Korean J Physiol Pharmacol
Vol 4: 219−226, June, 2000

Endotoxin-induced Acute Lung Injury is Mediated by PAF Produced via Remodelling of Lyso PAF in the Lungs

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In order to elucidate the role of platelet activating factor (PAF) in the acute lung injury induced by endotoxin (ETX), activities of phospholipase A2, lyso PAF acetyltransferase and oxidative stress by neutrophilic respiratory burst were probed in the present study. To induce acute lung injury, 100 μg of *E.coli* ETX (type 0127; B8) was instilled directly into the tracheae of Sprague-Dawley rats. Five hours after the ETX instillation, induction of acute lung injury was confirmed by lung leak index and protein contents in the bronchoalveolar lavage (BAL) fluid. At the same time, lung phospholipase A2 (PLA2) activity and expression of group I and II secretory type PLA2 were examined. In these acutely injured rats, ketotifen fumarate, known as lyso PAF acetyltransferase inhibitor and mepacrine were administered to examine the role of PAF in the pathogenesis of the acute lung injury. To know the effect of the ETX in the synthesis of the PAF in the lungs, lyso PAF acetyltransferase activity and PAF content in the lungs were measured after treatments of ETX, ketotifen fumarate and mepacrine. In addition, the role of neutrophils causing the oxidative stress after ETX was examined by measuring lung myeloperoxidase (MPO) and enumerating neutrophils in the BAL fluid. To confirm the oxidative stress in the lungs, pulmonary contents of malondialdehyde (MDA) were measured. After instillation of the ETX in the lungs, lung leak index increased dramatically ($p<0.001$), whereas mepacrine and ketotifen decreased the lung leak index significantly ($p<0.001$). Lung PLA2 activity also increased ($p<0.001$) after ETX treatment compared with control, which was reversed by mepacrine and ketotifen ($p<0.001$). In the examination of expression of group I and II secretory PLA2, mRNA synthesis of the group II PLA2 was enhanced by ETX treatment, whereas ketotifen and WEB 2086, the PAF receptor antagonist, decreased the expression. The activity of the lysoPAF acetyltransferase increased ($p<0.001$) after treatment of ETX, which implies the increased synthesis of PAF by the remodelling of lysoPAF in the lungs. Consequently, the contents of the PAF in the lungs were increased by ETX compared with control ($p<0.001$), while mepacrine ($p<0.001$) and ketotifen ($p<0.01$) decreased the synthesis of the PAF in the lungs of ETX treated rats. The infiltration of the neutrophils was confirmed by measuring and enumerating lung MPO and the neutrophils in the BAL fluid respectively. Compared with control, ETX increased lung MPO and number of neutrophils in BAL significantly ($p<0.001$) whereas mepacrine and ketotifen decrerased number of neutrophils ($p<0.001$) and MPO ($p<0.05$, $p<0.001$, respectively). The lung MDA contents were also increased ($p<0.001$) by ETX treatment, but treatment with mepacrine ($p<0.001$) and ketotifen ($p<0.01$) decreased the lung MDA contents. Collectively, we conclude that ETX increases PLA2 activity, and that the subsequently increased production of PAF was ensued by the remodelling of the lyso PAF resulting in tissue injury by means of oxidative stress in the lungs.

Key Words: Acute lung injury, Endotoxin, PAF, Oxidative stress

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INTRODUCTION

Acute respiratory distress syndrome (ARDS) is one of the morbid clinical manifestations of the systemic

inflammatory response syndrome (SIRS) and the underlying pathogenesis has not been clearly elucidated. As one of the etiologies of ARDS, sepsis is a grave and cryptogenic cause of acute edematous lung injury and the mortality rate of sepsis-induced ARDS is relatively high comparing with ARDS caused by other etiologies (Rangel-Fausto et al, 1995). Despite the continuous research on the sepsis-induced ARDS, the underlying mechanism of the acute edematous tissue injury, especially the vascular endothelium, has not been understood.

Recently, Delclaux et al (1997) reported that neutrophilic release of proteases, especially elastases, is the major factor to cause acute lung injury by endotoxin. In their report, free radicals were involved in the release of the protease in the vicinity of the activated neutrophils. As ETX provokes the inflammatory reaction in the tissue, some proinflammatory cytokines such as tumor necrosis factor (TNF) and interleukin-1 (IL-1) from macrophages are known to trigger the activation of phospholipase A2 (PLA2) (Vadas et al, 1991). In conjunction with acute lung injury and oxidative stress, xanthine oxidase (XO) has been presumed to be the prime oxidant generator (Terada et al, 1992).

But recently, Dana et al (1998) claimed that activation of the PLA2 is the prerequisite for the generation of the oxidants from neutrophils. In our previous studies with various ARDS models, the activation of the PLA2 is indispensable to the generation of oxidants from neutrophils (Lee et al, 1997; Lee et al, 1999). Needless to say, the activation of PLA2 in tissues ensues the increase in production of proinflammatory lipid mediators that are involved in the inflammatory reaction. However, the exact mechanism of the generation of the oxidants from neutrophils associated with PLA2 activation has not been clear.

One of the lipid mediators produced by the activation of PLA2 is lyso PAF. It is converted into PAF by lyso PAF acetyltransferase and the process of the binding of acetyl moiety to lyso PAF is called remodelling of lyso PAF. PAF is a well-known lipid molecule inducing inflammatory reactions through various pathways such as activation of adhesion molecules, chemotaxis and activation of NADPH oxidase in neutrophils (Hotter et al, 1997; Mitola et al, 1998). In conjuction with the pathogenesis of multiple organ failure (MOF) or systemic inflammatory response syndrome (SIRS), PAF has been presumed to have pivotal roles in tissue injury such as neutrophil-dependent and acid-induced acute lung injury (Nagase et al, 1999; Prescott et al, 1999). However, up to now, the precise role of PAF in sepsis-induced ARDS has not been confirmed in association with neutrophilic respiratory burst.

Based on these considerations, the role of PAF was probed in relation to the PLA2 and lyso PAF acetyltransferase in the sepsis-induced ARDS model of rats.

METHODS

Procedures for induction of acute lung injury by ETX

Acute lung injury was induced by the instillation of ETX (*E.coli* type: 0127; B8) to the Sprague-Dawley rats (B.W 300~350 g). Dissolved in normal saline, 100 μg of ETX was insufflated into the trachea directly. Five hours later, induction of acute lung injury was confirmed. To inhibit PLA2 or lysoPAF acetyltransferase, mepacrine (50 mg/kg) or ketotifen fumarate (1 mg/kg) was administered intraperitoneally immediately after the ETX treatment (Ninio & Jolly, 1991).

Measurement of protein leak in the lungs

To confirm the acute lung injury after the ETX instillation, lung leak index was determined as reported previously (Lee et al, 1997). Briefly, ^{125}I-BSA was used for detection of the protein leak from vascular lumen into the interstitium and the alveolar lumen. For detection of protein in the bronchoalveolar fluid, protein contents in the alveolar lumen was measured using bicinchoninic acid (Brown et al, 1989).

Assay of pulmonary PLA2

Pulmonary PLA2 activity was measured using the method by Katsumata et al (1986). Briefly, Blood-free excised lungs were homogenized with polytron homogenizer in 2.0 ml of 20 mM phosphate buffer (pH 7.4), and then sonicated for 90s. Two μCi of L-α-dipalmitoyl-2(9,10(N)^{3}H-palmitoyl) phosphatidylcholine (30 Ci/mmol) was incubated with 100 μl of the lung homogenate in 880 μl of 100 mM glycine buffer (pH 9.0) containing 200 mM EDTA, 10 g/L of BSA, 2.5 mM sodium deoxycholate, 0.1 mM dipalmitoyl-

phosphatidylcholine, 2.0 mM $CaCl_2$ and 1.75 M absolute ethanol. The reaction mixture was incubated for 60 min at 37°C. After the reaction was stopped by adding 200 μl of 5% Triton X-100 in 200 mM EDTA, 5.0 ml of hexane containing 0.1% acetic acid and 2.5 g of Na_2SO_4 were added. The radioactivity of separated hexane layer after vortexing was counted in the liquid scintillation counter. Snake venom PLA2 (*Crotalus adamanteus*) control samples (0.01 U/ml) were assayed with all other samples to confirm the reproducibility. One unit was defined as an activity to hydrolyze 1 μmol of substrate per min.

RT-PCR

Total RNAs were prepared with an RNA isolation kit (Gibco-BRL, Gaithersberg, MD) according to the manufacturer's instruction. After digestion with RNase-free DNase I, RT-PCR was performed by the method of Doehr et al (1995). Briefly, for cDNA synthesis, 1 μg of total RNA was heated in a final volume of 10 μl with 2 μg oligo$(dT)_{15}$ primer for 5 min at 60°C, chilled on ice and reverse transcribed in a final volume of 40 μl containing 1 mM of each dNTP, 8 μl of 5×M-MLV buffer, 60 units of RNase inhibitor, 10 mM DTT, and 400 units of M-MLV reverse transcriptase. Samples were incubated at 37°C for 2 h and subsequently denatured for 10 min at 70°C. PCR primers were synthesized with an Applied Biosystems 391 DNA synthesizer (Weiterstadt, Germany) and purified with NAP-5 columns (Pharmacia, Freiburg, Germany). Primer sequences were from published sources or chosen using a primer selection program (Oligo, National Bioscience, Plymouth, MN) and are given as: beta-actin forward and reverse primers: 5'-GTGGGGGCGCCCCAGGCACCA-3' and 5'-CTCCTTAATGTCACGATTTC-3' for the 541 base pair amplified fragments (Albinoni et al, 1991), rat group I PLA2 forward and reverse primers; 5'-GCAATATGATCAAGTGCACCATCCCC-3' and 5'-GTATTCCTTGTTGTAGGGGACCTTGG-3' for the amplification of 325 base pair fragments (Sakada et al, 1989), rat group II PLA2; forward primer and reverse primers; 5'-CCTGTTGCTAGCAGTTGTGA TCATGG-3' and 5'-CTCTTTCAGCAACTGGGCGT CTTCCC-3' to amplify 435 base pair DNA fragments (Ishizaki et al, 1989). PCR reactions were carried out in a final volume of 50 μl containing 2.5 μl RT sample, 5 μl of 10×TaqDNA polymerase and 1 μCi [α-^{32}P]dCTP. Amplifications were done using a DNA thermal cycler (Ericomp Inc., San Diego, CA) 4 min at 94°C before the first cycle, 1 min for denaturation at 94°C, 1 min for primer annealing at 54°C, 1 min for primer extension at 72°C and 7 min at 72°C after the last cycle. Linearity of the amplification was controlled by three different cycle numbers for one cDNA concentration. PCR products were electrophoresed on 10% (w/v) polyacrylamide gels, and gels were dried and autoradiographed.

Assay of lysoPAF acetyltransferase

LysoPAF acetyltransferase activity was measured in the lungs according to Bussolino et al (1986). Briefly, left lungs were immersed in 1.0 ml of 0.25 M sucrose solution containing 1 mM DTT and homogenized before sonication for 90s. Thereafter, 100 μl of sample solution was mixed well with 0.5 ml of 0.1 M Tris-HCl buffer (pH 6.9) containing 40 μM lyso PAF, 200 μM ^{3}H-acetyl CoA (0.5 μCi), 40 μg of BSA. Reactions were stopped by adding 2.0 ml of chlorform-methanol mixture (1 : 2, v/v). Lipids were extracted following the method of Bligh and Dyer (1959). The lipids in chlorform layer were dried under nitrogen, and radioactivity was measured with beta-scintillation counter.

PAF scintillation proximity assay

PAF contents were measured in the lungs by PAF scintillation proximity assay according to manufacturer's instructions. Briefly, left lungs were homogenized in 0.2 M citrate-normal saline solution and lipids were extracted from the samples by the method of Bligh and Dyer (1959). After separation of the organic layer, samples were dried under nitrogen. Then, samples were resuspended in assay buffer (0.05 M Tris-HCl buffer, pH 7.4 containing 0.9% NaCl, 0.01% Triton X-100, 0.1% gelatin and 0.0057% thimerosal). Resuspended samples were mixed with anti PAF antibody, SPA protein A reagent and tracer. Before measuring the radioactivity with beta-scintillation counter, the mixture was incubated for 18 h at room temperature with shaking.

Confirmation of pulmonary neutrophil infiltration: lung myeloperoxidase activity and enumeration of neutrophils in the bronchoalveolar fluid

To confirm the pulmonary infiltration of neutro-

phils, lung MPO activity was measured according to Goldblum et al (1985). In addition, the umber of neutrophils was counted as previously described (Lee et al, 1997).

Assay of malondialdehyde (MDA) in the lungs

In order to confirm the oxidative stress in the lungs, pulmonary contents of MDA was assayed following the method of Carraway et al (1998).

Sources of reagents

Dipalmitoyl-(2(9,10(N)-^{3}H)palmitoyl)-phosphatidylcho line, ^{125}I-bovine serum albumin (BSA), ^{3}H-acetyl CoA were purchased from Dupont NEN research product (Boston MA, USA). PAF scintillation proximity assay kit was purchased from Amersham International (Amersham UK), ketotifen fumarate was purchased from Sandoz Pharmaceutical Co. WEB 2086, a PAF receptor antagonist, was donated by Boeringer-Ingelheim (Germany). All other chemicals were purchased from Sigma Chemical Company (St. Louise, MO, USA).

Statistics

Data presented represent means ± SEM. Statistical significance at $p<0.05$ was assessed by Student-Newman-Keuls multiple comparison test.

RESULTS

Five hours after the instillation of ETX, the leak of the protein from the vascular compartment into the pulmonary interstitium and alveolar lumen was confirmed. Lung leak indices and BAL protein contents increased significantly ($p<0.001$) in ETX-treated group compared with control group. In contrast, mepacrine and ketotifen markedly decreased ($p<0.001$) the enhanced lung leak indices and BAL protein contents in ETX-treated rats (Table 1).

Likewise, pulmonary PLA2 activity increased ($p<0.001$) after the ETX treatment compared with control, and mepacrine and ketotifen decreased ($p<0.001$) PLA2 activity compared with ETX treatment (Table 2). The expression of the group II secretory PLA2 was increased by ETX, while inhibition of lysoPAF acetyltransferase with ketotifen and blocking of PAF receptor with WEB 2086 decreased the expression of

Table 1. Comparison of lung leak index and protein contents (mg/both lungs) in BALF after treatments of ETX, mepacrine and ketotifen

	Control	ETX	ETX + Mepa	ETX + ketotifen
Lung leak index	0.057 ± 0.0039 (n=6)	0.229 ± 0.0128* (n=8)	0.136 ± 0.0129† (n=8)	0.111 ± 0.0117† (n=8)
Protein content	2.28 ± 0.199 (n=8)	5.89 ± 0.363* (n=14)	3.23 ± 0.198† (n=9)	2.67 ± 0.207† (n=8)

Data represent mean ± SE. The number of animals tested are shown in the parentheses. *$p<0.001$: Control vs. ETX, †$P<0.001$: ETX vs. ETX + Mepa, †$p<0.001$: ETX vs. ETX + ketotifen. ETX: endotoxin, Mepa: mepacrine.

Table 2. Changes in PLA_2 activity (mU/g of lung) of the lung following treatments with ETX, mepacrine and ketotifen

	Control	ETX	ETX + Mepa	ETX + ketotifen
PLA_2 activity (mU/g of lung)	33.78 ± 4.679 (n=10)	128.61 ± 4.700* (n=11)	73.03 ± 8.463† (n=6)	12.57 ± 0.431† (n=9)

Data represent mean ± SE. The number of animals tested are shown in the parentheses. *$p<0.001$: Control vs. ETX, †$P<0.001$: ETX vs. ETX + Mepa, †$p<0.001$: ETX vs. ETX + ketotifen. ETX: endotoxin, Mepa: mepacrine.

group II PLA2 gene in the lungs. There was no change in the expression of group I secretory PLA2 (Fig. 1).

After the ETX treatment, the activity of lyso PAF acetyltrasferase was enhanced ($p<0.01$) conspicuously and this increase of the enzymatic activity was inhibited by mepacrine ($p<0.001$) and ketotifen ($p<0.01$). Accordingly, the pulmonary contents of PAF were increased ($p<0.01$) by ETX, which were completely reversed by mepacrine ($p<0.01$) and ketotifen ($p<0.001$)(Table 3).

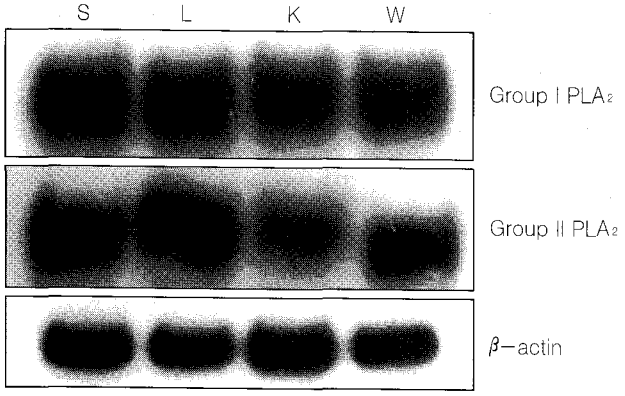

Fig. 1. RT-RCT revealed the enhanced expression of group II PLA_2 in the lung, whereas the expression of group I PLA_2 mRNA was not changed compared with sham rats after treatment of ETX. Ketotifen and WEB 2086 decreased the enhanced expression of group II PLA_2 in the lung of ETX-treated rats. S: sham, L: ETX treated, K: ETX+ketotifen, W: ETX+WEB 2086.

Lung MPO activity and BAL neutrophils, the parameters of pulmonary infiltration of neutrophils in the lungs, increased ($p<0.001$) after the ETX treatment. This increase was reversed partly by mepacrine and ketotifen (Table 4).

As an index of oxidative stress following lipid peroxidation, MDA contents in the lungs increased ($p<0.001$) after ETX treatment, whereas treatment of mepacrine ($p<0.001$) and ketotifen ($p<0.01$) decreased pulmonary MDA contents in ETX-treated rats (Table 5).

DISCUSSION

It has been well documented that neutrophilic

Table 3. Comparison of the activity of lysoPAF acetyltransferase (nmol PAF/min/g of ewt lung) and PAF contents (ng/g of ewt lung) in the lung after treatments with EFX, mepacrine and ketotifen

	Control	ETX	ETX+Mepa	ETX+ketotifen
lysoPAF acetyltransferase activity	0.294±0.0241 (n=5)	1.228±0.1770* (n=7)	0.334±0.0205† (n=7)	0.338±0.0113§ (n=7)
PAF contents	19.33±2.943 (n=5)	250±41.815* (n=8)	41.62±12.138† (n=7)	22.49±7.640‖ (n=8)

Data represent mean±SE. The number of animals tested are shown in the parentheses. *$p<0.01$: Control vs. ETX, †$P<0.01$, †$p<0.001$: ETX vs. ETX+Mepa, §$p<0.01$, ‖$p<0.001$: ETX vs. ETX+ketotifen. ETX: endotoxin, Mepa: mepacrine.

Table 4. Confirmation of the infiltration of neutrophils in the lungs following treatments with FTX, mepacrine and ketotifen

	Control	ETX	ETX+Mepa	ETX+ketotifen
MPO activity (mU/g of lung)	5.18±0.390 (n=20)	35.18±1.767* (n=20)	18.03±0.751† (n=15)	27.49±1.812† (n=10)
PMN number (millions/both lungs)	0.051±0.0035 (n=8)	3.498±0.4271* (n=8)	0.997±0.1436† (n=7)	1.368±0.1790§ (n=9)

Data represent mean±SE. The number of animals tested are shown in the parentheses. *$p<0.01$: Control vs. ETX, †$P<0.01$: ETX vs. ETX+Mepa, †$p<0.05$, §$p<0.01$: ETX vs. ETX+ketotifen. ETX: endotoxin, Mepa: mepacrine.

Table 5. Changes in the contents of MDA (nmole/g of lung) after treatments with ETX, mepacrine and ketotifen

	Control	ETX	ETX+Mepa	ETX+ketotifen
MDA content	108.1±14.47 (n=7)	301±30.59* (n=7)	145.5±15.99† (n=10)	151.6±19.78‡ (n=8)

Data represent mean±SE. The number of animals tested are shown in the parentheses. *$p<0.001$: Control vs. ETX, †$P<0.001$: ETX vs. ETX+Mepa, ‡$p<0.01$: ETX vs. ETX+ketotifen. ETX: endotoxin, Mepa: mepacrine.

respiratory burst plays a pivotal role in the pathogenesis of ARDS. According to Repine (1994), disruption of the balance between oxidants and antioxidants might be the cause of acute inflammatory pulmonary edema. In sepsis-induced ARDS, the massive infiltration of neutrophils, increased concentration of proinflammatory cytokines and PLA2 in the lungs are the cardinal features of ARDS (Endo et al, 1994). In the present study, the infiltration of neutrophils and acute pulmonary edema were demonstrated, which were diminished by the inhibition of PLA2 and lyso PAF acetyltransferase. van Helden et al (1997) insisted the biological relevance of rat ARDS model in which ETX was instilled directly into the trachea to human. In their model, they presented various reflecting signs of ARDS including the infiltration of neutrophils in the lungs after treatment with ETX. Although the neutrophil chemotactic factors such as interleukin-8, PAF and leukotriene B4 have been known to increase in lungs of patients with ARDS, the underlying mechanism altering the synthesis of these chemo-attractants are unclear as well as the mechanism of oxidant generation from neutrophils.

As one of the causes of the activation of infiltrated neutrophils, we demonstrated (Lee et al, 1997; Lee et al, 1999) that the activation of PLA2 is the prerequisite for the release of free radicals from neutrophils. As is well-known, the activation of PLA2 has phagocytes or tissue release diverse spectrum of lipid molecules such as arachidonic acid and lysophosphatides. According to Dana et al (1994), arachidonic acid is the causative molecules to activate NADPH oxidase in neutrophilic membranes. In addition, PLA2 metabolites are known to be able to induce the chemotaxis of phagocytes into the tissues (Aebischer et al, 1993).

PAF is one of the PLA2 products and has been believed as a responsible humoral factor to activate adhesion molecules and to generate free radicals by the activation of NADPH oxidase in neutrophils (Kurose et al, 1997). However, the mechanism of the activation of PLA2 has not been known and needs to be determined. According to Lee et al (1997), instillation of IL-1 activated PLA2 in the lungs and the inhibition of PLA2 with mepacrine diminished the acute lung injury. Since PLA2 is activated by proinflammatory cytokines such as TNF and IL-1 (Vadas et al, 1993), the increased concentration or expression of proinflammatory cytokines must occur prior to the activation of PLA2. In sepsis-induced ARDS, the infiltration of phagocytes and increased concentration of proinflammatory cytokines have been documented. In the BAL of ARDS patients, the increased concentrations of proinflammatory cytokines and increased PLA2 activity have been reported (Anderson et al, 1994). Therefore, the increased PLA2 activity by the mechanism unidentified yet seems to be the provoker of the activation of neutrophils. Suggesting this hypothesis in the present study, the instillation of ETX caused the infiltration of neutrophils and activated PLA2 in the lungs. RT-PCR analysis showed increased expression of group II secretory PLA2, which is consistent with the results by Ljungman et al (1996).

The acute lung injury was ensued after ETX treatment, which was proved by the increased protein leak into the alveolar lumen and interstitium. Interestingly, as previously reported by Lee et al (1997), the inhibition of PLA2 by mepacrine decreased the acute lung leak dramatically. These data suggest that the inhibition of PLA2 is effective enough to decrease the neutrophilic activation. Furthermore, the inhibition of lysoPAF acetyltransferase, a lyso PAF-remodelling enzyme, had the protein leak decrease effectively, indicating PAF plays a critical role in acute lung injury in sepsis. Pulmonary neutrophilic infiltration was induced by ETX, and the inhibition of PLA2 and remodelling of lysoPAF decreased the neutrophilc infiltration.

PAF is synthesized through *de novo* and remodelling pathway from lysoPAF that is one of the metabolites of PLA2 activation (Bussolino & Camussi, 1995). In the process of inflammation, it is known that remodelling of lysoPAF is the major pathway for PAF synthesis. As a source of PAF, neutrophils are known as one of the cells synthesizing PAF during inflammatory process (Wijkander et al, 1995). In the present study, the increased synthesis of PAF after PLA2 activation seems to be caused by increased activity of lyso PAF acetyltransferase. As lysoPAF acetyltransferase was inhibited by ketotifen very effectively, it is also not surprising that PAF content in the lungs was decreased by ketotifen. These data suggest that ETX activates PLA2, and the production of lysoPAF by the activated PLA2 stimulates the remodelling of lysoPAF by lysoPAF acetyltransferase.

The PAF produced by PLA2 activation seems to increase the neutrophilic respiratory burst resulting in oxidative stress in the tissues. As one of the end products of lipid peroxidation, MDA was increased in the lungs after treatment of ETX. Mepacrine and ketotifen decreased MDA contents effectively after ETX treatment. All these data imply that PAF is the lipid molecule that regulates the oxidative stress induced by neutrophilic respiratory burst.

Taken together, the results of the present study can be interpreted as follows. First, ETX activates PLA2 in the lungs, although the underlying mechanism is still unclear. Second, the products of PLA2 activation might induce chemotaxis of neutrophils and respiratory burst. PAF, one of the lipid molecules produced by the activation of PLA2, might be the major molecule that causes neutrophilic respiratory burst after ETX treatment. Third, PAF is synthesized by the remodelling process that was stimulated by ETX.

In conclusion, ETX-induced acute lung injury is mediated by oxidative stress as a result of neutrophils activation, at least in part, by PAF.

ACKNOWLEDGEMENT

The authors wish to acknowledge the financial support (98-198E-025) of Korea Research Foundation made in the program year of 1998.

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