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The Change of Secretory Activity of the Alveolar Type II Cell During Acute Alveolar Injury Induced by *N*-Nitroso-*N*-Methylurethane

Young Man Lee*, In Sook Bang and Suck Kang Lee

Department of Physiology, School of Medicine, Yeungnam University, Taegu 705-030
Taegu Catholic University, Taegu 705-034*

= Abstract =

In the animal model of acute respiratory distress syndrome (ARDS) induced by *N*-nitroso-*N*-methylurethane (NNNMU) the secretory activity of alveolar type II cells during acute alveolar injury was investigated by determining phospholipid and pulmonary surfactant associated proteins in crude surfactant. The mechanism of the secretory change was studied by determination of DNA and RNA levels in the lung tissue. After induction of acute alveolar injury with NNNMU, pulmonary hemorrhage , atelectasis and gross hypertrophy were observed. Seven days after NNNMU treatment the level of total DNA in lung homogenate was increased markedly indicating that a hypertrophy was induced by cellular proliferation. Although the total DNA level increased, the RNA/DNA ratio was gradually decreased after NNNMU treatment. Seven days after NNNMU treatment the RNA/DNA ratio returned to the normal control level. During the acute alveolar injury, phospholipid and surfactant associated proteins were reduced significantly as compared with the control, implying that the secretory activity of alveolar type II cells was altered during acute alveolar injury induced by NNNMU. The protein content in crude surfactant during peak injury(7 days after NNNMU) was decreased significantly but phospholipid/protein ratios were identical in both control and NNNMU treatment groups. SDS-PAGE of proteins in crude pulmonary surfactant showed a decrease in major surfactant associated protein(M.W. 38,000) during acute alveolar injury.

The present study may suggest that while alveolar type II cells proliferate markedly, transcription of alveolar type II cell gene was inhibited by an unknown mechanism such as DNA methylation induced by NNNMU. Such an inhibition of transcriptional activity is thought to be associated with the decreased secretory activity of alveolr type II cells, which may lead to pulmonary atelectasis and edema during the acute alveolar injury.

Key Words: ARDS, Alveolar type II cell, Pulmonary surfactant, NNNMU

INTRODUCTION

The alveolar type II cell is known to repair the lung injury and damage by its proliferation and differentiation into alveolar type I cells (Simnett, 1974). Besides their activity in repairing lung injury, alveolar type II cells have an important role in alveolar stabilization through secretion of pulmonary surfactant (Crapo et al, 1978)

The disruption of the pulmonary surfactant system leads to various kinds of diseases and adult respiratory distress syndrome(ARDS) is one such disease, in which an alveolar injury

accompanies respiratory insufficiency(Chander & Fisher, 1990). But up to now the exact pathogenesis of ARDS has not been elucidated clearly.

The acute alveolar injury caused by various conditions shows necrotic alveolar cells, hemorrhage and edema which produce respiratory insufficiency in humans and experimental animals (Wayne & Albert,1990).

In neonatal respiratory distress syndrome (NRDS) the abnormal secretory activity of alveolar type II cells is one of the causes of respiratory insufficiency and alveolar instability (Metcalfe et al, 1982). It has been suggested that the abnormal surface activity of surfactant is associated with the causes of NRDS (Camniti & Young, 1991).

While Ashbaugh and colleagues (1967) reported that the surface activity of pulmonary surfactant was decreased in ARDS, Pison et al (1989) suggested that the possibility of surfactant decrease in ARDS was unlikely.

Although several hypotheses, such as direct epithelial damage, derangement of phospholipid synthesis, alveolar edema, and destruction of surfactant by bacterial lecithinase have been proposed (Shepard et al, 1980; Pattle & Burgess, 1961), the mechanism of disruption of surfactant system in ARDS has not been identified clearly.

Recently, *N*-nitroso-*N*-methylurethane(NNNMU) has been introduced as an agent inducing an ARDS-like condition in experimental animals. The acute alveolar injury induced by a single subcutaneous injection of NNNMU resembles ARDS very closely(Ryan, 1990). Because of the similarities between ARDS and NNNMU induced alveolar injury, this NNNMU-induced ARDS model has been used widely in the study of ARDS.

Thus, in the present study we have attempted to evaluate changes in secretory activity of alveolar type II cells in NNNMU induced ARDS in rats. The secretory activities of alveolar type II cells during early and peak injury period were measured in crude surfactant. In addition, DNA and RNA in lung homogenates were determined to study the molecular mechanism associated with the secretory change in alveolar type II cells.

MATERIALS AND METHODS

The present study was carried out on adult Sprague-Dawley rats weighing 200 to 250 gm. The rats received a single subcutaneous injection of 6 mg NNNMU/kg of body weight (Ryan et al, 1981) in 2.0 ml of normal saline and were sacrificed 2, 4, or 7 days after the treatment. The control rats received no injection of saline, since 2.0 ml of normal saline was considered to cause negligible effect.

Rats were sacrificed by bleeding through abdominal aorta. The lungs were excised quickly and the trachea was cannulated. Then the lungs were lavaged eight times via the cannulated trachea with 8.0 ml of TN buffer (0.01 M Tris-HCl at pH 7.4, 0.15 M NaCl) and collected 6.0 ml of bronchoalveolar lavage fluid.

Lung weights were determined after extraction of lung fluid by compression with Whatman No. 2 filter paper and lung weight /body weight ratios were calculated. After that the lungs were stored in a deep freezer (Forma Scientific, USA) at −73℃ for the determination of DNA and RNA levels in the lung tissue.

Pulmonary surfactant was purified by centrifugation of bronchoalveolar fluid. Bronchoalveolar fluid was centrifuged at 200g for 10 minutes at 4℃ and the supernatant(supernatant I) was collected. The cellular pellet was then resuspended in TN buffer and centrifuged again at 200g for 10 minutes at 4℃ and again the supernatant(supernatant II) was collected. Supernatants I and II were pooled and then centrifuged at 27,000 g for 1 hour at 4℃. The resulting pellet was suspended in 3.0 ml of TN buffer. This material is referred to as crude surfactant.

To determine surfactant phospholipid and

surfactant associated proteins, total lipids and proteins were extracted by the method of Chan and Knowles(1973). The crude surfactant in TN buffer was extracted with a 3.0 ml solution of butanol/diisopropylether,4:6,v/v) at room temperature for an hour to partition the lipid into the organic phase and the protein into the aqueous phase.

After extraction the mixture was centrifuged at 2000 r.p.m. for 10 minutes at 4℃ to separate the aqueous and the organic phases. The organic phase was used for the determination of phospholipid phosphorus and the aqueous phase for protein analysis.

Phospholipid phosphorus was determined by the method of Chalvardjian and Rudnicki (1970) and the phospholipid content was calculated according to Corbet et al(1983).

The aqueous phase was dissolved by addition of 1 % SDS and 2.5% 2mer -captoethanol and warmed in boiling water for 10 minutes. Then the protein content was determined by the method of Bradford(1976).

SDS-PAGE was carried out by the method of Hames and Rickwood (1981). For the SDS-PAGE, each sample applied to the well contained 15 μg of protein. The stacking gel contained 4 % polyacrylamide and the sepa-rating gel contained 10 % polyacrylamide. Electrophoresis was performed at 20 mA per gel for 30 minutes followed by 40 mA for 60 minutes. For the estimation of molecular weights, low molecular weight standards (phosphorylase b: 94 KD, albumin: 67 KD, ovalbumin: 43 KD, carbonic anhydrase: 30 KD, Pharmacia) were used.

Lungs were homogenated with Virtis 45 homogenator in 2 % citrate buffer. DNA and RNA were extracted by Schneider's method (Schneider, 1945). DNA was determined by Burton's method(Burton 1956). RNA was determined by the method of Schneider(1945).

Statistics: Difference between mean values in the control and NNNMU treated rats were evaluated by the SPSS/PC+ program using Student's t-test.

RESULTS

The experimental animals were divided into a normal control group and experimental groups which were designated as day 2, day 4, or day 7 groups.

In table 1, the lung weight/body weight ratio (L/B ratio) and the total DNA level in the lung tissue are presented.

Seven days after injection of NNNMU, the L/B ratio and the total DNA level were increased significantly ($p<0.005$) compared with normal control rats implying that growth of the lung has been induced by NNNMU treatment. During the early injury, L/B ratios and total DNA were not different from normal control levels but seven days after injection of NNNMU, total DNA level and L/B ratio had

Table 1. Change of lung weight/body weight ratio (L/B ratio)$\times 10^{-3}$ and total DNA content of both lungs during acute alveolar injury induced by *N*-nitroso-*N*-methylurethane (NNNMU)

	Days post NNMU			
	NC (n=10)	2 (n=10)	4 (n=4)	7 (n=7)
(L/B)$\times 10^{-3}$	5.8±1.40	6.6±1.49	5.1±0.54	10.6±3.00*
Total DNA, mg/lung	3.2±0.68	5.0±0.77**	4.0±0.66	8.7±3.21*

Values are mean±S.D.

*$p<0.01$ vs normal control, **$p<0.001$ vs normal control.

NC: normal control.

Table 2. Phospholipid content of partially purified pulmonary surfactant during acute alveolar injury by *N*-nitroso-*N*-methylurethane

	Days post NNNMU			
	NC (n=10)	2 (n=10)	4 (n=4)	7 (n=7)
Phospholipid(μmol/gm of wet lung)	11.0±2.16	10.8±2.66	8.9±1.31*	5.2±1.31**

Values are mean±S.D.
*p<0.05 vs normal control, **p<0.001 vs normal control.
NC: normal control.

Table 3. Protein content (mg/gm of wet lung) and phospholipid/protein (μmol/mg) ratio of partially purified surfactant during acute alveolar injury induced by *N*-nitroso-*N*-methylurethane in rats

	Days after NNNMU			
	NC (n=10	2 (n=10)	4 (n=4)	7 (n=7)
Protein(mg/gm of wet lung)	5.4±1.55	4.0±1.47	4.8±1.03	2.4±0.85*
Phospholipid/Protein(μmol/mg)	2.1±0.83	3.0±0.97	2.0±0.39	2.7±0.85

Values are mean±S.D.
**p<0.001 vs normal control.
NC: normal control.

increased markedly($p<0.001$), which indicated the proliferation of alveolar type II cells caused by the acute alveolar injury.

As shown in table 2, there was time-dependent decrease of phospholipid in the crude surfactant. While non-significant decrease of phospholipid was observed 2 days after the treatment, a significant gradual reduction of phospholipid was seen at 4 and 7 days after NNNMU treatment. In particular, seven days after NNNMU injection, the gross pulmonary hemorrhage, general atelectasis and hypertrophy were noted(data not illustrated).

Table 3, shows the amount of the protein and the phospholipid/protein ratio. Seven days after NNNMU injection, protein content in the crude surfactant was reduced significantly($p<0.05$)as compared with normal control, however the phospholipid/protein ratios were identical in all groups.

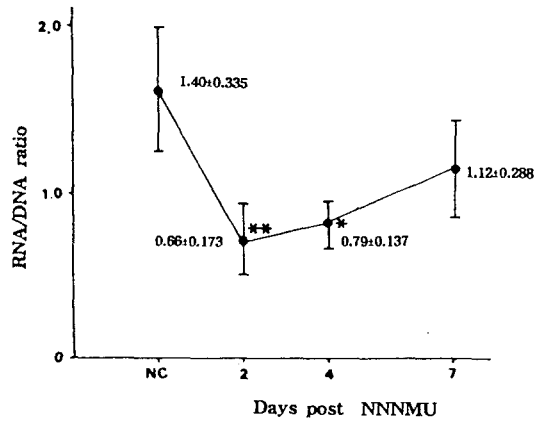

Fig. 1. RNA/DNA ratio during acute alvcolar injury induced by NNNMU(N-nitroso-N-methylurethan) in rats. RNA/DNA ratio decreased gradually after NNNMU injection but at day post 7 RNA/DNA ratio returned to normal control level.
**p<0.01 vs normal control, **p<0.001.*
NC: normal control

As a parameter of transcriptional activity the RNA/DNA ratio was determined. As depicted in Fig. 1. the ratio decreased significantly($p< 0.001$) 2 and 4 days after NNNMU treatment, but it had returned to the control level 7 days after the treatment.

Fig. 2. shows a representative SDS-PAGE slab gel electrophoretogram of crude surfactant protein.

The major surfactant apoprotein(M.W. 38, 000), referred to as surfactant protein A, and two minor bands were observed in all phases of injury. From the electrophoretogram it was evident that the decrease of surfactant associated proteins after NNNMU was time-dependent. It is interesting to note that seven days after NNNMU injection, the density of surfactant associated proteins in the gel was nadir. Though the amount of surfactant associated proteins was not determined in the gel, it seems that phases of acute alveolar injury are correlated with the changes of the phospholipid content, morphology and protein content.

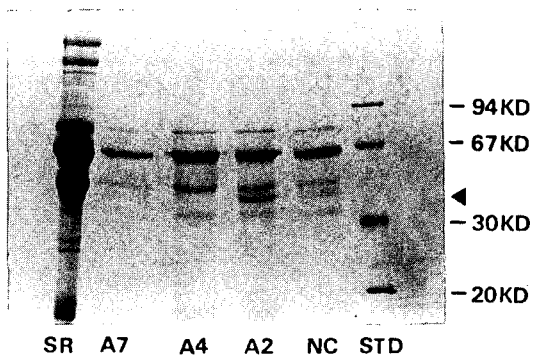

Fig. 2. *Representative SDS-polyacrylamide slab gel electrophoretogram, stained with Coomassic blue, of crude surfactants during acute alveolar injury induced by NNNMU.*
Each surfactant protein sample contained 15 μgm of total protein.
The major apoprotein(M.W. 38KD) was decreased time-dependently during acute alveolar injury.
NC; normal control.
2; 2days post NNNMU.
4; 4days post NNNMU.
7; 7days post NNNMU.
SR; serum.
STD; M.W. standard

DISCUSSION

To mimic conditions of ARDS, acute alveolar injury in rats was induced by *N*-nitroso-*N*-methylurethane (NNNMU). In this model of ARDS the secretory activity of alveolar type II cells was evaluated by measuring surfactant phospholipid and surfactant associated proteins.

Seven days after NNNMU injection gross pulmonary hemorrhage, atelectasis and hypertrophy were observed in all NNNMU treated rats(data not shown). According to Ryan(1990) a single subcutaneous injection of 5-8 mg/kg, body weight of nitrosourethane to a dog causes acute alveolar injury indistinguishable by light and electronmicroscopy from that seen in humans. Along with these gross findings in ARDS, it was revealed that L/B ratio and total DNA were increased markedly seven days after NNNMU injection.

As the proliferation and differentiation of type II cells have been well documented as a cellular mechanism of repair after lung injury (Clement et al, 1990), the increase in L/B ratio and total DNA content in the present study would indicate that proliferation and differentiation of the alveolar type II cell into type I cell were facilitated.

The quantity of surfactant phospholipid decreased during the early and peak injury period. These findings are very interesting in association with the atelectasis followed by pulmonary hypertrophy. Such a decrease in surfactant phospholipid might be associated with atelectasis and pulmonary edema and ultimately may lead to pulmonary hypertrophy.

The pulmonar surfactant system has been reported to be disrupted in various pathological conditions including NRDS and ARDS (Mendelson & Boggaram, 1991). Thus the atelectasis and hypertrophy observed in this

animal model appear to be closely related to the decrease of surfactant secretion. In addition to a decrease in phospholipid content, a decrease of surfactant associated protein indicated the altered secretory activity of the type II cells.

Timothy and colleagues(1991) have recently insisted that compositional change, decreases in total phospholipids and protein in crude surfactant altered the surface activity of the surfactant in ARDS patients.

Functionally surfactant associated proteins were reported to be closely related to the adsorption and recycling of phospholipid in alveoli (Chander & Fisher, 1990). According to Haagman and van Golde(1991) surfactant associated proteins enhance the adsorption of phospholipids to an air-fluid interface. Young and colleagues(1989) suggested that surfactant protein A and lipids are taken up *in vivo* and preferentially incorporated into lamellar bodies of alveolar type II cells by endocytosis. In this regard, decrease of surfactant associated proteins might cause the derangement of surfactant surface activity and metabolism in alveolar type II cells in acute alveolar injury induced by NNNMU in the present study.

Although protein content of crude surfactant may be contaminated with plasma protein, SDS-PAGE electrophoretograms showed a time-dependent decrease of surfactant associated proteins after NNNMU injection. It has been demonstrated that pneumonectomy causes alveolar type II cell hyperplasia and to increase the secretion of surfactant from alveolar type II cells (Lee et al, 1991). However, in this study, the secretion of surfactant was decreased in spite of the alveolar type II cell proliferation. It should be noted that NNNMU is one of the DNA methylation agents which can cause mismatching of base pairs during DNA synthesis (Herrold, 1967; Albert et al, 1989). It is thus possible that methylation of DNA may cause inhibition of transcription and consequently decrease the RNA/DNA ratio in the early phase of acute alveolar injury. Accordingly, reduction of transcription in alveolar type II cells during the early phase of injury may be related to decreased secretion of surfactant associated proteins and phospholipid.

Reduction of transcription during alveolar type II cell proliferation might be one of the molecular mechanisms associated with ARDS, yet there are a variety of factors causing ARDS (Jobe & Ikegami,1987). It is difficult to confirm whether the reduction of transcription in the alveolar type II cell is the sole mechanism of the decreased surfactant secretion in the present study.

In conclusion, the present study suggests that a reduced secretion of surfactant phospholipid and proteins might cause atelectasis and pulmonary edema in NNNMU induced acute alveolar injury. The reduction of phospholipid and surfactant associated proteins might be due to a reduced transcriptional activity during the early phase of NNNMU induced acute alveolar injury.

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