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ANP Inhibits Surfactant Secretion from Isoproterenol Stimulated Alveolar Type II Cells

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In order to investigate the effect of ANP on surfactant secretion from alveolar type II cell(AT II cell) during circulatory derangement in adult respiratory distress syndrome (ARDS), the secretion of surfactant from AT II cells was evaluated in purely isolated AT II cultures from rat lungs. For the simulation of sympathetic stimulation during circulatory derangement, primary AT II cultures were incubated with isoproterenol and IBMX. In this isoproterenol stimulated AT II cells, ANP were added in the media for the investigation of effect of ANP on surfactant secretion from AT II cells. For the evaluation of surfactant secretion, [^{3}H]-methylcholine was incorporated and the level of radiolabelled choline chloride secreted from the cells was determined. As previously reported, isoproterenol and IBMX stimulated surfactant secretion from AT II cells. Isoproterenol showed synergistic increase of surfactant secretion with IBMX in AT II cells. In isoproterenol stimulated AT II cells, physiological level of ANP inhibited the secretion of surfactant in primary cultures of AT II cells. On the basis of these experimental it is suggested that, in association with ciculatory change during ARDS, increased secretion of ANP by the pulmonary edema, hypoxia and congestive heart heart failure might aggravate the symptoms of ARDS by reduction of surfactant secretion from AT II cells.

Key Words: Alveolar type II cells, Surfactant, ARDS, ANP

INTRODUCTION

Pulmonary surfactant, a substance which is synthesized and secreted by the alveolar type II cells, stabilizes alveoli by reducing the surface tension of air-fluid interface (Mason & Voelker, 1988). Decreased secretion of surfactant or disruption of surfactant system can be caused by various conditions; such as pulmonary immaturity (Jobe & Ikegami, 1987) sepsis, multiple organ system failures (Tighe et al., 1987). These kinds of factors cause the adult respiratory distress syndrome (ARDS). While it has been well documented that the main cause of infant respiratory distress syndrome (IRDS) is decreased secretion of surfactant from AT II cells in neonate (Jobe & Ikegami, 1987), the relation between decreased secretion of surfactant from AT II cells and ARDS has not well been documented.

Recently some reports suggested that decreased secretion of surfactant or disruption of surfactant system prior to pulmonary edema or formation of hyaline membrane might cause ARDS (Caminiti & Young, 1991). For the treatment of IRDS and ARDS, investigators have tried various agents which have been partially effective in IRDS and ARDS (Hollingworth & Gifillan,1984). For IRDS, steroid hormone is one of the most effective agent and beta agonists have also been known to be effective to increase the secretion of surfactant from AT II cells (Liggins, 1969).

On the other hand, treatment of ARDS still has not been established because the causes of ARDS has not confirmed and multifactorial. Recently decreased secretory activity of AT II cells has been proposed as one of the causes of ARDS (Lewis & Jobe, 1993). In this regard beta-agonists have been commonly used to increase the secretion of surfactant from AT II cells and to improve cardiovascular function during ARDS. In many cases of ARDS, circulatory failure ensues and this could be one of the causes of death in ARDS. During circulatory failure, increased ANP

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level and increased sympathetic tone for the compensation of the volume overloading in the heart has been well established (Wilson, 1991). But in this condition, little is known about the effect of the increased level of ANP on the secretory activity of AT II cells in the lung during ARDS.

To study the secretion of surfactant from AT II cells with secretagogues, various kinds of experimental models have been suggested (Hollingworth & Gifillan, 1984). *In vitro* study, the interpretation of secretion study with whole animal is very difficult by the cellular heterogeneity of the lung. The lung contains more than 40 different cell types (Breeze & Wheeldon, 1977). Even with lung slice or intact excised lung, it is very difficult to evaluate the effect of secretagogues on surfactant secretion. Since Kikkawa and Yoneda (1984) developed the isolation and primary culture of AT II cell, several modified methods for AT II cell culture have been introduced for surfactant study (Dobbs, 1990). Even with cultured AT II cell it has been very difficult to obtain consistent result of secretion study partly because of the contamination of other cells during primary culture of AT II cells. In addition, Dobbs and Mason(1979) suggested that the digestive enzyme trypsin which is popular in dissociating lung tissue affects the secretory activity of AT II cells. To solve above mentioned shortcomings the primary AT II cell culture was carried out by the method of Dobbs'(1986) with which almost pure AT II cells were harvested in the present study.

In these primary AT II cultures, the effect of ANP on the secretory activity stimulated by isoproterenol was studied to evaluate the effect ANP on the secretion of surfactant from AT II cells during ARDS.

METHODS

Sprague-Dawley male rats (B.W. 150~170 gm) were used for isolation of AT II cells from the lung. All chemicals otherwise stated were from Sigma Chemical Co (USA). For the isolation of AT II cells elastase was purchased from Worthington (USA). Pentobarbital from Abbot (USA), nylon mesh from Tetko Inc. (USA). Falcon bacteriologic culture plate from Becton Dickinson(USA). For the culture of AT II cells, cell culture media and balanced salt solutions (BSS) were from Gibco. ^{3}H-methylcholine was from New England Nuclear (Boston, MA, USA). Alveolar type II cells were isolated by panning on IgG coated (rat IgG) plates as decribed by Dobbs et al.(1986).

Pathogen free male Sprague-Dawley rats were anesthetized intraperitoneally with an injection of pentobarbital 10 mg/kg/body weight and 300 unit of heparin. The trachea was cannulated and the heart was cut diagonally. Then the lungs were perfused via pulmonary artery with balanced salt solution consisting of 140 mM NaCl, 5 mM KCl, 2.5 mM sodim phosphate buffer, 10 mM HEPES and 6 mM glucose (pH 7.4 at 22°C). The lungs and trachea were excised en bloc and lavaged 8 times to total lung capacity with balanced salt solution containing 0.2 mM EGTA(pH 7.4, at 22°C), then lavaged with balanced salt solution containing 2 mM $CaCl_2$, and 1.3 mM $MgSO_4$. A solution of porcine pancreatic elastase suspended at 150 units/40 ml in the $CaCl_2$, $MgSO_4$ supplemented with balance salt solution was instilled into the trachea over 20 minutes shaking with orbital shaker at 37°C in warm room. Using sterile technique, the trachea and large airways were dissecting away and lung parenchyma was minced in the solution of 0.2 ml of 0.5% DNase I (w/v, in Ca, Mg supplemented BSS)and 5.0 ml of 10% fetal calf serum in 50ml conical tubes.

The resulting lung minces and cell suspension were filtered sequentially through 200 μm, 100 μm, and 20 μm nylon mesh. The filtered cells were collected at 500 g for 10 minutes, resuspended in MEM and incubated in bacteriological IgG coated plastic dishes at 37°C for 60 minutes in CO_2 incubator. The bacteriological IgG coated dishes were prepared by coating of IgG (4.0 ml of rat IgG at 400 μgm/ml in 50 mM Tris, pH 9.5) for 4 hours and then washed 6 times with PBS, pH 7.4). Thereafter, the non-adherent AT II cells were aspirated from the plates, then pelleted by centrifugation and counted immediately and placed in culture in MEM supplemented with 10 % fetal bovine serum at 10^6 cells/ml of tissue culture plastic dishes. The cell yield averaged 30~40 million AT II cells/lung. Isolated cells were cultured in 5% CO_2 in air at 37°C. The medium contained vitamins, non-essential amino acids, L-glutamine, sodium pyruvate and penicilline/streptomycin. For the simulation of sympathetic stimulation, isoproterenol and IBMX (3-isobutyl-1- methylxanthine) were used as secretagogues. After confirmation of the stimulatory effect on secretion from AT II cells, ANP was added in the media of isoproterenol stimulated AT II cultures.

For the assay of surfactant secretion isolated AT II cells were suspended in MEM containing 10% FCS with 1 μCi/ml of [^{3}H]-choline chloride at a million cells/ml and plate 500 μl in 16mm well and incubate

for 16 hours in an atmosphere of air with 5% CO_2. After incubation cells were washed two times with 1.0 ml of MEM containing pyruvate, 40 mM HEPES, 3 mg/ml of BSA, pH 7.4 and add 480 μgm of the same media at 37°C then incubate for 30 min. The point of post 30 min incubation was regarded as zero time. After 30 min incubation media and cells were extracted with CM (chloroform/methanol 2:1, v/v), dried under nitrogen. The residues were disssolved in 3.0 ml of scintillation fluid and count the radioactivity.

To the other wells for study of the effect of secretagogues, 20 μl of secretagogues of known concentration in same media or media alone were added to wells and were incubated for predetermined time at 37°C in CO_2 incubator. After incubation for predetermined time, the media and cells were analyzed for radioactivity. Percent secretion of radioactivity were calculated by radioactivity in media/radioactivity in media+radioactivity in cells.

Statistics: All data were given as mean ± S.E. and difference in all data were analyzed by non-paired, two-tailed Student's *t* -test.

RESULTS

In the present study, preparations of AT II cells in each experiment was from same rats, ie., AT II cells from same animals were divided into each experimental groups. Experimental groups were designated as control which was subdivided into 0 time basal, 120 min basal, and 180 min basal groups. AT II cells with secretagogue groups were divided into isoproterenol, IBMX (3-isobutyl-1-methylxanthine), isoproterenol with IBMX and isoproterenol with ANP group.

The isoproterenol was used as concentration of 10^{-6}, 10^{-5}, 10^{-4} molar concentration. IBMX used as 10^{-5} molar concentration. In isoproterenol with ANP group, the concentration was fixed at 10^{-5} molar concentration and the concentrations of ANP were varied as 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} molar concentrations.

Fig. 1 shows the effect of isoproterenol and IBMX on surfactant secretion of AT II cells. Compared with control, isoproterenol and IBMX treated cells showed increased secretion of surfactant significantly ($p < 0.05$). In addition, isoproterenol and IBMX acted synergistically on surfactant secretion of AT II cell. These data confirmed the stimulatory effect of beta agonist on surfactant secretion from AT II cells. The

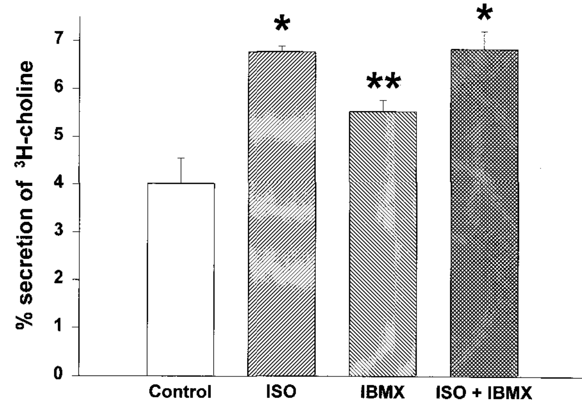

Fig. 1. The effect of isoproterenol and IBMX on the secretion of radiolabeled [^{3}H]-choline from primary AT II cultures. Increases of radiolabeled [^{3}H]-choline secretion in isoproterenol and IBMX- treated cultures were shown, compared to control cultures. The concentrations of secretagogues were fixed at 10^{-5} M and cultures were incubated for 180 min.
Values are given as mean ± S.E. and determination of radioactivity was carried in 5 cases of each group.
*$p < 0.01$ control vs ISO and ISO + IBMX
**$p < 0.05$ control vs IBMX
ISO: isoproterenol
IBMX: 3-isobutyl-1-methylxanthine

percent secretion in the control was 4.02 ± 0.53% (mean ± S.E.) but isoproterenol, IBMX and isoproterenol with IBMX groups showed increased secretion of 6.67 ± 0.32, 5.53 ± 0.24, 6.83 ± 0.37 respectively. In the Fig. 2 the secretory activity of AT II cell as a function of time were shown. The secretion of surfactant from AT II cell increased time-dependently in control and isoproterenol treated groups and the higher percent secretion of 6.67 ± 0.22% was showen compared with percent secretion of 4.35 ± 0.51% in the control group ($p < 0.05$).

Fig. 3 presents the effect of concentration of isoproterenol on the secretion of surfactant from AT II cell. At the concentration of 10^{-5} molar concentration, the secretory activity of the AT II cell reached at the plateau phase and increase in the secretion of AT II cell was concentration-dependent till the concentration of isoproterenol reached at 10^{-5} molar concentration.

Effect of ANP on the secretion of isoproterenol stimulated AT II cell was shown in Fig. 4. As shown

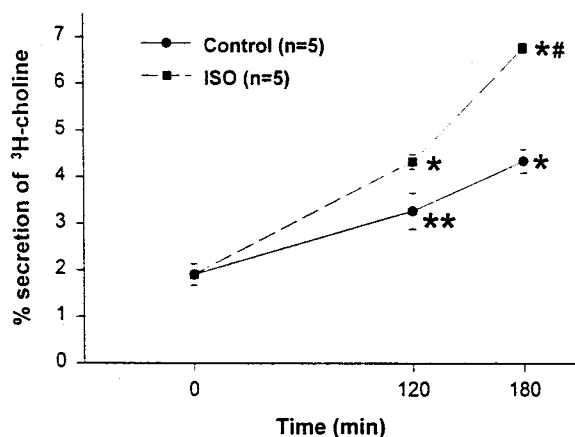

Fig. 2. Comparison of [^{3}H]-choline secretion from primary AT II cultures and isoproterenol treated AT II cultures. It shows time-dependent increase of secretion in the control and isoproterenol treated groups. After 180 min the secretion of [^{3}H]-choline reached at plateau phase. The concentration of isoproterenol was fixed at 10^{-5} M. Zero time indicates incubation of cultures for 30 min without secretagogues.
Values are given as mean±S.E. and determination of radioactivity was carried out in five cases of each group.
*$p<0.001$ time 0 vs time 120, 180 min.
**$p<0.05$ time 0 vs time 120, 180 min.
#$p<0.001$ control time 180 vs ISO time 180 min.

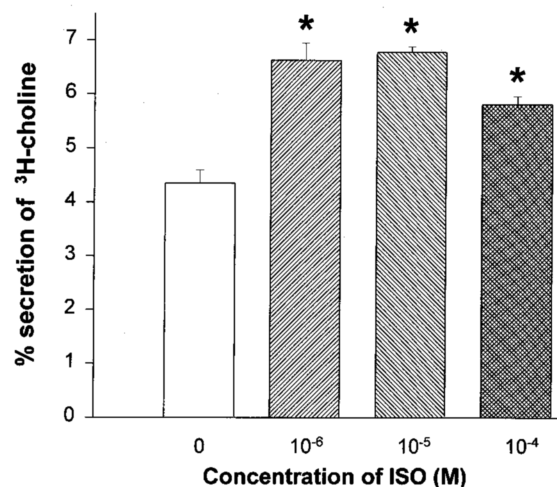

Fig. 3. Effects of isoproterenol on the secretion of [^{3}H]-choline in primary AT II cultures. Percent secretion of [^{3}H]-choline showed concetration-dependent increases. The percent secretion has reached up to maxima at 10^{-5} M.
Values are given as mean±S.E. and determination of radioactivity was carried out in five cases of each group.
*$p<0.001$ concentration 0 vs 10^{-4}, 10^{-5}, 10^{-6} M
**$p<0.01$ concentration 0 vs 10^{-4}, 10^{-5}, 10^{-6} M

in fig 4, ANP decreased the secretion of surfactant from isoproterenol stimulated AT II cell significantly($p<0.01$) as compared with only isoproterenol treated AT II cultures. In isoproterenol treated groups the percent secretion from AT II cultures was 6.67± 0.11% but in isoproterenol with ANP group showed significantly decreased percent secretion ($p<0.001$). This experimental result is very interesting because pulmonary edema and circulatory derangement is common which increses the secretion of ANP in the course of ARDS.

DISCUSSION

The secretory activity of alveolar type II cells is affected by various factors. Mechanical, hormonal and pharmacological agents can change the surfactant secretion from AT II cells (Chander & Fisher, 1990). As is well known, phospholipds are the main component of pulmonary surfactant which stabilize the alveolus by reducing the surface tension in air-fluid interface (Hills, 1990).

In the case of IRDS, the premature lung can not secrete sufficient amount of surfactant from AT II cell which leads to alveolar collapse (Jobe & Ikegami, 1987). Currently the hypothesis of decreased secretion of surfactant has been introduced to explain the cause or aggaravating factor of the symptoms of ARDS (Pison et al., 1990). But experimentally, as the lung has over 40 types of cells it is very difficult to evaluate the secretory activity of AT II cells by the heterogeneity of cells *in vivo*. Since Kikkawa and Yoneda (1984) succeeded in the isolation of alveolar type II cells from the rat lung, many investigators have tried to isolate and culture pure uncontaminated AT II cells. From the early years of AT II cell isolation from the lung, trypsin has been used as a popular dissociating enzyme but Dobbs and Mason (1979) insisted that the isolated type II cells dissociated by trypsin showed inconsitent responses to secretagogues by the cellular impairing effect of the trypsin itself. And the procedure of gradient centrifugation after filtration of cells through molecular sieve could impair the viability of the AT II cells.

To avoid these shortcomings Dobbs et al (1986) introduced the elastase, and the panning of the iso-

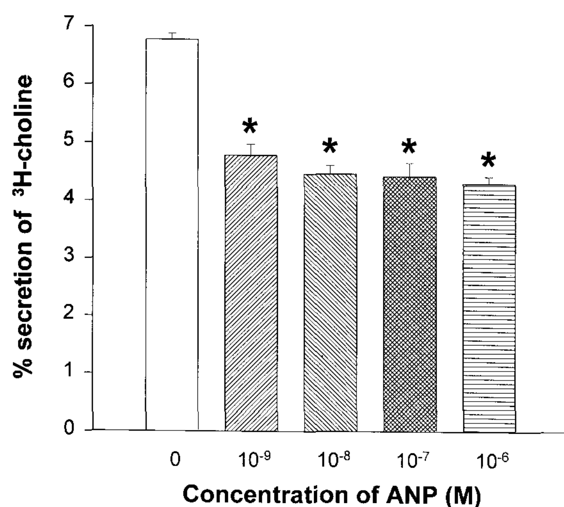

Fig. 4. Effects of ANP on [^{3}H]-choline secretion in the isoproterenol-stimulated AT II cultures. The concentration of isoproterenol was fixed at 10^{-5} M. The AT II cultures were incubated for 180 min.
Values are given as mean±S.E. and determination of radioactivity was carried out in eight cases of ANP treated group.
*$p<0.001$, ISO vs ANP
ISO: isoproterenol

lated, contaminated AT II cells on the IgG coated plates. With this method pure and unimpaired AT II cells could be obtained.

Effect of neurohumoral control on AT II cells has been widely investigated because of correlation between lung immaturity and surfactant secretion. Among those, cholinergic and adrenergic stimulation were widely studied but the constant results from these studies obtained recently (Post & Smith, 1992).

Pharmacologically beta agonists have been known to increase the secretion of surfactant from AT II cells (Mettler et al., 1981). The basic mechanism of increased secretion is activation of adenyl cyclase of cell membrane which induces increase of cAMP in the cytoplasm of AT II cells. By the activation of cAMP, exocytosis of phospholipid from lamellar bodies in the cytoplasm of AT II cell increases (Caniggia et al., 1990). In the present study isoproterenol and IBMX increased surfactant secretion and isoproterenol and IBMX acted synergistically on surfactant secretion of AT II cells.

By this effect of increasing surfactant secretion, beta agonists have been tried to relieve the symptoms of IRDS and ARDS. Hypothetically beta agonists are thought to be effective to relieve the symptoms of ARDS but as the causes of ARDS are multifactorial, usually supportive therapy has been tried (Friedli & Strebel, 1991).

One of the most troublesome factors aggaravating ARDS is circulatory failure which leads to pulmonary edema and respiratory insufficiency. In the course of ARDS the permeability of pulmonary capillaries increase to cause transudation of plasma protein into alveolar space. Decreased secretion of surfactant from AT II cells auguments this transudation (van Golde et al, 1988). Plasma fibrinogen is the main hazardous protein which inhibits surfactant function in alveoli (Seeger et al, 1985).

Besides the direct injury of AT II cell during ARDS, hypoxia and chemically toxic materials can cause disruption of surfactant system of the lung (Bunnel & Pacht, 1993). All these factors aggaravate pulmonary edema which leads to increased secretion of ANP.

While it has been well established that circulatory failure increases ANP secretion from atrial wall, recently Kawashima suggested hypoxia only could increase ANP secretion without circulatory change (Kawashima et al, 1992). Taken all these factors together, i.e. pulmonqry hypertension, pulmonary edema, and hypoxia may cause increased secretion of ANP in ARDS.

But in the present study to assess the effect of ANP on surfactant secretion of AT II cell, the result was ominous to improve pulmanary edema or respiratory insufficiency. ANP decreased the surfactant secretion significantly at physiological level in isoproterenol stimulated AT II cell.

In consideration of increased sympathetic tone during early phase of circulatory derangement and hypoxia, this experimental result implies that ANP might not improve the symptoms of circulatory derangement and respiratory insuffiency in ARDS. On the basis of present experimental results, increased level of ANP by congestive heart failure, and pulmonary edema might aggarave the respiratory insuffiency and pulmonary edema by reduction of surfactant secretion from AT II cells. As ANP is known to inhibit sympathetic stimulation *in vivo* (Wilson, 1991) which might cause to reduce surfactant secretion further more.

Though it is not certain why ANP decreases the secretion of surfactant from AT II cells, in some

tissues ANP inhibits basal and hormone stimulated adenylate cyclase activity (Wilson, 1991). So this inhibition of adenylate cyclase might cause decrease of cAMP level in the cytoplasm of AT II cell, which leads to decreased secretion of surfactant. Therefore it seems to be very important to reduce volume expansion in order to reduce ANP secretion which decrease surfactant secretion from AT II cells. Moreover ANP is thought to be ineffective in sustained volume overloading, reduction of circulatory volume is to be prerequisite to improve respiratory insuffiency associated with decreased secretion of surfactant from AT II cells in ARDS on the basis of the present study.

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