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Inhibition of Phospholipase A_2 Diminishes the Acute Alveolar Injury Induced by Interleukin-1α

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In an attempt to investigate the role of phospholipase A_2(PLA_2) in interleukin-1 (IL-1) induced acute lung injury, mepacrine was tried to inhibit PLA_2 in IL-1 induced ARDS rats. For confirmation of acute lung injury by IL-1, and to know the role of neutrophils in this injury, lung leak index, lung myeloperoxidase(MPO), number of neutrophils and protein content in the bronchoalveolar lavage (BAL) and wet lung weight were measured. At the same time lung PLA_2 was measured to know the effect of IL-1 on PLA_2 activity. Pulmonary surfactant was also measured for an investigation of type II alveolar cell function. Neutrophil adhesion assay was performed to know the effect of PLA_2 inhibition *in vitro* with human umbilical vein endothelial cells (HUVEC). For precise location of injury by IL-1, morpholgical study was performed by electron microscopy. Five hours after instillation of IL-1 (50 ng/rat), lung leak index, protein content, number of neutrophils, lung MPO and wet lung weight were increased significantly. Five hours after IL-1 instillation lung PLA_2 activity was increased significantly, and increased surfactant release was observed in IL-1 induced ARDS rats' BAL. In contrast, in rats given mepacrine and IL-1, there was decrease of acute lung injury i.e. decrease of lung leak index, wet lung weight, protein content, number of neutrophils in BAL and decreased lung MPO activity. Mepacrine decreased surfactant release also. Interestingly, inhibition of PLA_2 decreased adhesion of human neutrophils to HUVEC *in vitro*. Morphologically, IL-1 caused diffuse necrosis of endothelial cells, type I and II epithelial cells and increased the infiltration of neutrophils in the interstitium of the lung but after mepacrine treatment these pathological findings were lessened. On the basis of these experimental results it is suggested that PLA_2 has a major role in the pathogenesis of acute lung injury mediated by neutrophil dependent manner in IL-1 induced acute lung injury.

Key Words: Acute lung injury, Interleukin-1, Neutrophils, PLA_2, Mepacrine, Reactive Oxygen Species(ROS)

INTRODUCTION

Ever since Ashbaugh et al (1967) described Adult Respiratory Distress Syndrome(ARDS), many documents have been issued. Up to now, however, the underlying pathogenesis remains unclear. Recently it has become evident that the pulmonary cytokines are implicated in the pathogenesis of ARDS, i.e. interleukin-1(IL-1) and tumor necrosis factor(TNF) are elevated in bronchoalveolar fluid of ARDS patients (Suter et al, 1992; Jacobs et al, 1993). Although the underlying mechanisms of ARDS are not clear, these cytokines are thought to cause the acute inflammatory lung injury.

Leff et al (1994) demonstrated the effect of direct instillation of interleukin-1 on rats. In their study, they demonstrated that IL-1 caused acute alveolar injury which was very similar to the findings of ARDS in human beings. According to Guidot et al. (1993), in this IL-1 induced acute lung injury model, increased production of reactive oxygen species (ROS) is the main cause of acute inflammatory lung injury. In spite of the diversity of underlying causes of ARDS, the histological findings and the progress of this syndrome is amazingly similar to the IL-1 model.

In this context it is rather interesting to con-

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sider the PLA_2 as a causative factor to evoke acute alveolar injury, since authors (Vadas et al, 1991) described the elevation of PLA_2 in ARDS patients as a result of increased interleukin-1 and TNF. Meyer and Marshall (1993) described the effect of the cytokines, IL-1 and TNF, on phospholipase A_2 release from the neutrophils. PLA_2 increases the release of arachidonic acid, leukotrienes and platelet activating factor(PAF).

Moreover some authors (Forehand et al, 1993) directly related PLA_2 to ROS since stimulation of PLA_2 by IL-1 or TNF caused the activation of NADPH oxidase in the neutrophils. Pruzanski and Vadas (1991) insisted that PLA_2 is a mediator between proximal and distal effectors of inflammation. Increased arachidonic acid metabolites are followed by increased production of ROS. For such reasons, the activation of PLA_2 might be one of the causes of ARDS in associated with the increased production of ROS from inflammatory cells.

Therefore, in this experiment the role of PLA_2 was investigated in acute inflammatory lung injury by inhibiting PLA_2 with mepacrine. Mepacrine was chosen based on the efficacy of PLA_2 inhibition (Turner & Wood, 1994).

METHODS

Source of reagents

Recombinant human interleukin-1(IL-1, endotoxin level $<$ 1 EU/mg) was purchased from the R&D systems(Mineapolis, MN, USA), frozen in aliquots, and thawed daily before use. ^{125}I-labelled bovine serum albumin(^{125}I-BSA) from ICN radiochemicals (Irvine, CA, USA), L-α-dipalmitoyl-(2-palmitoyl-9.10-3H(N))phosphatidylcholine and ^{51}Cr were obtained from Dupont NEN Research Products (Boston, MA, USA). All other reagents were purchased from Sigma Chemical Company(St. Louis, MO, USA).

Grouping

For this study experimental animals (Sprague-Dawley B.W. 250~300 gm) were divided into control, IL-1 treated, and IL-1 with mepacrine treated groups. For induction of acute alveolar injury in experimental animals, IL-1 was insufflated into the trachea (50 ng/rat) according to Leff et al (1994). In the IL-1 with mepacrine group, mepacrine was injected intraperitonealy(50 mg/kg) immediately after IL-1.

Measurement of lung leak index and MPO activity

Lung leak was measured by administering an intravenous injection of 1.0 μCi of ^{125}I-BSA at 4.5 hours after initial intratracheal insufflation of IL-1. Rats were anesthetized with ketamine and xylazine intraperitoneally. At 5 hours lungs were perfused blood free and 1.0 ml of blood was withdrawn from the right atrium. The right lung and blood were used for measuring radioactivity. Lung leak was determined as radioactivity in the lung/radioactivity in the blood. The left lung was frozen at -70°C and used later to determine myeloperoxidase(MPO) activity according to Goldblum (1985). MPO estimates the accumulation of neutrophils in the lung tissue. In addition to the lung leak index, protein content in bronchoalveolar lavage(BAL) was determined using bicinchoninic acid (Brown & Jarvis, 1989).

Count of polymorphonuclear leukocytes in BAL fluid

Bronchoalveolar lavage was performed as follows; 8.0 ml of cold normal saline was instilled through trachea and washed 3 times, 6.0 ml of BAL fluid being obtained through this procedure. BAL fluid was centrifuged at 1000 g for 10 minutes for sedimentation of cellular components. The supernatant after centrifugation was stored at -70°C for isolation of pulmonary surfactant and th pellet was used to count neutrophils according to the previous protocol (Leff et al, 1994). Briefly, cellular pellet was resuspended with distilled water and 1.0 ml of HBSS for a couple of seconds and centrifuged again. After discard of supernatant, the pellet was suspended in 0.1 ml of normal saline. With 0.2 ml of suspended solution, cytospin was performed for differential count and the cells were Wright stained.

Polymorphonuclear leukocyte count was performed with hemocytometer and the fraction of neutrophils was calculated by the percentage of neutrophils in differential count.

Measurement of wet lung weight

Wet lung weights were measured after perfusion with PBS, i.e. the lungs were excised and blotted with filter paper and weighed.

Measurement of phospholipase A_2 activity

According to Katsumata et al (1986), PLA_2 activity was measured. Briefly, after perfusion, the lungs were excised from the chest cavity and right lungs were homogenated with Virtis homogenator in 20 mM potassium phosphate, pH 7.4. After homogenation, the homogenate was sonicated for 90 seconds. The radioactive substrate L-α-dipalmitoyl-2(9,10(N)-3H)palmitoyl)-phosphatidylcholine(30 Ci/mmol) was incubated with 100 μl of the lung homogenate in 880 μl of a glycine buffer, 100 mM at pH 9.0 containing 10 gm/L of BSA, 2.5 mM sodium deoxycholate, 0.1 mM DPPC(dipalmitoyl phosphatidylcholine), 2.0 mM $CaCl_2$ and 1.75 M absolute ethanol. The reaction mixture was incubated for 60 minutes in waterbath at 37°C. The reaction was stopped by adding 200 μl of 5% Triton X-100 containing 200 mM EDTA. The fatty acids released by hydrolysis were extracted by 5.0 ml of hexane containing 0.1 % acetic acid and 2.5 gm of Na_2SO_4. After vortexing, the hexane layer was separated and counted in the liquid scintillation counter. Snake venom PLA_2(Crotalus adamanteus) control samples (0.01 U/ml) were assayed with all other samples to ensure the reproducibility. One unit of enzyme activity was defined as the ability to hydrolyze 1 μmol of substrate per minute.

Measurement of phospholipid in surfactant

Phospholipid content in surfactant was measured according to Liau et al (1987). Briefly, crude surfactant was isolated by centrifugation. In order to isolate crude surfactant, BAL was centrifuged at 150 g for 10 minutes and the supernatant was centrifuged for 120 minutes at room temperature at 20000g. The pellet was resuspended in normal saline and lipids were extracted by the method of Folch et al (1957). The phosphorus content was measured according to Hess and Derr (1975) and phosphorus content was converted into phospholipid according to Corbet et al (1983).

Measurement of neutrophil adhesion to endothelial cells

The adhesion to cultured monolayers of human umbilical vein endothelial cells of ^{51}Cr prelabelled neutrophils that had been previuosly isolated from the peripheral blood of normal human donors was determined *in vitro* (Terada et al, 1992).

Histological analysis

For ultrastructural study, small blocks(1 mm^3) of tissue were rapidly fixed by immersion in cold (4°C) 2.5% glutaraldehyde and then degassed. Following rinsing, storage overnight in 0.1 M patassium phosphate buffer, postfixation with osmium tetroxide, dehydrated in graded ethanols, and embedding in Epon 812 resin, block sections, 60~80 nm thick, were cut with a diamond knife on a Sorvall MT-

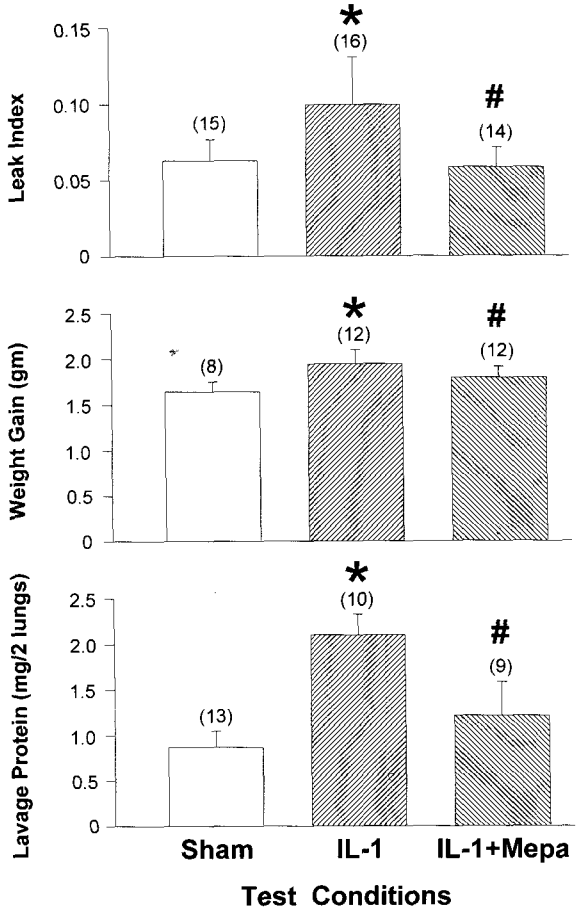

Fig. 1. Effect of mepacrine on lung leak, lung weight gain, and lung lavage protein concentrations. Lung leak index, lung weight, and lung lavage protein concentrations were increased ($p<0.05$) in IL-1 treated rats compared to control rats. By comparison, lungs from rats given mepacrine and IL-1 had decreased($p<0.05$) lung leak index, lung weight and lung lavage protein concentrations compared to rats given only IL-1 intratracheally. Each bar represents the mean±SE. The number of determinations was shown in the parentheses.

7000 ultramicrotome, collected on grids coated with isoamyl acetate, and stained with uranyl acetate and lead citrate. Electron micrographs were taken with a Hitachi H-600 transmission electron microscope at 75 kV.

Statistical analyses

Data were analyzed using a one-way analysis of variance with a Turkey-Kramer multiple comparison test. A p value of <0.05 was considered statistically significant.

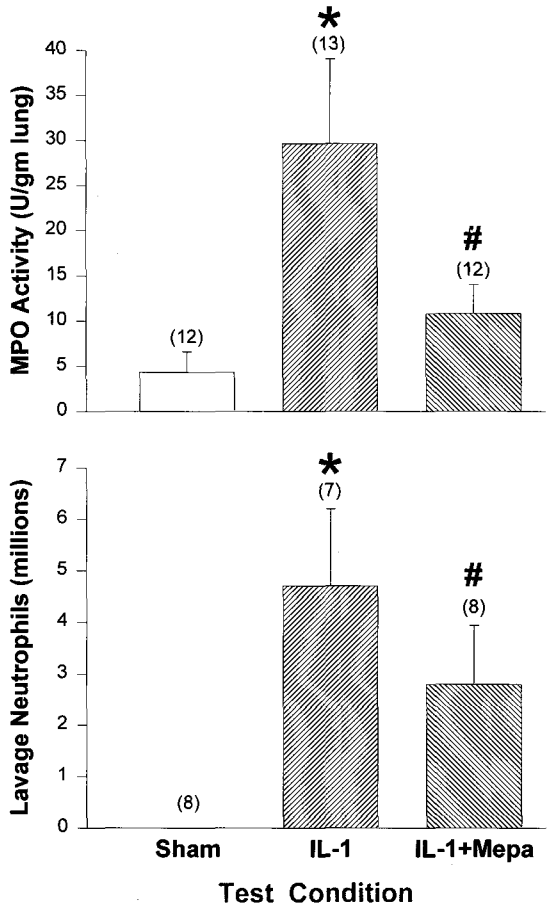

Fig. 2. Effect of mepacrine on lung MPO activity and lung lavage neutrophils.
Lung MPO and lung lavage neutrophils were increased ($p<0.05$) in rats given IL-1 intratracheally compared to control rats. By comparison, lungs from rats given mepacrine and IL-1 had decreased ($p<0.05$) MPO activity and lung lavage neutrophil numbers compared to lungs from rats given only IL-1 intratracheally. Each bar represents the mean ± SE. The number of determinations was shown in the parentheses.

RESULTS

Rats given IL-1 intratracheally had increased lung leak, lung weight and lung lavage protein concentrations (Fig 1.) compared to control rats. By comparison, rats pretreated with PLA_2 inhibitor, mepacrine, and given IL-1 intratrachealy had decreased lung leak, lung weight and lung lavage protein concentrations compared to rats given IL-1 only.

Rats given IL-1 intratracheally had increased lung MPO activity and lavage neutrophils compared to lungs from control rats. In contrast, rats pretreated with mepacrine and IL-1 intratracheally had decreased lung MPO activity and lung lavage neutrophils compared to rats given IL-1 intratracheally (Fig 2.)

Rats given IL-1 intratracheally 5 hours before had increased lung PLA_2 activity. By comparison, rats pretreated with mepacrine and then given IL-1

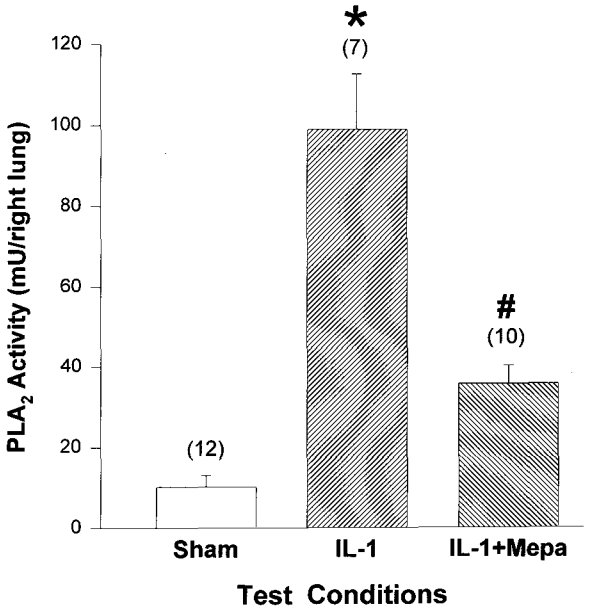

Fig. 3. Effect of mepacrine on PLA_2 activity. PLA_2 activity was increased ($p<0.05$) in lungs of rats given IL-1 intratracheally compared to control rats. By comparison, lungs from rats given mepacrine and then IL-1 had decreased ($p<0.05$) PLA_2 activity compared to lungs from rats given only IL-1 intratracheally. Each bar represents the mean ± SE. The number of determinations was shown in the parentheses.

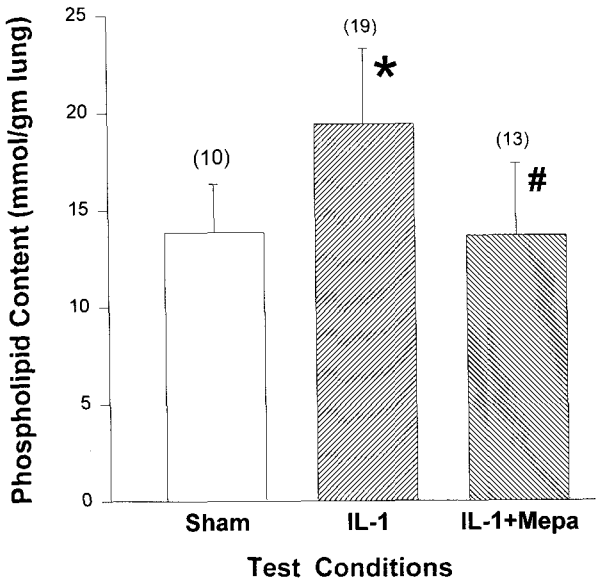

Fig. 4. Effect of mepacrine on surfactant phospholipid content. Surfactant phospholipid content was increased ($p<0.05$) in lungs of rats given IL-1 intratracheally compared to control rats. By comparison, lungs from rats given mepacrine and IL-1 had decreased ($p<0.05$) phospholipid content compared to lungs from rats given only IL-1 intratracheally. Each bar represents the mean±SE. The number of determinations was shown in the parentheses.

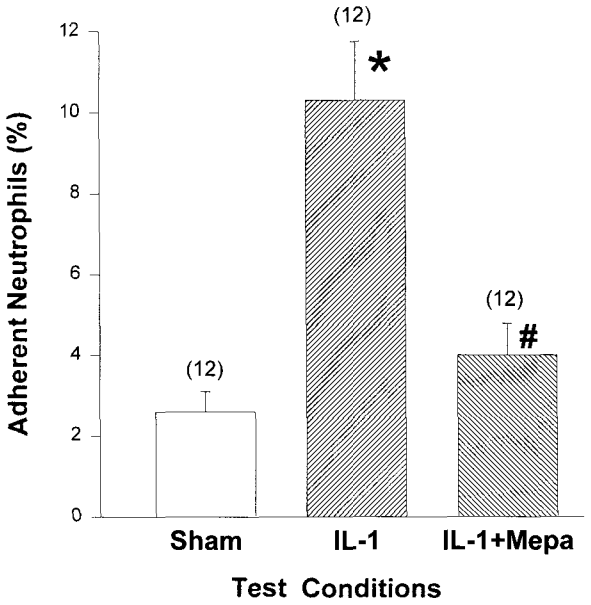

Fig. 5. Effect of mepacrine on neutrophil adherence to endothelial cells *in vitro*. Human neutrophil adherence was increased in IL-1 treated human umbilical vein endothelial cells(HUVEC) compared to control HUVEC. By comparison, HUVEC treated with mepacrine and IL-1 had decreased ($p<0.05$) neutrophil adherence compared to HUVEC treated only with IL-1. Each bar represents the mean±SE. The number of determinations was shown in the parentheses.

intratracheally had decreased lung PLA_2 activity (Fig. 3).

Rats given IL-1 intratracheally 5 hours before had increased surfactant phospholipid in BAL. In contrast, rats pretreated with mepacrine and then IL-1 intratracheally had decreased surfactant phospholipid content in BAL compared to rats given IL-1 intratracheally (Fig. 4).

Human umbilical vein endothelial cell(HUVEC) monolayers that were pretreated with IL-1 (50 ng/ml, 2hours) had increased numbers of adhering human neutrophils compared to control HUVEC($p<0.05$). By comparison, HUVEC treated with mepacrine and then IL-1 had fewer ($p<0.05$) neutrophils adhering than HUVEC treated with IL-1 only($p<0.05$)(Fig 5.).

In morphological study, infiltration of neutrophils were increased in IL-1 treatred lungs and generalized necrosis of type I and type II epithelial cells were found. Blebbing of endothelial cells and thinnng of the basement membrane were observed. In contrast, in mepacrine treated rats these pathological changes were diminished (Fig 6. panel a, b, c).

DISCUSSION

Direct insufflation of interleukin-1(IL-1) into the trachea causes acute alveolar injury followed by protein leak in the interstitium and alveolar lumen (Repine, 1994). The change after IL-1 instillation into the trachea is very similar to the morphology and progress of ARDS in the human. According to Suter et al (1992) and Jacobs et al (1993) IL-1 and TNF were elevated in the BAL of ARDS patients.

In the present study to investigate the role of PLA_2 on IL-1 induced injury, acute lung leak was induced by IL-1. Lung leak and wet weights of the lungs were increased after instillation of IL-1. As an index of leukostasis, MPO in the lung and numbers of neutrophils in BAL were increased significantly after IL-1 compared to the control

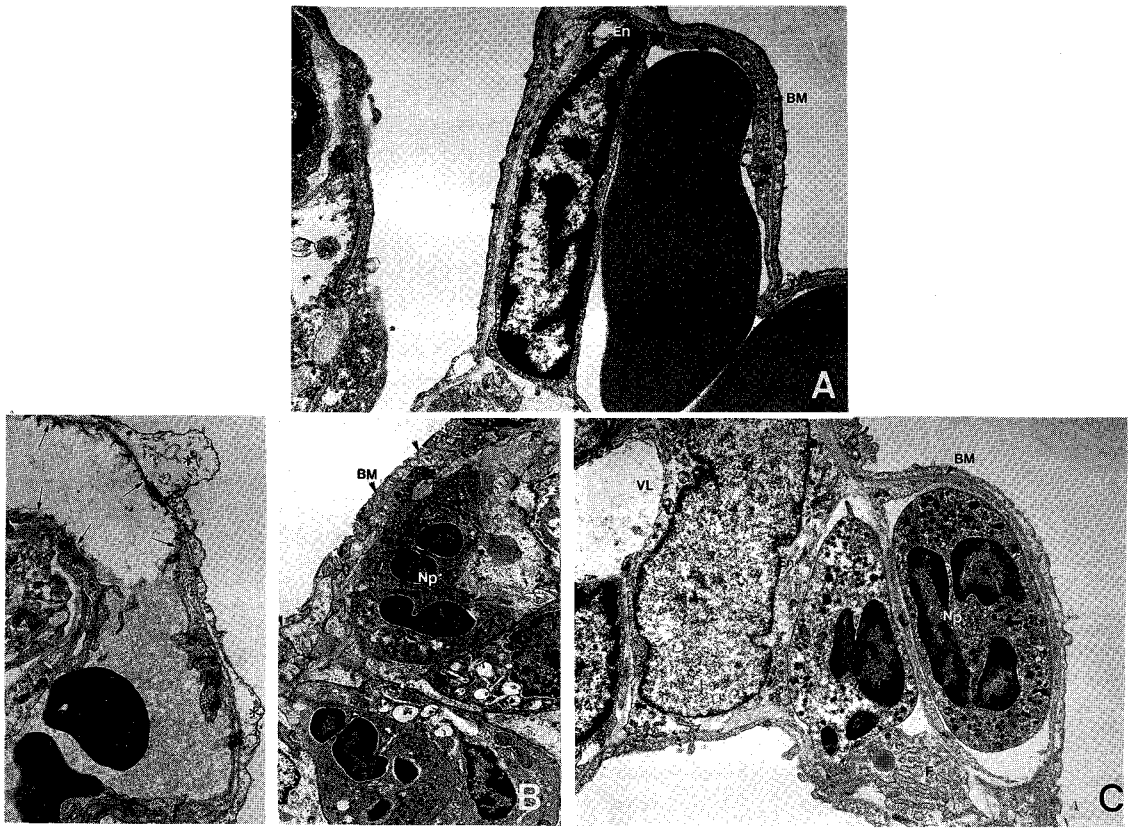

Fig. 6. Effect of mepacrine on ultrastructure of the lung. Panel A represents a normal structure of the lung. Panel B represents IL-1 treated lung structure. It shows increased infiltration of neutrophils and early evidence of alveolar injury; blebbing of endothelial cells and destruction of type I and II alveolar cells. Panel C represents mepacrine and IL-1 treated lung. Basement membrane of endothelial cell is intact and it shows decreased number of neutrophils in the interstitium. Np: neutrophil, EC: endothelial cell, BM: basement membrane.

group, but decreased after IL-1 with mepacrine. The protective effect of mepacrine was evident as shown in results.

It has been well documented that IL-1 caused PLA_2 induction in pulmonary endothelial cells (1991). Increased PLA_2 activity is said to increase the release of lipid mediators because PLA_2 is a rate limiting enzyme in lipid mediator synthesis (Marshall et al, 1995). According to Vadas and Pruzanski (1990), IL-1 induces PLA_2 in 5-6 hours after IL-1 treatment and this PLA_2 is produced by *de novo* synthesis in the cytoplasm. Also, some authors (Leslie et al, 1988; Lin et al, 1992) note that TNF and IL-1 activate the cytosolic PLA_2 which starts the arachidonic acid release from cell membrane immediately. In the present study, there was significant increase of PLA_2 activity after IL-1 instillation. Mepacrine treatment decreased PLA_2 activity in IL-1 treated rats. Some authors (Maridonneau-Parini & Tauber, 1986; Dana et al, 1994) insist that PLA_2 acts on neutrophils to produce reactive oxygen species(ROS) through the activation of NADPH oxidase in the cell membrane. The inhibition of PLA_2 by mepacrine might then decrease ROS production from accumulated neutrophils in this study.

ROS can oxidize the phospholipid of the membrane. Oxidized phospholipids are liable to be hydrolyzed by PLA_2 (Salgo et al, 1993). Mepacrine could prevent this harmful effect by inhibition of PLA_2. As is shown in the results, there was an increase of phospholipid content after IL-1. Das et al (1987) and Ballis et al (1988) reported the effect of pancreatic phospholipases on phospholipid content in BAL. In their report the toxic effect of PLA_2 could injure the type II cells directly. By

observing the ultrastructural change the increase of phospholipid content appeared to be caused by direct type II cell injury by ROS or other chemicals released during IL-1 insult i.e. increased phospholipid content was caused by necrosis of the cell membrane of alveolar type II cells.

Moreover, the increased phospholipid content would be harmful because PLA_2 can use the phospholipid as a substrate. Especially phosphatidylcholine can be source of lysophosphatidylcholine. In the present study the effect of lysophospholipid was not confirmed but it is natural to conjecture that there must be an increase of lysophospholipid by the increased activity of the PLA_2 after IL-1 insult. The chemotactic and cytolytic effect of lysophospholipid could be inhibited by mepacrine in the present study.

In this context the decrease of protein content after mepacrine treatment seems to contribute to the maintenance of alveolar barrier. Cockshutt et al (1991) reported that leaked protein in alveoli could intensify the disruption of the surfactant layer. Therefore decreased protein leak by mepacrine treatment might maintain a surfactant system which stabilizes alveoli and decreases the protein leak into the alveolar lumen. Besides these effects of mepacrine there might be the decrease in the production of ROS, which inhibits the adherence of neutrophils to the endothelium. In the present study mepacrine decreased the number of adherent neutrophils to the HUVEC. On the basis of this data it is highly probable that mepacrine decreased the number of infiltrated neutrophils in the alveoli and interstitium.

In the ultrastructural findings, there was prominent destructive changes after IL-1 instillation and these changes were very similar to morpholgical changes of ARDS in humans (Schlag & Redl, 1985). After IL-1 instillation diffuse necrosis of type I and type II epithelial cells and endothelial cells were observed. Especially in the proximity of neutrophils, the necrosis and interstitial edema was prominent. In contrast these patholgical findings were lessened by mepacrine.

Taken together, the inhibition of PLA_2 in IL-1 induced lung injury resulted in diminished protein leak and maintenance of alveolar and endothelial structure. Therefore the experimental results in this study suggest that the activation of PLA_2 is one of the major factor to cause acute alveolar injury in IL-1 induced ARDS model and this injury was thought to be neutrophil dependent.

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