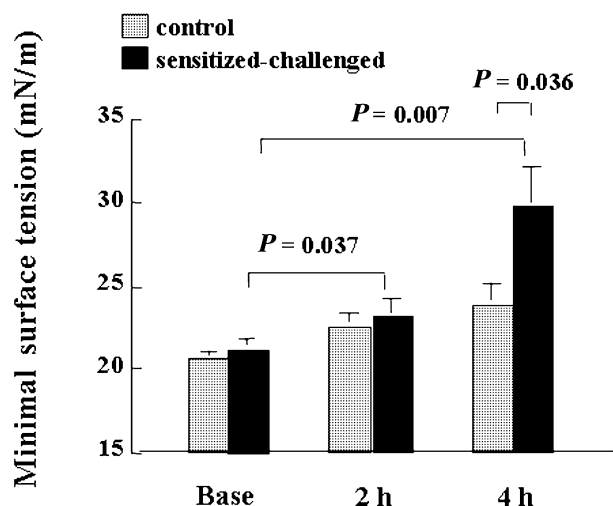


Figure 1 Minimal surface tension of BALF after 5 min of pulsation in the non-sensitized, saline-challenged control group ($n = 6$) and the sensitized antigen-challenged group ($n = 6$), measured before and after incubation at 37°C for 2 h and 4 h. Values are mean $\pm$ SEM.

Time course of changes in surfactant activity in BALF at 37°C

Surface activities of BALF as expressed by $ST_{\min}$ were not significantly different between the control and antigen-challenged groups at baseline (Fig. 1). Controls showed no significant increase in $ST_{\min}$ during the 4-h incubation at 37°C. However, the $ST_{\min}$ of BALF from antigen-challenged animals was significantly increased after 2 h and even more after 4 h of incubation at 37°C, indicating that surfactant activity was significantly decreased after the 2-h and 4-h incubations. Changes in $ST_{\min}$ plateaued after 4 h (data not shown).

The effect of temperature on surfactant dysfunction of BALF from antigen-challenged animals

To examine the nature of surfactant degradation activity of BALF from antigen-challenged animals, BALF was incubated for 4 h at 37°C and at 4°C. $ST_{\min}$ was significantly increased when incubated at 37°C, but remained at a similar level when incubated at 4°C after 4 h (Fig. 2).

Surfactant degradation factor in the pellet and supernatant of BALF from allergen-challenged animals

The study then investigated whether the decrease in surfactant activity of BALF from antigen-challenged animals resulted from changes in the surfactant or factors in the supernatant. As shown in Figure 3, reconstitution of the BALF pellet with saline and

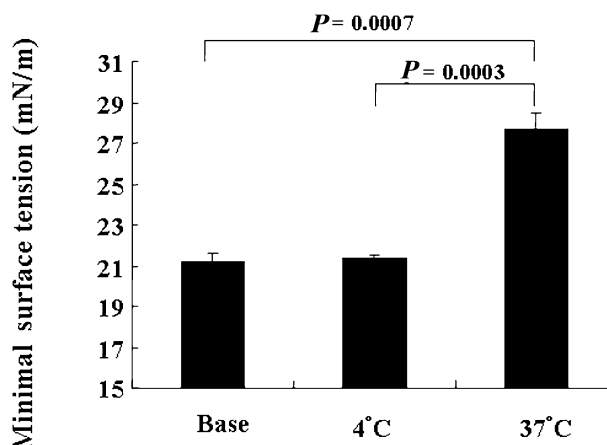


Figure 2 Minimal surface tension of BALF after 5 min pulsation in the sensitized antigen-challenged group ($n = 6$), measured before and after incubation at 4°C and at 37°C for 4 h. Values are mean $\pm$ SEM.

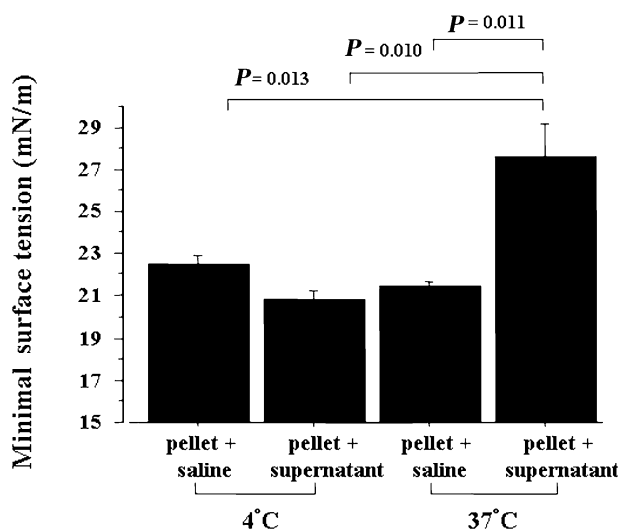


Figure 3 Minimal surface tension of BALF from the sensitized antigen-challenged group ($n = 6$) after incubation at 4°C and at 37°C for 4 h. BALF was separated by centrifugation into a pellet and supernatant, and reconstituted as 'pellets + supernatant' or 'pellets + saline'. Values are mean $\pm$ SEM.

incubation at 4°C for 4 h showed a similar $ST_{\min}$ as that of the 'pellet + supernatant'. After 4 h of incubation at 37°C, the 'pellet + supernatant' showed a significant decrease in surface tension activity, but the 'pellet + saline' showed surface activity similar to incubation at 4°C.

Influence of sPLA2 and protease inhibitors on change of surfactant activity

The surfactant degradation was partially inhibited by dose-dependent incubation with the sPLA2 inhibitor indoxam. Incubation with protease inhibitors also

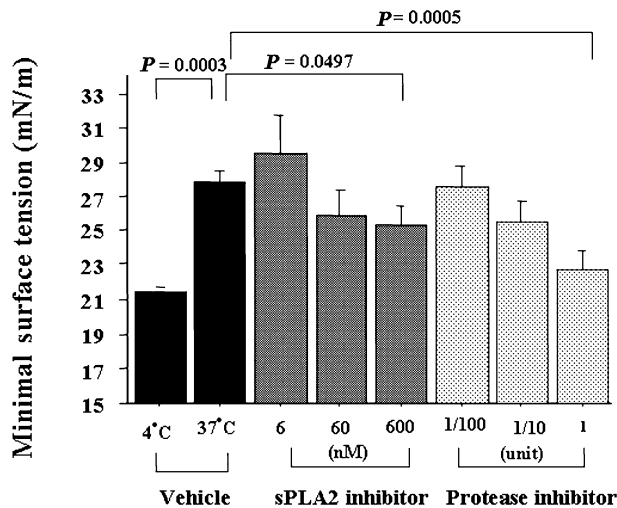


Figure 4 Effect of sPLA2 inhibitor and protease inhibitors on surfactant degradation activity in BALF from the sensitized, antigen-challenged group ($n = 6$). Values are mean $\pm$ SEM.

inhibited the degradation of surfactant activity in a dose-dependent manner (Fig. 4).

DISCUSSION

This study showed that BALF from guinea pigs challenged with aerosolized antigen displayed pulmonary surfactant degradation. This degradation activity was inhibited by the specific group II sPLA2 inhibitor indoxam and by protease inhibitors.

Surfactant inactivation by sPLA2 or proteolytic enzymes has previously been studied by incubating surfactant directly with enzymes.^{9,16,26} To our knowledge the present study is the first to demonstrate surfactant degradation activity in the airways of antigen-challenged animals. Inactivation of surfactant was observed only in antigen-challenged animals and it disappeared when BALF was incubated at 4°C. No surfactant degradation activity occurred in BALF from non-sensitized and saline-challenged control animals. Furthermore, surfactant degradation activity was documented in the soluble fraction of BALF supernatant, and surface tension activity of surfactant in the pellet from antigen-challenged animals was not changed by incubation at 37°C for 4 h. These results suggest that surfactant inactivation was the result of a specific enzymatic reaction and not the result of natural degradation of surfactant-associated lipids or protein components.

One well-known mechanism of surfactant dysfunction in experimental asthma is surfactant inhibition by protein leakage into the airway. Liu *et al.* demonstrated rapid inactivation of surfactant in antigen-challenged guinea pigs, and showed that severe surfactant dysfunction could be due to the proteins which leak into the airways.²² This mechanism of surfactant dysfunction has been demonstrated clinically in asthmatic patients after segmental antigen chal-

lenge.^{27,28} In this study, there was no significant increase in the protein concentration of BALF from antigen-challenged animals compared with non-sensitized saline-challenged animals (Table 1). Consistent with this result, the ST_{min} of BALF at baseline was not significantly different between the groups. One possible reason for this result may be the magnitude of the antigen-induced reaction, which was kept to a minimum in order to ensure survival of the guinea pigs for 24 h after antigen challenge. On the other hand, an acute antigen-induced airway response was observed, reflected by the increase in airway resistance and airway eosinophilia 24 h after the last antigen challenge.

With the small amount of BALF available from guinea pigs it was impossible to concentrate BALF to the physiological level of surfactant necessary to exhibit zero ST_{min} .²⁹

Each sample for analysis consisted of BALF from two animals in order to obtain a higher concentration of surfactant, but this method could not be extended to more animals as approximately one-third of the liquid was lost for each animal. It is possible that a small difference in surfactant activity may not be detectable at low concentrations of surfactant.²⁹ However, it was considered that the ST_{min} of the BALF observed in this study was sufficiently low for the study purpose because differences could be detected after incubation at 37°C.

BALF from asthmatic patients demonstrated increased amounts of sPLA2 activity after allergen challenge,¹⁵ but the type of PLA2 has not been previously identified. While the specific source of sPLA2 is speculative, mast cells are highly plausible as they contain group II sPLA2 and release this enzyme following antigen challenge.³⁰ Indoxam has a powerful inhibitory potency for group II sPLA2 activity ($IC_{50} = 1.2$ nM), whereas the other isoforms (I, V and X) are far less sensitive to indoxam, with over 100-fold higher IC_{50} values.³¹ The results of this study suggest that the inhibitory effect of indoxam on surfactant degradation activity cannot be fully explained by the inhibition of group II sPLA2. However, the contribution of other isoforms of sPLA2 is likely to be small because most of the inhibitory effects of indoxam appeared at 60 nM, although it did not reach a level of significant difference from the control group.

Finally, the effect of a non-specific protease inhibitor cocktail (AEBSE, EDTA, bestatin, E-64, leupeptin, aprotinin) was examined. This cocktail inhibited most of the surfactant degradation activity, which further confirms the hypothesis that surfactant degradation occurs in antigen-challenged airways. However, there is no specificity with respect to which protease is responsible for the surfactant degradation. In addition, the possibility that this protease cocktail might also inhibit sPLA2 cannot be excluded, as sPLA2 generally requires millimolar concentrations of Ca^{2+} .³¹

In conclusion, our study suggests that surfactant is metabolized in the airway by group II sPLA2 and other enzymes after allergen challenge, and implies that rapid recruitment or production of surfactant is necessary to maintain the normal surfactant pool. sPLA2 may be a candidate for bronchial asthma therapy.

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