Original Article

A Study on the Mechanism of Immunomodulating Effects of Moxifloxacin in Oleic Acid-Induced Acute Lung Injury

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Background: It was hypothesized that the immunomodulating effects of moxifloxacin contribute to ameliorate oleic acid (OA)-induced acute lung injury (ALI) by suppression of cytosolic phospholipase A2 (cPLA2). This was based on observations from experiments on rats associated with neutrophilic respiratory burst, cPLA2 activity, and expressions of cPLA2, TNFα, and COX-II in the lung.

Methods: ALI was induced by intravenous injection of OA in male Sprague-Dawley rats. Five hours after OA injection, protein content in bronchoalveolar lavage (BAL), lung myeloperoxidase (MPO) activity, and numbers of BAL neutrophils were measured. As an index of oxidative stress-induced lung injury, the content of malondialdehyde (MDA) in lung tissues was also determined. Lung histology, immunohistochemistry and determination of activity of cPLA2 in lung tissues were carried out. In addition, Western blotting of TNFα and COX-II in lung tissues was performed.

Results: The accumulation of neutrophils in the lungs was observed after OA injection. BAL protein was increased along with neutrophilic infiltration and migration by OA. Moxifloxacin decreased all of these parameters of ALI and ameliorated ALI histologically. The increased malondialdehyde (MDA) in the lung by OA was also decreased by moxifloxacin. Moxifloxacin not only suppressed cPLA2 expression in the lungs and neutrophils but also decreased cPLA2 activity in lung tissues of rats given OA. The enhanced expressions of TNFα and COX-2 in the lung tissues of rats given OA were also suppressed by moxifloxacin.

Conclusion: Moxifloxacin inhibited cPLA2 and down-regulated TNFα and COX-2 in the lungs of rats given OA, which resulted in the attenuation of inflammatory lung injury.

Key Words: Moxifloxacin; Oleic Acid; Neutrophils; Acute Lung Injury

Introduction

Acute lung injury (ALI) and its severe form of acute respiratory syndrome (ARDS) are characterized by acute hypoxemic respiratory failure, low pressure pulmonary edema, and massive neutrophilic infiltration in the lung[1]. The fundamental pathogenesis that initiates and intensifies this intractable inflammatory process has not

been elucidated. Phospholipase A_2 (PLA$_2$) has long been known to participate in ALI, and various studies have been carried out to develop new therapeutic modalities for acute lung injury[2,3]. Until now, however, the results are discouraging and the heterogeneity of etiologies increases the difficulties to explain the exact pathogenesis of ALI[4].

Fat embolism syndrome (FES) is one of the etiologies causing ALI and oleic acid in the bone marrow or other part of the body is the main source of acute inflammatory lung injury in the FES[5]. OA not only directly injures endothelial cells of the lung[6], but also initiates inflammatory reaction through the activation of PLA$_2$[7].

In spite of the unsuccessful outcome to develop new therapeutic drugs against ALI, the recent discovery of new types of PLA$_2$s and the novel knowledge on these

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97

new PLA$_2$s have prompted studies on PLA$_2$ expecting to discover new therapeutic modalities of ALI.

Though PLA$_2$s have been studied widely in conjunction with ALI[8] and its new therapy, the efforts have been in vain still. However, in FES-induced ALI, cytosolic PLA$_2$ (cPLA$_2$) might play a crucial role for the initiation of acute inflammation in the lung, as cPLA$_2$s are abundant in the lung and mononuclear phagocytes are enriched with cPLA$_2$[9]. Besides, cPLA$_2$ mediates arachidonic acid-induced tissue injury which comprises the activation of NADPH oxidase and inflammatory reaction irrespective of free radicals[10]. In OA-induced ALI, neutrophils play a crucial role along with its accumulation and migration to the lung provoking epithelial and endothelial injury by oxidative stress and release of proteases[11].

Recently, fluoroquinolones were discovered to have immunomodulatory functions, especially anti-inflammatory effects[12,13] by suppressing the migration of phagocytes in some clinical conditions. Considering the possibility that moxofloxacin's immunomodulating effects are linked to cPLA$_2$ activity, effects of moxifloxacin on OA-induced ALI were tested in conjunction with the cPLA$_2$-activated neutrophilic oxidative stress and the activation of TNFα and COX-II in the lung.

Materials and Methods

1. Experimental animals and reagents

Male *Sprague-Dawley* rats were purchased from Sasco Company (Sasco Korea, pathogen free, 250~300 g). Goat anti-human cPLA$_2$ polyclonal antibody and anti-COX-II antibody were purchased from Santa Cruz Biochem (Santa Cruz, CA, USA). Anti-TNF-α-polyclonal antibody was obtained from Cell Signaling (Danvers, MA, USA). L-α-dipalmitoyl-[2-palmitoyl-9,10-^{3}H(N)-phosphatidylcholine was obtained from Dupont NEN Research Products (Boston, MA, USA). Moxifloxacin hydrochloride was graciously provided by Bayer (Bayer Leverkusen, Germany). Not otherwise mentioned, all other reagents were purchased from Sigma Aldrich Company (St. Louise, MO, USA).

2. Induction of ALI

Animals were fed *ad libitum* standard chow and water till 24 hours before experiments. ALI was induced in rats by being given oleic acid intravenously (30 μL of oleic acid with 270 μL of 10% bovine serum albumin) as previously described[11,14].

3. Treatments

Rats were divided into three treatment groups

1) Sham group: rats were given 300 μL of 10% bovine serum albumin intravenously via femoral vein; 2) Oleic acid control group (OA group): rats were given 30 μL of oleic acid with 270 μL of 10% bovine serum albumin intravenously; 3) Moxifloxacin group (MO group): rats were given moxifloxacin hydrochloride (100 mg/kg) with 30 μL of oleic acid in 270 μL of 10% bovine serum albumin. Moxifloxacin hydrochloride was given intraperitoneally just after the injection of oleic acid.

4. Bronchoalveolr lavage (BAL)

BAL was performed 5 hours after oleic acid injection. The rats were anesthetized with enflurane and tracheotomized and cannulated. BAL was performed with 8.0 mL of PBS for each rat and approximately 6.0 mL of BAL was recovered. BAL fluid was collected into 10 mL sterile tubes, centrifuged to collect cell free supernatant and stored at -20°C for protein assay. The sediments were resuspended with PBS for counting of leucocytes.

5. BAL protein

Determination of BAL protein was performed as previously described by Brown et al.[15] with modification. Stored BAL supernatant was used for protein assay.

6. BAL neutrophil count

Ten μL of resuspended sediment solution was applied on hemocytometer and leukocytes were counted. Around 100 μL of BAL suspension was cytospinned and slides were prepared before Wright staining. Percentage of neutrophils was calculated by differential count and

total numbers of neutrophils were calculated.

7. Lung myeloperoxidase (MPO) assay

The MPO activity (U/g of wet lung) was determined to estimate the accumulation of neutrophils in the lung. The assay was performed as described by Goldblum et al.[16] with modification. Lungs were weighed and homogenized with Polytron homogenizer (Polytron, Switzerland) in 4.0 mL of 20 mM PBS at pH 7.4. An aliquot was mixed with an equal volume of 1% hexadecytrimethyl ammonium bromide in PBS and rehomogenized for 90 s in ice cold water. Then it was centrifuged 15,000 g for 5 minutes at $4^\circ C$. The supernatant (0.1 mL) was added to 2.9 mL of PBS (50 mM potassium phosphate, pH 6.0) containing 0.0005% H_2O_2 and 0.168 g/L of o-dianisidine. The samples were analyzed for change in absorbance on spectrophtometer at 460 nm over 3 minutes. MPO activity was expressed as U/g of wet lung by multiplying 13.5 to the changes of absorbance/min/g of wet weight tissue.

8. Determination of lung malondialdehyde (MDA); thiobarbituric acid reactive substance (TBARS) assay

TBARS assay was carried out as described previously[17] to measure lipid peroxidation as a marker of ALI. The same supernatant used for MPO assay was used. Aliquots of supernatant were mixed with equal volume of 3.5% sodium dodecyl sulfate (SDS) and 0.13 mL of 100% acetic acid in 15 mL-glass centrifuge tubes. The pH was adjusted to 3.5 with 1 N NaOH. Five mL of 0.6% thiobarbituric acid was added to each tube and they were heated in boiling water bath at $95^\circ C$ for 30 minutes. After cooling, 1.5 mL of n-butanol/pyrimidine (15 : 1) mixture was added to each tube and vortexed vigrously for 30 s followed by centrifugation at 4,000 rpm for 10 minutes. The absorbance of top layer of each sample was measured by spectrophotometer at 525 nm.

9. Determination of cPLA₂ activity

cPLA₂ activity was determined by the hydrolysis of radioactively labelled phospholipid (^{3}H-dipalmitoyl-phosphatidylcholine, ^{3}H-DPPC) as previously described by Katsumata et al.[18] with modification. Lung tissues were homogenized in a polytron in ice-cold buffer (1 : 5, wt/vol) containing Tris- HCl (pH 7.6), EDTA (1 mM), EGTA (1 mM) and DTT (5 mM) to abolish sPLA₂ activity. The phospholipid substrate solution (pH 9.0) was composed of ^{3}H-DPPC, lecithin (0.1 mM), glycine (100 mM), deoxycholate (2.5 mM), BSA (10 g/L) and 1.75 mM ethanol. The reaction was initiated by the addition of 100 μL of lung homogenate to the phospholipid substrate solution and incubated at $37^\circ C$ in a shaking water bath for 60 minutes. *Crotalus adamanteus* PLA₂ was used as a positive control. The reaction was stopped by adding 200 μL of 5% Triton X-100 containing 200 mM EDTA and 5 g of anhydrous Na_2SO_4. For the extraction of hydrolysis product, 5.0 mL of 0.1% glacial acetic acid-hexane was added to each sample. The extract was scanned with β-scintillation counter. 1 unit of PLA₂ activity was defined as 1 μM production of fatty acid per minute.

10. Lung histological preparations

Lungs were fixed by inflation with paraformaldehyde (pH 7.4, 4%) at pressure of 20 cm H_2O and sliced before putting into paraformaldehyde, then degassing and fixed. Tissue slices were stained with hematoxylin-eosin for examination of lung histology.

11. Immunohistochemistry of cPLA₂ in rat lung tissue

Microtomer sections were subjected to staining for fluorescence and diaminobenzidine (DAB) for cPLA₂ in rat lung tissues. Goat anti-human-cPLA₂ polyclonal was used as first antibody. The antibody complex was visualized by propidium iodide (fluorescense) and DAB staining.

12. Protein expression of TNFα and COX-2

Proteins of TNFα and COX-2 were identified in homogenized lung tissue (100 μg of protein) using standard western blotting. Specific polyclonal antibodies, anti-TNF and anti-COX-II were diluted 1 : 1,000 (Tris buf-

fer, Saline Tween 20 [TBST] buffer with 0.1% BSA).

13. Statistical analyses

Data were expressed as mean±standard error (SE). Data were analyzed using a one-way analysis of variance with Tukey-Kramer multiple comparisons test. A p-value of <0.05 was considered statistically significant.

Results

1. Effects of moxifloxacin on lung leak

The assay of BAL protein revealed the protective effect of moxifloxacin on lung leak in rats. As shown in Table 1, OA had increased the protein content (mg/two lungs) in BAL of rats given OA, as compared with that of sham-treated rats ($p<0.001$). Moxifloxacin had decreased the protein content in BAL of rats given OA ($p<0.01$).

Table 1. Effect of moxifloxacin on lung leak in the OA-induced acute lung injury in rats

	Sham (n=13)	OA (n=7)	OA+MO (n=7)
BAL protein, mg/two lungs	1.9±0.3	6.3±0.5*	3.3±0.2[†]

Values are given as mean±SE.
*p$<$0.001; Sham vs. OA, [†]p$<$0.01; OA vs. OA+MO.
BAL: bronchoalveolar lavage; OA: oleic acid; MO: moxifloxacin; SE: standard error.

Table 2. Effect of moxifloxacin on the infiltration and migration of neutrophils in the lungs of oleic acid given rats

	Sham	OA	OA+MO
Lung MPO, U/g of lung	2.5±0.4 (n=8)	34.7±2.4* (n=14)	13.9±1.3[†] (n=8)
BAL PMNs, millions/two lungs	0.2±0.1 (n=12)	9.1±2.5[‡] (n=9)	1.6±0.4[§] (n=8)

Values are given as mean±SE.
*p$<$0.001; Sham vs. OA, [†]p$<$0.001; OA vs. OA+MO, [‡]p$<$0.001; Sham vs. OA, [§]p$<$0.001; OA vs. OA+MO.
MPO: myeloperoxidase; OA: oleic acid; MO: moxifloxacin; BAL: bronchoalveolar lavage fluid; PMN: polymorpho nuclear neutrophil; SE: standard error.

2. Effects of moxifloxacin on the accumulation and migration of neutrophils in the lungs of rats given the OA

As noted in Table 2, moxifloxacin reduced the accumulation of neutrophils in the interstitium of the lung and suppressed the migration of neutrophils into the alveoli of the OA group ($p<0.001$). As expected, the OA caused dramatic mobilization of neutrophils manifested by the drastic increase of lung MPO ($p<0.001$). The accumulation of neutrophils in the lung interstitium caused the migration of neutrophils into the alveoli as a process of alveolar flooding. Moxifloxacin was effective to decrease the effects of OA on the mobilization of neutrophils ($p<0.001$).

3. Estimation of protective effects of moxifloxacin by TBARS assay

As an index of lung injury, the TBARS in the lung was measured. The MDA content (nmol/g of lung) was higher in the lungs of OA given rats compared with that of sham treated rats ($p<0.001$). The MDA content in

Table 3. Effect of moxifloxacin on the MDA content in the lung reflecting oxidative lung injury

	Sham (n=7)	OA (n=9)	OA+MO (n=7)
MDA content, nmol/g of lung	141.6±15.1	370.9±21.5*	241.2±30.9[†]

Values are given as mean±SE.
*p$<$0.001; Sham vs. OA, [†]p$<$0.001; OA vs. OA+MO.
OA: oleic acid; MDA: malondialdehyde; MO: moxifloxacin; SE: standard error.

Table 4. Effect of moxifloxacin on the cytosolic PLA$_2$ activity in the lungs of oleic acid given rats

	Sham (n=10)	OA (n=9)	OA+MO (n=8)
cPLA$_2$ activity, mU/g of lung	15.1±5.3	30.2±14.1*	12.4±4.2[†]

Values are given as mean±SE.
*p$<$0.001; Sham vs. OA, [†]p$<$0.05; OA vs. OA+MO.
cPLA$_2$: cytosolic phospholipase A$_2$; OA: oleic acid; MO: moxifloxacin; SE: standard error.

the lungs of moxifloxacin given to rats was lower than that of the rats given OA reflecting the protective effect of moxifloxacin ($p < 0.001$) (Table 3).

4. cPLA₂ activity in the lung tissue

As shown in Table 4, the cPLA₂ activity was higher in the lungs of OA-treated rats compared with that of sham-treated rats ($p < 0.001$). The cPLA₂ activity was lowered in the lung tissue by moxifloxacin in OA-treated rats ($p < 0.05$).

5. Lung histology and cPLA₂ immunohistochemistry of lung tissue

While examining the histopathology of the lung of rats given OA, many phagocytes, mainly neutrophils, were observed. Perivascular cuffing and alveolar flood-

ing were also observed, as these were the results of the OA injection (Figure 1A and B). Moxifloxacin ameliorated these inflammatory reactions dramatically, even if the remnants of migrated phagocytes, mainly alveolar monocyte/macrophages remained (Figure 1C). Along with these histopathological changes, the immunohistochemistry revealed the inhibitory effects of moxifloxacin on cPLA₂ in the lung. The DAB and fluorescent staining of cPLA₂ in the lung demonstrated the diminished expression of cPLA₂ by moxifloxacin (Figures 2, 3). Note the deep staining of cPLA₂ in the intra-alveolar neutrophils with DAB in the OA-treated rats. Interestingly, this phenomenon disappeared by moxifloxacin almost completely. In the lung tissue, the fluorescent staining of the alveolar septa was shown in OA-treated rats, and again, moxifloxacin suppressed this phenomenon
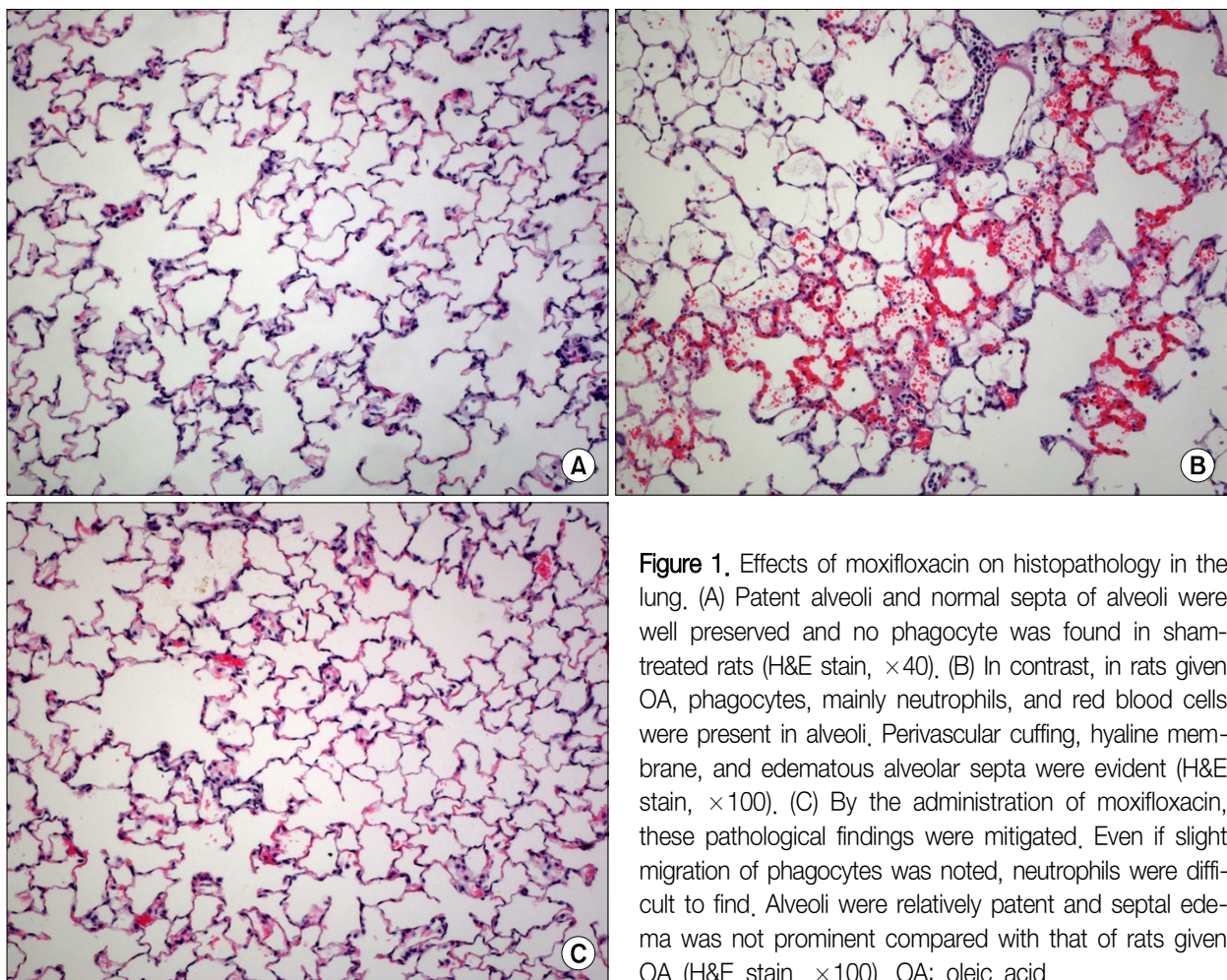

Figure 1. Effects of moxifloxacin on histopathology in the lung. (A) Patent alveoli and normal septa of alveoli were well preserved and no phagocyte was found in sham-treated rats (H&E stain, ×40). (B) In contrast, in rats given OA, phagocytes, mainly neutrophils, and red blood cells were present in alveoli. Perivascular cuffing, hyaline membrane, and edematous alveolar septa were evident (H&E stain, ×100). (C) By the administration of moxifloxacin, these pathological findings were mitigated. Even if slight migration of phagocytes was noted, neutrophils were difficult to find. Alveoli were relatively patent and septal edema was not prominent compared with that of rats given OA (H&E stain, ×100). OA: oleic acid.
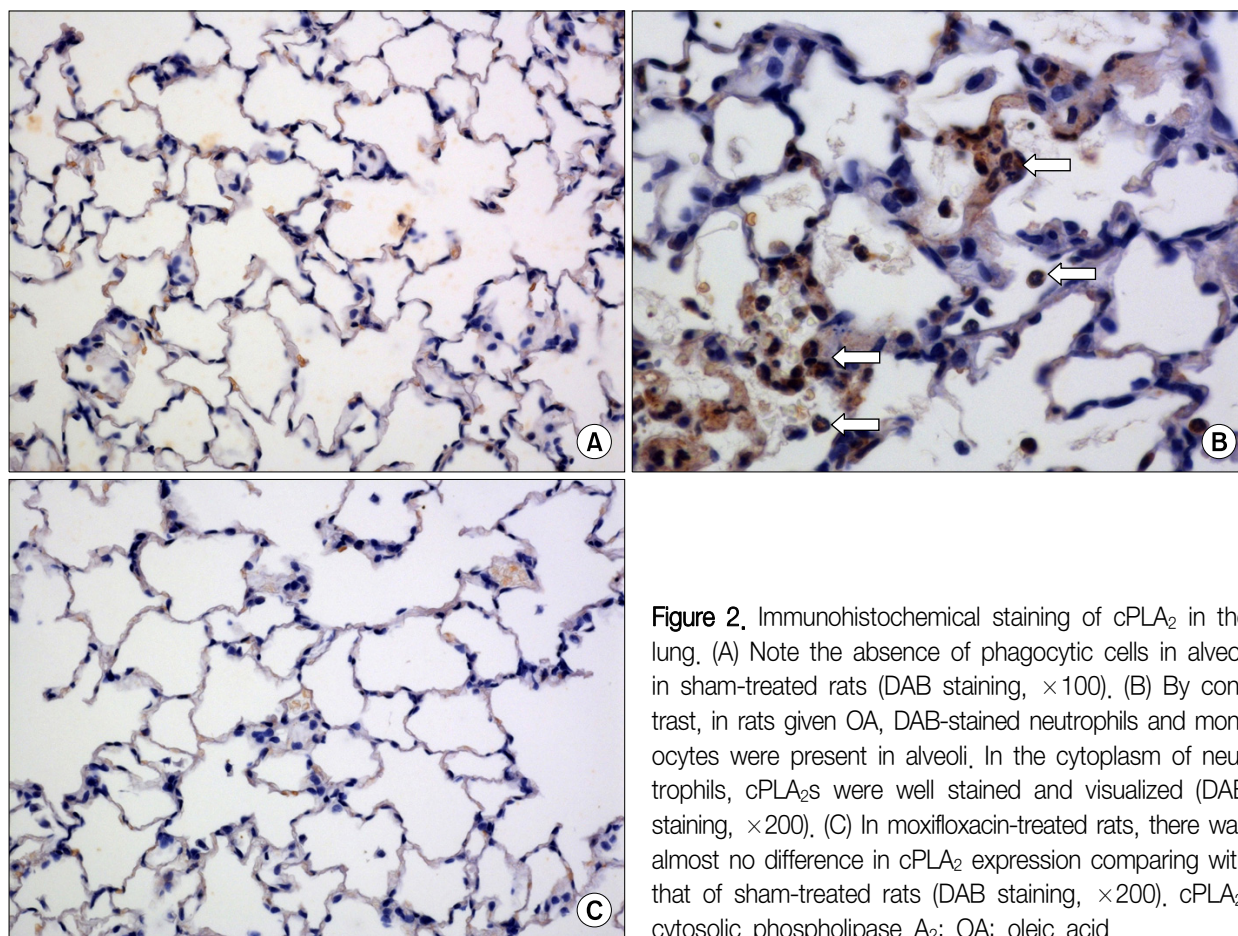
Figure 2. Immunohistochemical staining of cPLA$_2$ in the lung. (A) Note the absence of phagocytic cells in alveoli in sham-treated rats (DAB staining, ×100). (B) By contrast, in rats given OA, DAB-stained neutrophils and monocytes were present in alveoli. In the cytoplasm of neutrophils, cPLA$_2$s were well stained and visualized (DAB staining, ×200). (C) In moxifloxacin-treated rats, there was almost no difference in cPLA$_2$ expression comparing with that of sham-treated rats (DAB staining, ×200). cPLA$_2$: cytosolic phospholipase A$_2$; OA: oleic acid.

(Figure 3).

6. Western blotting of TNFα and COX-2 in the lung tissue

The immunoprecipitation of TNFα and COX-2 was carried out to estimate the anti-inflammatory effects of moxifloxacin associated with the pro-inflammatory cytokine and rate-limiting enzyme of inflammation. The OA increased the expression of TNFa and COX-2, which was normalized by moxifloxacin (Figure 4).

Discussion

Moxifloxacin is a fluoroquinolone that acts against gram positive and gram negative bacteria[19]. It has a dual role of antimicrobial and protective anti-inflammatory effects in clinical conditions[20].

In the present study, the property of immunomodu-lating effect of moxifloxacin was evaluated on OA-induced ALI in terms of protection from neutrophilic inflammatory reaction. As expected, moxifloxacin was shown to exert a protective effect on OA-induced lung leak and to suppress the accumulation and migration of neutrophils in the lung.

The OA is one of the derivatives of fatty acids and is responsible for acute inflammatory injury of the lung in FES, that is, OA induces lung leak, accumulation of neutrophils, and oxidative stress leading to ALI in experimental animals and human beings[21]. Moxifloxacin was effective to suppress lung leak by the decrease of neutrophilic oxidative stress or neutrophil-induced tissue injury evidenced by reducing lung MPO, number of BAL neutrophils, MDA content in the lung of rats given OA. Specifically, the lower content of MDA in the lung by moxifloxacin signifies the anti-oxidative stress effect of moxifloxacin.
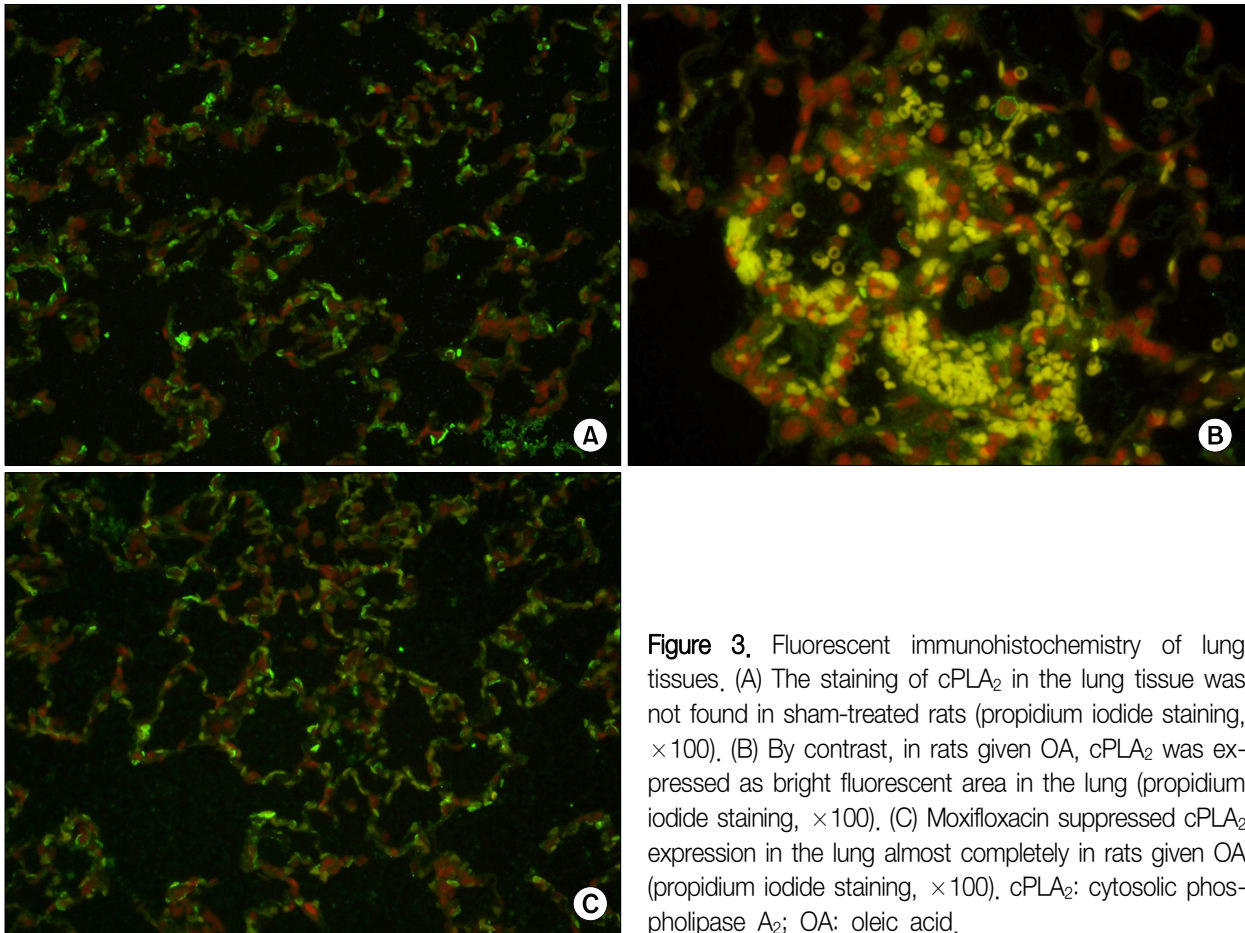
Figure 3. Fluorescent immunohistochemistry of lung tissues. (A) The staining of cPLA$_2$ in the lung tissue was not found in sham-treated rats (propidium iodide staining, ×100). (B) By contrast, in rats given OA, cPLA$_2$ was expressed as bright fluorescent area in the lung (propidium iodide staining, ×100). (C) Moxifloxacin suppressed cPLA$_2$ expression in the lung almost completely in rats given OA (propidium iodide staining, ×100). cPLA$_2$: cytosolic phospholipase A$_2$; OA: oleic acid.

According to Shalit and associates[13], moxifloxacin was effective to inhibit the migration of neutrophils on endothelial cells resulted in the decrease of cellular injury. The results in this experiment are consistent with their report.

In addition, moxifloxacin's anti-inflammatory effects were demonstrated prominently in lung histology. Although the mechanism is obscure, the decreased activity of cPLA$_2$ and the expression in the lung might be one of the clues to understand the anti-inflammatory property of moxifloxacin.

Until recently, the relationship between the role of PLA$_2$ and the immunoregulatory effect of moxifloxacin has not been elucidated. Since the role of cPLA$_2$ on ALI is predominant in provoking acute pulmonary edema, the decrease of the pulmonary edema by moxifloxacin might be originated from the down regulation of cPLA$_2$. Because the activation of cPLA$_2$ by OA was evident and the activation of cPLA$_2$ would be followed by the synthesis of pro-inflammatory lipid molecules[22], it is natural to envisage that the moxifloxacin's anti-inflammatory effect was derived from the inhibition of cPLA$_2$.

In western blot analysis, the expression of TNF α was stimulated by OA, and moxifloxacin depressed the increased expression of TNF α by OA. According to the classical process of the inflammation, the activation of cPLA$_2$ could be initiated by the increased expression of TNF α, one of the pro-inflammatory cytokines which induced inflammatory reaction in ALI[23]. The decreased expression of TNF α by moxifloxacin might subsequently decreased the cPLA$_2$ activity in the lung and suppressed the expression of cPLA$_2$ in the lung and neutrophils.

One more interesting result was that the OA increased expression of COX-2, one of the rate-limiting enzymes of inflammation. The increased expression of
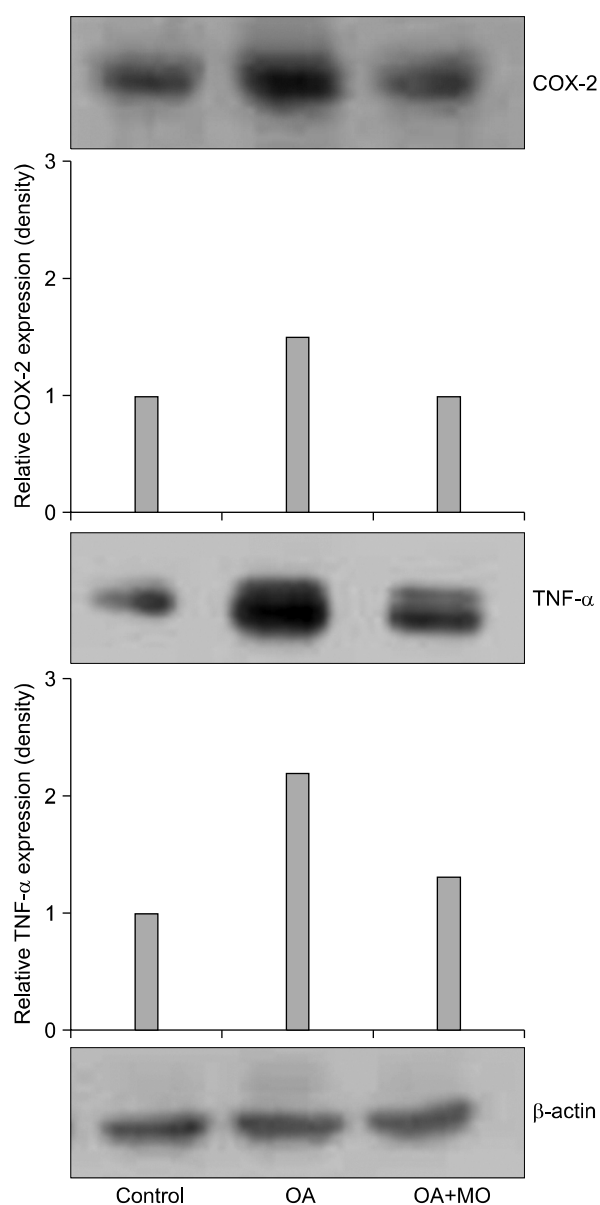
Figure 4. Western blot of TNFα and COX-2 in the lung tissue. The OA increased the expression of TNFα and COX-2. Moxifloxacin effectively suppressed the expression of TNFα and COX-2 which had been upregulated by OA. OA: oleic acid; MO: moxifloxacin.

COX-2 by OA was lowered by moxifloxacin, which implied the anti-inflammatory property of moxifloxacin in ALI. Moxifloxacin seemed to block the downstream of the inflammatory process by disintegration of positive feedback loop of neutrophilic oxidative stress[24].

In other words, moxifloxacin suppressed secretion of TNFα from phagocytes which down regulated cPLA$_2$

and was followed by the decreased expression of COX-2. From the inflammatory reaction's point of view, the block or suppression of pro-inflammatory cytokine presumed to lower the cPLA$_2$ activity in the lung by moxifloxacin.

Conclusively, though further studies are to be desired to understand the precise mechanism of the effects of moxifloxacin on FES-induced ALI, based on the present study, I would like to suggest that the suppression of cPLA$_2$ by moxifloxacin might be protective against injurious neutrophilic oxidative stress in rats.

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