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Investigation and Development of Artificial Lung Surfactant Preparations with a Novel Amphiphilic Peptide for Respiratory Distress Syndrome (RDS)

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CHAPTER IV

The correlation between *in vitro* surface activity and *in vivo* lung function of synthetic surfactant preparations

IV-1 Introduction

Until now, the author has reported about several new pulmonary surfactant preparations containing a synthetic peptide and their evaluation from the view-point of their surface behavior (Chapters I, II, and III).

Langmuir film properties (π–A, ΔV–A isotherms and the temperature dependence of π–A, ΔV–A isotherms) have revealed that the pulmonary surfactant preparations used in the previous study (DPPC/PG/PA/Hel 13-5: Chapter III) have better pulmonary functions than binary system (DPPC/Hel 13-5: Chapter I) and ternary systems (DPPC/PG/Hel 13-5 and DPPC/PA/Hel 13-5: Chapter II). The role in squeeze-out mechanism for individual component was made clear by Langmuir film and FM. In addition, the hysteresis loop was also observed. Finally, a comparison of our preparations ($X_{\text{Hel 13-5}}$ = 0.05 and 0.1) with Surfacten (Surfactant TA: what is clinically used for neonatal distress syndrome in Japan) in terms of Langmuir film properties and FM micrographs reveals that both preparations are similar in surface behavior and morphology within the spread monolayer. These results observed above indicate that our present preparations containing a small amount of Hel 13-5 have a high potential of clinical use for RDS patients.

It is necessary to extend the study to compare them with *in vivo* analysis using an animal such as a rat.

First of all, it is essential to measure the surface activity of a novel synthetic surfactant preparation (DPPC/PG/PA/Hel 13-5) and bovine-derived surfactant (Surfacten) in biological conditions such as in saline and at 37^{o}C. Next, to make clear the *in vivo* ability for these preparations, a sequential dynamic compliance and pressure (P)-volume (V) curves were measured. In this chapter, the author will test the *in vivo* efficacy of the DPPC/PG/PA/Hel 13-5 preparation using a surfactant-deficient rat model to improve lung compliance by comparison with Surfacten.

IV-2 Results and Discussion

IV-2-1 Surface Adsorption

Surface adsorption is the process where surfactant molecules move from a liquid subphase to an air/liquid interface to form a monolayer(1). As a result, the adsorbed surfactants decrease surface tension accordingly. That is, monitoring a change in surface tension against time after addition of surfactants to an agitating subphase provides information on the molecular dynamics corresponding to the motion of native pulmonary surfactants from the tubular myelin structure to the surface. A substantial improvement in adsorption is found when SP-B and SP-C are added independently or together to mixtures of synthetic lipids(2-5). The activity of facilitating adsorption correlates with the ability for SP-B and SP-C to disrupt and fuse phospholipid bilayers and to promote the insertion and transfer of phospholipids into vesicles and surface films(6,7). Adsorption measurements for the surfactant preparations of the DPPC/PG/PA lipid mixture (PL), PL plus 1 wt% Hel 13-5, PL plus 2.5 wt% Hel 13-5, PL plus 5 wt% Hel 13-5, PL plus 10 wt% Hel 13-5, and Surfacten (Surfactant TA) at 37 ^{o}C are shown in Figure 33A. PL alone shows very slow adsorption to the surface due to the absence of proteins or peptides(8). The addition of Hel 13-5 improves an ability of adsorption of the PL mixture. All of the PL plus Hel 13-5 preparations except for PL plus 1 wt% Hel 13-5 adsorb to equilibrium values within 2 min after injecting the preparation into subphase. The surfactants are adsorbed more rapidly, and the equilibrium values (40.7, 34.2, and 21.6 mN m^{-1}) decrease with increasing Hel 13-5 contents from 2.5 to 10 wt%, respectively. DPPC, which is the major component in PL, generates high surface tension because it has a high energy barrier to leave subphase aggregates and to enter the interface. As a result of the addition of Hel 13-5, the barrier is reduced and then more surfactant molecules are adsorbed to the interface. On the other hand, PL plus 1 wt% Hel 13-5 indicates poor adsorption similarly to PL without Hel 13-5, meaning that the addition of 1 wt% Hel 13-5 is too small to break down the barrier for the ability of adsorption. For Surfacten, its surface pressure reaches the equilibrium value of 24.4 mN m^{-1} within 2 min. The equilibrium surface tension value, which directly reflects surface activity of surfactants, depends on a weight basis (or a molecular weight) of contained proteins and peptide in the preparations(1). In terms of the time to reach the equilibrium values, therefore, the preparations containing more than 2.5 wt% Hel 13-5 are effective to improve the adsorption kinetics of the PL mixture and are comparable to Surfacten.

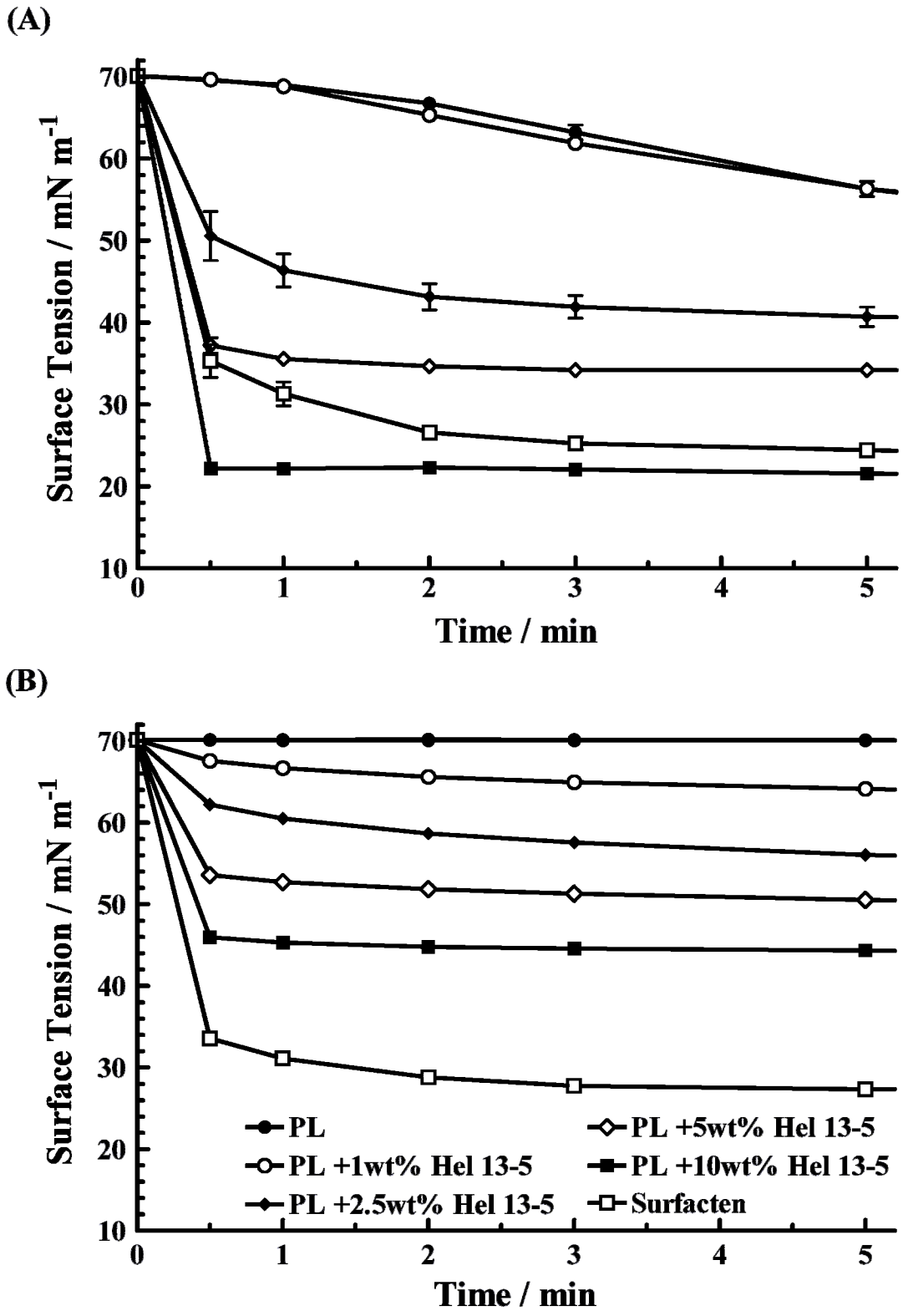

Figure 33. Adsorption (A) and Spreading (B) of the DPPC/PG/PA mixture (PL; ●), PL plus 1 wt% Hel 13-5 (○), PL plus 2.5 wt% Hel 13-5 (♦), PL plus 5 wt% Hel 13-5 (◊), PL plus 10 wt% Hel 13-5 (■), and Surfacten (Surfactant TA; □) at 37°C. Data are surface tension values (mean ± SD, n = 5 – 8) as a function of time.

IV-2-2 Surface Spreading

A lateral diffusion of surfactants at the air/liquid interface after placing a droplet of surfactant dispersions onto the surface from the air is known as a surface spreading. In clinical treatments for NRDS, the dispersion containing surfactant preparations is practically instilled through the trachea, and then results in contacting an air/alveolar liquid interface to spread rapidly. As a result, the spread of surfactants induces a decrease in surface tension(8). Thus, the process is possible to be simply investigated from the *in vitro* surface spreading measurement. Rapid spreading of lipids is a prerequisite for formulation of an effective surfactant preparation(9). Surface spreading measurements for the surfactant preparations (PL, PL plus 1 wt% Hel 13-5, PL plus 2.5 wt% Hel 13-5, PL plus 5 wt% Hel 13-5, PL plus 10 wt% Hel 13-5) and Surfacten (Surfactant TA) at 37 ^{o}C are shown in Figure 33B. Surface tension values of PL are almost constant (~70 mN m^{-1}) against time. This result is also because of the existence of the energy barrier for DPPC molecules to move laterally from vesicular suspensions to the surface. However, the surface activity is enhanced by addition of a small amount of Hel 13-5 as the same as the above-mentioned surface adsorption. The improvement depends on additional amounts of Hel 13-5, supporting its good spreading ability. In addition, all of the preparations with Hel 13-5 spread rapidly to reach equilibrium values within 2 min after placing the preparations onto the surface. On the other hand, Surfacten shows the rapid spreading after 2 min with similar tendency of the preparations with Hel 13-5. The surface activity of Surfacten is the highest of all data obtained here. In this respect, it present seems that Hel 13-5 is less effective than SP-B and SP-C. As mentioned above, however, the difference in equilibrium values can be elucidated by the fact that the molecular weight of Hel 13-5 (2.2 kDa) is quite small compared to SP-B (8.7 kDa)(10,11) and SP-C (4.2 kDa)(12,13). Furthermore, the similar behavior has been reported for the surfactant substitute containing different peptides(9,14).

IV-2-3 Dynamic Lung Compliance

Lung compliance is an index of the softness and allows us to understand the mechanical state of lung function, the efficacy of surfactant preparations, and the spreading rate of the preparations onto the air/alveolar interface. From the practical perspective, the patients suffered from RDS are treated with mechanical ventilation in the presence of positive end-expiratory pressure (PEEP) after instillation of surfactant preparations. In the experimental aspect, Hawgood and co-workers(15) reported that the surfactant preparation containing an exogenous analogue peptide did not improve

dynamic compliances unless PEEP was loaded. Ogawa and colleagues(16) showed that neither natural surfactants nor synthetic lipid mixtures without surfactant proteins improve compliances of preterm rabbits in the absence of PEEP. Therefore, PEEP amount was kept constant (4 cm H_2O) throughout the ventilation periods in this study. From the results of the *in vitro* surface activity as mentioned above, it is suggested that the PL preparations containing more than 2.5 wt% Hel 13-5 have a capability of effective pulmonary substitutes. As clinical and commercial demands, however, the substitutes with a small amount of mimic peptides are needed. Therefore, the surfactant preparations (with 2.5 and 5 wt% Hel 13-5) are representatively focused in the present *in vivo* study. Dynamic compliances as a function of time for control (saline), PL alone, PL plus 2.5 wt% Hel 13-5, PL plus 5 wt% Hel 13-5, and Surfacten in ventilated surfactant-depleted rats are shown in Figure 34. After instillation, control group indicates a sharp reduction in compliance and then retains the value of ~0.30 mL/cm H_2O/kg. The similar behavior has been previously reported for saline-instilled rabbits(17). In contrast, all of the groups treated with the surfactant preparations generate compliance of more than 0.37 mL/cm H_2O/kg. The lung functions for these groups are improved more than that for the control group, and the differences are statistically significant ($p < 0.001$). It is suggested that administration of the surfactants significantly improves lung function. Among the PL preparations, the compliance of PL plus 5 wt% Hel 13-5 is significantly different from that of PL alone ($p < 0.01$). On the other hand, there is no statistic difference between PL plus 2.5 wt% Hel 13-5 and without the peptide. In terms of the dynamic compliance, the addition of more than 5 wt% Hel 13-5 is needed to exert the effect of Hel 13-5. In comparison to Surfacten, the efficacy of PL alone is significantly inferior in lung compliance ($p < 0.01$). However, the dynamic compliances are not significantly different among PL plus 2.5 wt% Hel 13-5, PL plus 5 wt% Hel 13-5, and Surfacten. Nevertheless, it is noticeable that the efficacy of PL plus 2.5 and 5 wt% Hel 13-5 in enhancing lung function is comparable to that of Surfacten.

IV-2-4 Pressure (*P*)–Volume (*V*) Curve

The quasi-static *P*-*V* curve has been measured to evaluate lung mechanics. In previous study (Chapter III), we had measured the *in vitro* hysteresis curves (or the cycled surface pressure-molecular area isotherms) for the spread films of the DPPC/PG/PA (68:22:9, wt/wt/wt) mixtures with Hel 13-5 and Surfacten(18). These results demonstrated that the lung surfactant or PL preparations of DPPC/PA/PG/Hel 13-5 could be equivalent to Surfacten in surface activity and behavior. However, it is

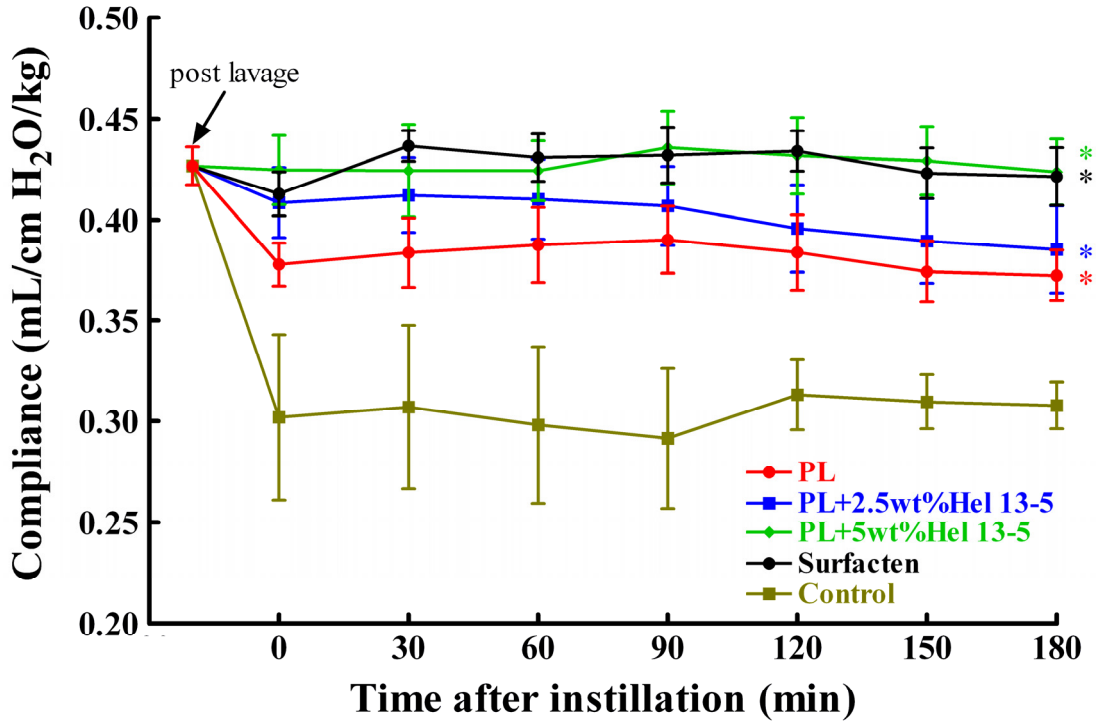

Figure 34. Dynamic compliances of rats ventilated with 4 cm H_2O PEEP and treated with 120 mg/kg of PL (●), PL plus 2.5 wt% Hel 13-5 (■), PL plus 5 wt% Hel 13-5 (♦), Surfacten (Surfactant TA; ●), and saline (control; ■) during the course of the experiment. Data values are given as mean ± SEM for $n = 6 – 8$. *: $p < 0.001$ versus control.

still necessary to carry out the direct (*in vivo*) experimental measurements due to the crucial differences between *in vivo* and *in vitro* experiments. Figure 35 shows the *P-V* curves for control (saline), PL alone, PL plus 2.5 wt% Hel 13-5, PL plus 5 wt% Hel 13-5, and Surfacten. The inclination of *P–V* curves means static lung compliance. Therefore, the inclination of *P–V* curves shows sharply when the compliance is restored. That is, the *P–V* results can be elucidated using maximum lung volumes at a certain higher pressure. The *P–V* curves of the groups treated with PL alone, PL plus 2.5 wt%, and 5 wt% Hel 13-5 are not significantly different from that treated with Surfacten. It is indicated that the PL preparations exert a similar effect in static lung mechanics to Surfacten. Considering the results dynamic lung compliances (Figure 35), it is suggested that a smaller additional amount of Hel 13-5 (2.5 wt%) to the PL mixture would be sufficient in designing pulmonary substitutes, because very small contents of synthetic peptides for the surfactant designing is desired in the clinical field as well as commercial scene. On the other hand, the control group has a static lung volume of 24.4 ± 3.3 mL/kg at an airway pressure of 35 cm H_2O and decreases lung compliance as

indicated by a change in the slope of the *P-V* curve during inflation at higher pressures compared to the other curves. At 35 cm H_2O, the maximum volumes of PL alone, PL plus 2.5 wt%, 5wt % Hel 13-5, and Surfacten are significantly different from that of control ($p < 0.01$ for PL and $p < 0.001$ for the others). In addition, the difference in the volumes between PL plus 5 wt% Hel 13-5 and PL without the peptide is significant ($p < 0.05$). However, there is no significant difference between the PL preparations and Surfacten. These results also support the above-mentioned suggestion for the additional amount of Hel 13-5. During lung inflation, the *P-V* curve of the control group has an infection point at 20 cm H_2O, where a recruitment of lung units begins to occur(19). In contrast, the other groups show the different infection points at 15 cm H_2O. The difference suggests that administration of surfactant preparations used in this study eases the alveolar recruitment, and accordingly generates better compliances and larger lung volumes at 35 cm H_2O.

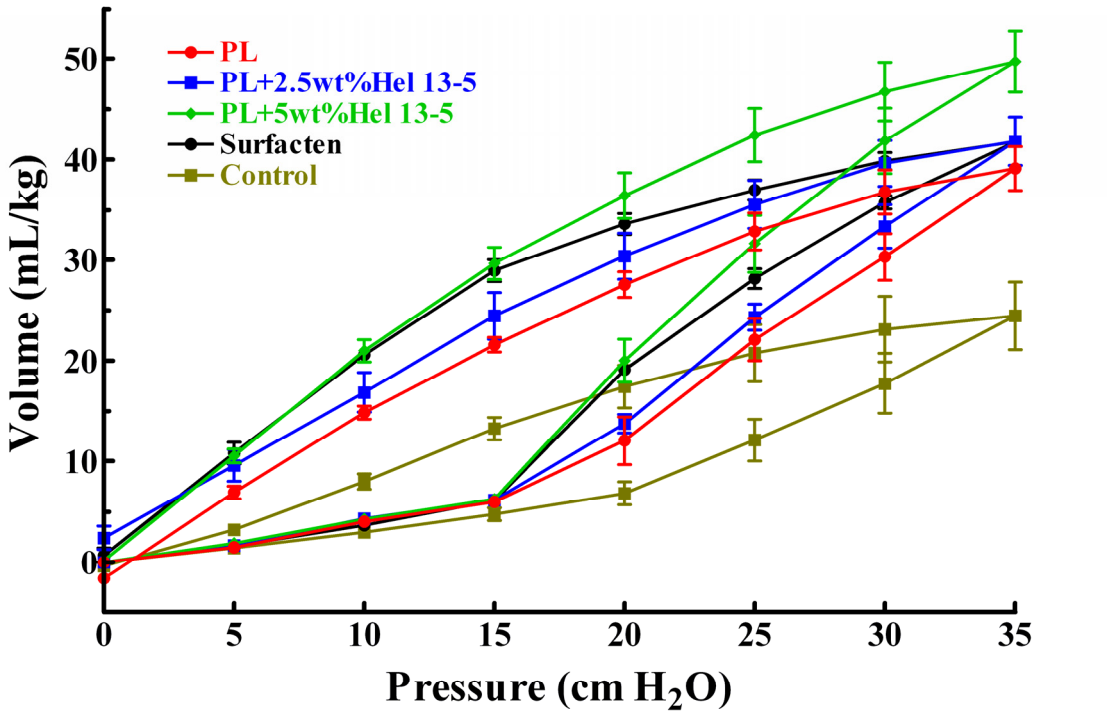

Figure 35. Inflation and deflation pressure (*P*) – volume (*V*) curves of surfactant-depleted rats treated with 120 mg/kg of PL (●), PL plus 2.5 wt% Hel 13-5 (■), PL plus 5 wt% Hel 13-5 (♦), Surfacten (Surfactant TA; ●), and saline (control; ■) after a period of ventilation. Data values are given as mean ± SEM for $n = 5 – 7$.

IV-3 Conclusion

Synthetic surfactant peptides (Hel 13-5) based on the human sequence of SP-B confer surface activity on the DPPC/PG/PA lipid mixtures (68:22:9, wt/wt/wt). The *in vitro* studies on surface activity can represent essential ground work for the application of the surfactant preparations with Hel 13-5 to *in vivo* models of surfactant deficiency. The DPPC/PG/PA dispersions containing 2.5 wt% or 5 wt% Hel 13-5 result in improvement of dynamic lung compliance comparable to the commercial surfactant preparation [Surfacten (Surfactant TA), an analogue preparation to Survanta] in ventilated surfactant-depleted rats. In addition, quasi-static *P*-*V* curves support the efficacy of the surfactant preparations containing Hel 13-5. Based on the clinical and commercial point of view, this study demonstrated that the DPPC/PG/PA preparation containing 2.5 wt% (5 wt%) Hel 13-5 can be safely administered intratracheally to improve lung function in the surfactant-deficient rat. The Hel 13-5 peptide may be important for the formulation of clinical surfactant preparations.

Experimental Procedures

Animal Protocol

All animal experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals as adopted and promulgated by the Declarations of Helsinki and of Faculty of Pharmaceutical Sciences, Nagasaki International University and were approved by the Committee of the Ethics of Animal Experimentation of Nagasaki International University. Male SPF-designed Wistar/ST rats (Japan SLC, Inc., Hamamatsu, Japan) weighing 250 to 350 g were anesthetized by injecting pentobarbital sodium, 25 mg/kg, intraperitoneally. After placement of a tracheal cannula, the rats were supported on an infant ventilator (Bear Cub, model BP 2001; SOMA Technology, Inc., CT) with 100% oxygen, a tidal volume (V_T) of 8 mL/kg body weight, positive end-expiratory pressure (PEEP) of 4 cm H_2O, and a respiration rate of 40 breaths/min (Scheme 8). A tracheal pressure and V_T were monitored using an A/D transducer (YTS, Tokushima, Japan). The lungs were gently lavaged 10 times with 5 ml of 0.15 M NaCl warmed to body temperature. Sixty minutes after the last lavage, surfactant preparations were instilled intratracheally at a dose of 120 mg lipids/kg body weight in a volume of 4 mL/kg body weight. The same volume of 0.15 M NaCl was administered to control animals in a similar fashion. Subsequently, a peak inspiratory pressure (PIP) was

measured for a period of time up to 180 min after surfactant instillation at 15-min intervals. Throughout the experiment period, V_T and PEEP were kept constant at 8 mL/kg body weight and at 4 cm H_2O, respectively. Each group contained six to nine animals. Tracheal pressures were corrected for body weight. Dynamic lung compliance was calculated by dividing V_T by (PIP–PEEP) and body weight (mL/kg/cm H_2O).

Pressure–Volume curves

After ventilation periods, the animals were killed with overdose of pentobarbital sodium, and the abdomen was opened to inspect the diaphragm for evidence of pneumothorax. Then, the diaphragm was opened and the lungs were allowed to degas *in situ*. Quasi-static pressure (P)–volume (V) curve measurements were made by stepwise inflating and deflating the lungs from 0 to 35 cm H_2O by 5 cm H_2O with 5- or 20-mL syringes via three-way stopcock. After 10-s stabilization at least at each step, the corresponding volumes were expressed as mL/kg body weight.

Statistical Analysis for Chapter IV

Surface-activity data are presented as mean ± SD, with five measurements at least for each data point. Data from the lung compliances and P–V curves are given as mean ± SEM, with five rats at minimum in each experimental group. The significance of differences among multiple experimental groups was compared through one-way analysis of variance (ANOVA). In such cases as the F test showed a significant difference ($p < 0.05$) among groups, comparisons of different groups were made with the Student–Newman–Keuls test. A value of $p < 0.05$ was considered to indicate a significant difference.

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