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Rutin Ameliorates Neutrophilic Oxidative Stress-Induced Acute Lung Injury by Intratracheal IL-1 Insufflation in Rats

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Rutin, a group II phospholipase A_2 (PLA_2) inhibitor, was tested on interleukin-1 (IL-1) induced acute lung injury (ALI) in male Sprague-Dawley rats. Rutin did not alter the increased lung myeloperoxidase activity by IL-1. However, the number of neutrophils in bronchoalveolar lavage fluid (BALF) and IL-1 induced lung leak were decreased by rutin ($p<0.001$). Simultaneously, rutin decreased lung PLA_2 activity, which was increased by IL-1 ($p<0.001$). The reduction of neutrophilic respiratory burst by the inhibition of PLA_2 was confirmed by group II PLA_2 inhibitors such as rutin, manoalide and scalaradial. The increased level of cytokine-induced neutrophilic chemoattractant (CINC) in BALF by IL-1 was not affected by rutin. Ultrastructural changes of ALI and increased generation of free radicals in the lung by IL-1 were found, and rutin ameliorated these pathological findings. Taken together, rutin seems to be effective in decreasing IL-1 induced ALI through inhibition of group II PLA_2.

Key words : Group II phospholipase A_2, neutrophils, free radicals, acute lung injury

Introduction

Acute respiratory distress syndrome (ARDS) is a type of acute inflammatory edema caused by various etiologies [20]. The pathogenesis of ARDS has been studied extensively, but the exact mechanism is still cryptogenic.

Since the inflammatory reaction in the lung is evident regardless of the etiologies, investigators have agreed that phospholipase A_2 (PLA_2), a rate limiting enzyme of inflammatory reaction, is one of the critical causes to provoke the acute pulmonary inflammation [17]. In an association with the pulmonary inflammation, the massive infiltration of phagocytes especially neutrophils has been confirmed [18].

The so called collateral damage caused by the activated neutrophils seems to be linked to the activation of PLA_2 in the lung [1]. As Dana et al. [6] has insisted, the activation of NADPH oxidase for neutrophilic respiratory burst needs the lipid mediators produced by activation of PLA_2. On the basis of these reports, the inhibition of PLA_2 has been tested to examine the possibility as the therapeutic modality and possible pathogenic mechanism of ARDS. However, up to now, the underlying mechanism of the ARDS has been discovered partially associated with oxidative stress and inflammatory cytokines.

Recent studies have discovered that there are various types of PLA_2s and they appear to have specific roles in inflammation [5,19]. Among these PLA_2s, group II PLA_2 is one of the candidates to provoke the inflammatory reaction in ARDS. Therefore, the inhibition of group II PLA_2 has been tried to elucidate the role of secretory PLA_2 ($sPLA_2$) in acute lung injury (ALI), which resulted in unsatisfactory outcome. In this regards, we would like to inspect the effect of rutin, one of the flavonoids and $sPLA_2$ inhibitors, on the neutrophilic respiratory burst in the IL-1 induced ALI. We admit that present study is not the first experiment to figure out the role of PLA_2 inhibition in ALI, but we tried to understand the effect of rutin mainly on the production of free radicals associated with neutrophilic chemotaxis and respiratory burst.

Materials and Methods

Source of reagents

Recombinant human interleukin-1 (IL-1, endotoxin level<1 EU/mg) was purchased from R & D systems (Minneapolis, MN, USA) frozen in aliquots and thawed before use. L-α-dipalmitoyl-(2-palmitoyl-9,10-^{3}H(N))-phosphatidylcholine was obtained from Du Pont NEN research product (Boston, MA, USA). All other chemical reagents, otherwise mentioned, purchased from Sigma Chemical Company

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(St. Louis, MO, USA).

Source of rats and experimental protocols for IL-1 *treatment in vivo*

Male Sprague-Dawley rats (250-300 g, 10 weeks old) were obtained from Bio-Korea Co. (Osan, Korea). Rats were fed standard chow and water *ad libitum*. Experimental protocols were permitted by the Ethical Committee of Animal Experiments in Daegu Catholic University, School of Medicine in accordance with NIH guidelines for the use of laboratory animals.

Induction of ALI and inhibition of group II PLA_2

In order to induce ALI simulating ARDS in human being, rats were given IL-1 (50 ng) intratracheally in 0.5 ml of normal saline while rats were anesthetized with enflurane. In sham treated rats, 0.5 ml of normal saline was given intratracheally. Rutin (100 mg/kg; Sigma Chemical, St. Louis, MO, USA) was given intraperitoneally as suspension in normal saline.

Measurement of lung myeloperoxidase (MPO) activity

Lung tissue MPO activity was measured using o-dianisidine as the chromogenic substrate. H_2O_2 was used for the initiation of the reaction. Light absorbance at 460 nm was proportional to MPO activity according to the elapse of time [7].

Enumeration of bronchoalveolar lavage neutrophils

Bronchoalveolar lavage was performed by cannulating the trachea and instilling 8.0 ml of normal saline with a syringe 3 times. Approximately 6.0 ml of bronchoalveolar lavage fluid (BALF) was obtained from each rat. After the recovered BALF was centrifuged (1,000 rpm, 10 min at room temperature), the supernatant was collected and stored at -20°C for protein measurement. The sedimented cellular pellet was resuspended in 1 ml of distilled water and 1 ml of balanced Hank's salt solution (HBSS) for two seconds to lyse erythrocytes, and then centrifuged again. The supernatant was discarded and the pellet was resuspended in 1 ml of normal saline. Using 200 µl of suspended solution, cytospin slide preparations were prepared and Wright stained for differential cell count. Total leukocyte count was performed with a hemocytometer and the fraction of neutrophils was calculated by determining percentage of neutrophils.

Determination of protein content in bronchoalveolar lavage fluid

To evaluate the protein leak from the alveolar capillary, the protein content in BALF was measured according to the method of Brown et al. [4].

Measurement of lung PLA_2 activity

To evaluate the PLA_2 activity in the lung tissue, lungs were excised after perfusion with normal saline. Right lungs were homogenized in 20 mM potassium phosphate (pH 7.4). The homogenate was then sonicated 90 s on ice. PLA_2 activity was measured by incubating the radioactive substrate was tritiated DPPC (30 Ci/mmol) with 100 µl of the lung homogenate in 889 µl of glycine buffer (100 mM, pH 9.0) containing 10 g/l BSA, 2.5 mM sodium deoxycholate, 0.1 mM DPPC, 2.0 mM $CaCl_2$, and 1.75 M ethanol. After the mixture was incubated for 60 min at 37°C, the reaction was stopped by adding 200 µl of 5% (v/v) Triton X-100 containing 200 mM EDTA. Subsequently, fatty acids released by PLA_2 hydrolysis were extracted by 5.0 ml of hexane containing 0.1% acetic acid and 2.5 g of Na_2SO_4. After vortex mixing, the hexane layer was separated and counted in a liquid scintillation counter. Snake venom (*Crotalus adamanteus*) PLA_2 control samples were assayed along with all other experimental samples for standardization. One unit of enzyme activity was defined as the ability to hydrolyze 1 µmol of substrate per minute [10].

Cytochrome-c reduction assay

Neutrophils were isolated from fresh human blood. Briefly the mixture of 15 ml of pentaspan and 0.1 ml of heparin (1,000 IU) were mixed with 30 ml of fresh human blood before standing for 40 min at room temperature. Plasma layered on the 75%, 55% isotonic percoll gradient after separation from erythrocytes, centrifuged (15,000 rpm, 20 min) and neutrophils were collected from the 55%, 75% interface of percoll. Collected suspension of neutrophils was hypotonic lysed for the elimination of erythrocytes and washed twice with normal saline. For cytochrome-c reduction assay, save control samples, phorbol myristate acetate (PMA, 2.5 µg/ml), rutin (100 µM), manoalide (20 µM), scalaladial (200 µM) were added to the neutrophil suspension (2×10^6 cell/ml) before incubating for 15 min at 37°C. Additionally, to know the effect of group II PLA_2 on the generation of free radicals from neutrophils, *Crotalus adamanteus* PLA_2 (1.0 unit) was added to the neutrophil suspension before incubat-

ing for 60 min at 37°C. Rutin was added to know the effect on the generation of free radicals in PLA_2-stimulated neutrophils. Light absorbance at 550 nm was proportional to the reduction of cytochrome-c by free radicals [2,8].

Examination of the ultrastructural changes with electron microscopy

After small blocks (1 mm^3) of the lung tissue were rapidly fixed in the cold 2.5% glutaraldehyde, the lung tissue was degassed by the addition of pressurized air into the sealed vial. The prefixed tissues were rinsed with 1.0 mM phosphate buffer (pH 7.4) and post fixed with 1% osmium tetroxide. After dehydrated with graded alcohol-propylene, the tissues were embedded in epoxy-resin. The embedded tissues were polymerized at 37°C for 12 hr and at 60°C for 24 hr before ultra thin sectioning. The sections were stained with uranyl acetate and lead citrate before electron microscopy.

Localization of hydrogen peroxide in the lung

Five hours after IL-1 insufflation, rats were anesthetized with enflurane. Subsequently, thoracotomy was performed, lungs were removed and the small sections of the tissue were reacted with cerium chloride. Briefly, after the air was removed from the lung tissue, samples were incubated for 1 hr at 37°C in 0.1 M Tris-maleate buffer (pH 7.5) with 7% sucrose, 10 mM 3-amino-1,2,4-triazol, 2 mM cerium chloride, and 0.0002% Triton X-100. Then the samples were successively rinsed in 0.1 M Tris-maleate buffer (pH 7.5) with 7% sucrose and then 0.15 M sodium cacodylate buffer (pH 7.4). Subsequently, samples were fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 hr at room temperature, dehydrated through a graded series of ethanol to propylene oxide and embedded in epoxy resin. Block sections, 60-80 nm thick, were stained with uranyl acetate and then examined quantitatively using TEM at 75 kV [9].

Measurement of cytokine-induced neutrophil chemoattractant (CINC) in BAL

The concentration of CINC in BALF was measured by the method of sandwich ELISA according to Wittwer et al. [21].

Statistical analyses

Data are expressed as mean±SD and were analyzed using a one way analysis of variances with Student-Newman-Keuls multiple comparison test. A p value of <0.05 was considered significant.

Results

Effect of rutin on the accumulation and migration of neutrophils

The rats given IL-1 had increased the lung MPO activity compared to sham-treated rats ($p<0.001$). And the increased lung MPO activity was not altered by the treatment of rutin in IL-1 given rats (Table 1). Interestingly, the number of neutrophils in BALF, as an index of neutrophilic migration from the vascular compartment into the alveoli, showed a different pattern compared with that of the lung MPO activity. IL-1 rats had increased ($p<0.001$) the number of neutrophils in BALF compared with sham-treated rats. However, rats given IL-1 and rutin showed a decrease in the number of

Table 1. Effects of rutin on the lung MPO activity, neutrophils and protein content in BALF, and lung PLA_2 activity in experimental rats

Treatment	Sham	IL-1	IL-1+Rutin
MPO activity (U/g of lung)	7.44±4.05 (n=14)	36.78±9.45[a] (n=18)	33.31±7.74 (n=9)
BALF neutrophils (millions/ml of BALF)	0.233±0.070 (n=7)	0.780±0.323[a] (n=15)	0.359±0.092[b] (n=5)
Protein content (mg/ml of BALF)	0.36±0.37 (n=10)	3.07±0.41[a] (n=8)	1.77±0.53[b] (n=7)
PLA_2 activity (mU/ml of lung homogenate)	1.32±0.55 (n=8)	4.70±1.59[a] (n=8)	2.54±0.81[b] (n=6)

Values are given as mean±SD.
n indicates number of experiments.
MPO: myeloperoxidase, BALF: bronchoalveolar lavage fluid
a, $p<0.001$, Sham vs. IL-1; b, $p<0.001$ IL-1 vs. IL-1+Rutin

neutrophils in BALF ($p<0.001$) compared to rats given IL-1 only (Table 1).

Effect of rutin on lung leak

IL-1 rats had increased protein content in BALF compared to sham-treated rats ($p<0.001$). Rutin significantly decreased this increase of protein content in the BALF in IL-1 rats (Table 1).

Effect of rutin on the PLA2 activity

IL-1 rats had increased lung PLA_2 activity ($p<0.001$) compared to sham rats. And the rats given IL-1 and rutin had decreased ($p<0.01$) the lung PLA_2 activity compared with IL-1 rats (Table 1).

Production of free radicals from neutrophils

The generation of free radicals from neutrophils was assessed by the cytochrome-c reduction assay. The neutrophils treated with PMA had increased the amount of reduced cytochrome-c compared with control neutrophils ($p<0.001$). The neutrophils treated with PLA_2 inhibitors had reduced the production of free radicals which was shown as the decreased amount of reduced cytochrome-c ($p<0.001$) (Table 2). Similarly, neutrophils treated with $sPLA_2$ had increased ($p<0.001$) the reduction of cytochrome-c compared with control neutrophils. Likewise in PMA-treated neutrophils, rutin had decreased the reduction of cytochrome-c in PLA_2 activated neutrophils ($p<0.001$) (Table 3).

CINC concentration in BALF

IL-1 rats had increased the concentration of CINC in BALF compared with sham rats ($p<0.001$). Rutin did not affect the increased concentration of CINC in the BALF of rats given IL-1 (Table 4).

Examination of the ultrastructural changes of the lung

In the lung of sham rats, basement membranes of the endothelial and epithelial cells were intact and alveolar lumen was well preserved (Fig. 1A). However the lungs of rats given IL-1 showed various pathological findings. Endothelial and epithelial blebbing and necrosis were noted (Fig. 1B). Vacuolization of lamellar bodies and denaturation of surfactant in the cytoplasm of type II pneumocytes, and interstitial edema were shown (Fig. 1C,D). Adhesion and merging of

Table 2. Evaluation of production of free radicals from isolated human neutrophils inhibited by Group II PLA_2 inhibitors

Treatment	Control (n=14)	PMA (n=9)	PMA+Rutin (n=8)	PMA+Mano (n=8)	PMA+Scala (n=7)
cytochrome-c reduced (nmol/2×10^6 PMNs)	11.16 ± 9.82	91.51± 4.62[a]	trace[b]	10.59± 9.17[c]	trace[d]

Values are given as mean±SD.
n indicates number of experiments.
PLA_2: phospholipase A_2, Mano: manoalide, Scala: scalaradial, PMN: polymorphonuclear neutrophil, PMA: phorbol myristate acetate
a, $p<0.001$, control vs. PMA; b, $p<0.001$ PMA vs. PMA+Rutin; c, $p<0.001$, PMA vs. PMA+Mano; d, $p<0.01$ PMA vs. PMA+Scala

Table 3. Effect of rutin on the generation of free radicals from isolated human neutrophils stimulated by group II PLA_2

Treatment	Control (n=9)	PLA_2 (n=9)	PLA_2+Rutin (n=8)
Cytochrome-c reduced (nmol/2×10^6 PMNs)	7.94±5.64	50.55±3.93[a]	12.31±4.43[b]

Values are given as mean±SD.
n indicates number of experiments.
PMN: polymorphonuclear neutrophil
a, $p<0.001$, Control vs. PLA_2; b, $p<0.01$, PLA_2 vs. PLA_2+Rutin

Table 4. Comparison of CINC concentration in experimental rats

Treatment	Sham (n=6)	IL-1 (n=8)	IL-1+Rutin (n=11)
CINC concentration (ng/ml of BALF)	0.04±0.03	0.41±0.16[a]	0.39±0.14[b]

Values are given as mean±SD.
n indicates number of experiments.
CINC: cytokine-induced neutrophil chemoattractant
a, $p<0.001$, Sham vs. IL-1; b, $p<0.001$, Sham vs. IL-1+Rutin

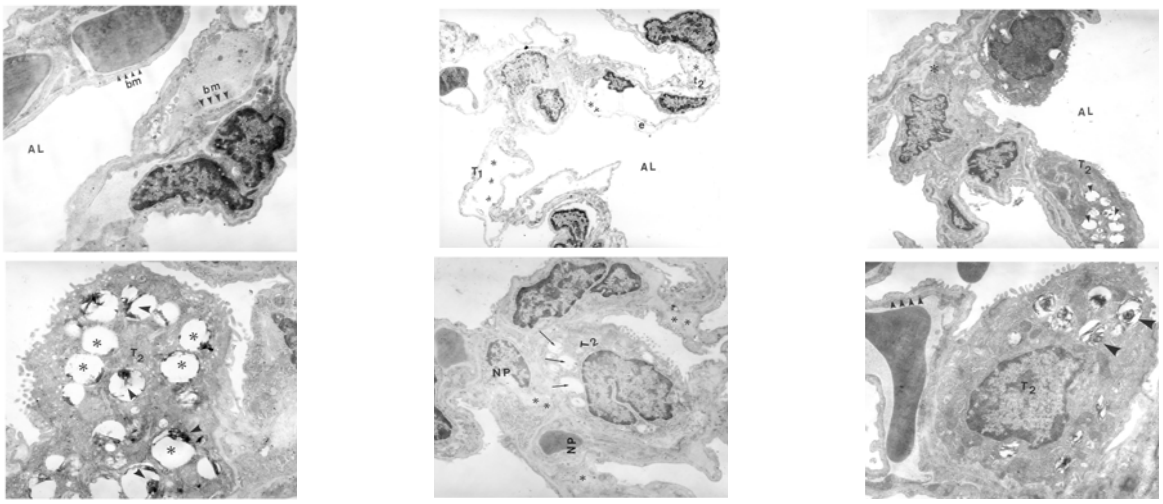

Fig. 1. Electron microscopic finding of the lung of experimental rats. In normal lung of rat, basement membrane (BM) was well preserved and alveolar lumen was intact (original mag. ×8,000, 1A). In IL-1 insufflated rats, ultrastructural changes of the lung were shown. Endothelial and epithelial blebbing and necroses were noted (asterisks, original mag. ×3,000, 1B). Vacuolization of lamellar bodies in the cytoplasm of the alveolar type II pneumocyte (arrow heads) were shown (original mag. ×5,000, 1C). Vacuolization of lamellar bodies (asterisks) and denaturation of surfactant (arrow heads) in the lamellar bodies in the alveolar type II pneumocyte were noted (original mag. ×8,000, 1D). Neutrophilic infiltration in the interstitium of the lung, vacuolization of the lamellar bodies in the alveolar type II cells (arrow) and interstitial edema (asterisks) were found. Adhesions and merging of neutrophil and epithelial membrane were evident (original mag. ×6,000, 1E). Attenuation of the IL-1 induced injury was demonstrated by the treatment of rutin in the lungs of rats (original mag. ×8,000, 1F). The basement membrane (arrow heads) and lamellar bodies were intact and interstitial edema was diminished. e; endothelial cell, T1; alveolar type I pneumocyte, T2; alveolar type II pneumocyte, AL; alveolar lumen, NP; neutrophil.

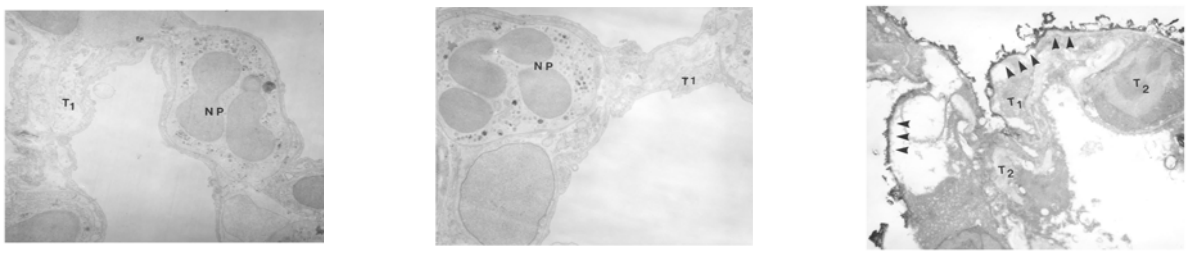

Fig. 2. Localization of the hydrogen peroxide in the lung by the method of cytochemical electron microscopy. No deposit of cerrous perhydroxide was found (original mag. ×4,000, 2A). In contrast, in the lungs of rats given IL-1, dense deposits of cerrous perhydroxide granules (arrow heads) were found along the epithelial lining, signifying the production of hydrogen peroxide in the lung (original mag. ×5,000, 2B). In the lungs of rats given IL-1, rutin inhibited the production of hydrogen peroxide and resulted in the devoid of cerrous perhydroxide in the lung in spite of the infiltration of neutrophils (original mag. ×5,000, 2C). T1; alveolar type I pneumocyte, T2; alveolar type II pneumocyte, NP; neutrophil

neutrophils and epithelial membrane were evident (Fig. 1E). Rutin attenuated ALI caused by IL-1 (Fig. 1F). Basement membrane and lamellar bodies were intact and interstitial edema was diminished. Localization of hydrogen peroxide in the lung (Fig. 2): No deposits of cerrous perhydroxide (reactive product of cerium chloride and hydrogen peroxide) was found in the lungs of sham-treated rats (Fig. 2A). In contrast, in the lungs of IL-1 rats, dense deposits of cerrous perhydroxide granules were found along the epithelial lining which signified the production of hydrogen peroxide in the lung (Fig. 2B). However the treatment of rutin to the rats given IL-1 inhibited the generation of hydrogen peroxide and resulted in absence of cerrous perhydroxide granules in the lung in spite of the infiltration of the neutrophils in the interstitium (Fig. 2C).

Discussion

Neutrophilic oxidative stress is one of the important pathogenic mechanisms of ARDS. Among the variable origins, phagocytes including neutrophils have been known to play a key role in generating free radicals which is responsible for the oxidative stress in the lung [22]. In the present study, we could confirm the fact that IL-1 had increased lung MPO activity and increased the number of neutrophils in BALF. At the same time, IL-1 had increased the protein content in BALF implicating the endothelial injury by IL-1. As Leff et al. [15] insisted, these changes were induced by neutrophilic oxidative stress by IL-1. Rutin, a derivative of flavonoids, effectively attenuated protein leak in the lung even though it could not inhibit the infiltration of neutrophils in the lung. The migration of neutrophils from vascular lumen into alveoli was suppressed by rutin and it might be the cause of the amelioration of lung leak associated with the decrease of endothelial injury. Rutin is known as an oxidant scavenger and effective inhibitor of group II secretory PLA_2 [3]. In conjunction with this fact, antioxidant effects of rutin have acted on the decrease of lung leak caused by IL-1. As is well known, IL-1 provokes ALI in human and experimental animals through the activation of PLA_2 resulting in the release of proinflammatory lipid mediators [14]. Among these lipid mediators, arachidonic acid or PAF activates NADPH oxidase on the neutrophilic membrane, by which superoxide anions can be released from neutrophils [11].

We observed that rutin had decreased the PLA_2 activity which had been enhanced by IL-1. Though rutin has long been known to decrease the oxidative stress in various experimental and clinical situations, still the exact mechanism of reducing oxidative injury has not been elucidated. According to Lee et al. [13], IL-1 increased PLA_2 activity in the lung and adhesion of neutrophils to endothelial cells. It was revealed that mepacrine, a non-specific PLA_2 inhibitor, did decrease the PLA_2 activity and neutrophilic adhesion to endothelial cells, and subsequently decreased lung leak. In relation to these reports and our data, the inhibition of PLA_2 by rutin might be the cause of the lessening of ALI. The inhibition of group II PLA_2 with manoalide and scalaradial denoted the reduced production of free radicals from neutrophils. And the amount of free radicals produced by the PLA_2-activated neutrophils was diminished by rutin also. Collectively, these experimental results suggest that the inhibition of PLA_2 by rutin contributed to the decrease the ALI by IL-1.

CINC is a strong chemoattractant for neutrophils but CINC itself is not able to provoke oxidative stress from neutrophils [12]. CINC was increased by IL-1. Rutin could not affect the concentration of CINC in the BALF of IL-1 rats. From this result, we deduced that rutin decreased the migration of neutrophils from vessel to alveoli by the inhibition of oxidant production. Even if there is a possibility of scavenging effect of rutin, it is not strong enough to inhibit explosive oxidative stress. Thus, it appears that the inhibition of PLA_2 by rutin could be the principal cause of decreased lung leak in this study. These suggestions were even more evident in our morphological study. As was shown in our data on the ultra structural changes, IL-1 caused typical pathological changes such as endothelial and epithelial injuries. The pathognomonic signs of oxidative stress [16], in the lung, i.e. vacuolization and hypertrophy of lamellar bodies with denaturation of pulmonary surfactant were evident after IL-1. Again, these changes were lessened by rutin. In addition, the increased generation of free radicals from neutrophils was reduced to normal by rutin. Morphological, cytochemical confirmation of the effect of rutin on the neutrophilic oxidative stress is direct evidence of rutin's antioxidant property associated with PLA_2 inhibition. Recently many types of PLA_2s have been discovered and their functions have been under investigation in conjunction with anti-inflammatory pulmonary edema. Though some clinical trials of anti-sPLA_2 antibody turned out to be unsuccessful, still it is possible that a certain type of specific PLA_2 inhibitor might be effective for ARDS treatment. Since most of the chemotherapeutic agents for ARDS have been proven unsuccessful, present results might contribute to the understanding and development of new therapeutic paradigm for ALI/ARDS.

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초록 : Interleukin-1으로 유도된 급성폐손상에서 rutin의 효과

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흰쥐에서 Interleukin-1 (IL-1)으로 유도된 급성폐손상에서의 group II phospholipase A_2 (PLA_2) 억제제인 rutin의 효과를 알아보기 위하여 본 연구를 시행하였다. Rutin은 IL-1에 의해 증가한 폐장내의 myeloperoxidase의 활성도를 감소시키지는 못하였으나 폐포세척액 내의 호중구의 수 및 모세혈관의 손상지표로 알려져 있는 폐장 모세혈관에서의 단백질 누출양을 감소시켰다. 동시에 rutin은 IL-1에 의하여 증가한 폐장의 염증조절효소인 PLA_2의 활성도를 감소시키고 결과적으로 호중구에서의 산소기의 생성을 감소시켰다. Rutin 뿐만 아니라 manoalide, scalaradial 같은 group II PLA_2의 억제제도 호중구의 respiratory burst를 감소시킴을 확인하였다. IL-1에 의하여 증가한 폐포세척액 내에서의 cytokine induced neutrophil chemoattractant의 농도는 rutin에 의해 영향을 받지 않았다. 형태학적으로는 IL-1에 의한 폐장조직에서의 산소기의 형성이 관찰되었고 rutin은 이러한 산소기의 생성을 현저히 감소시켰다. 이러한 결과로 미루어 group II PLA_2 억제제인 rutin은 호중구에서의 활성 산소기의 생성을 효과적으로 억제함으로써 IL-1에 의한 급성폐손상의 감소를 가져 오는 것으로 결론지을 수 있다.