J Pharmacol Sci **91**, 8 – 14 (2003)

Journal of Pharmacological Sciences

Full Paper

Lysophosphatidic Acid Enhances In Vivo Infiltration and Activation of Guinea Pig Eosinophils and Neutrophils via a Rho/Rho-Associated Protein Kinase-Mediated Pathway

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Received August 5, 2002; Accepted September 26, 2002

Abstract. Lysophosphatidic acid (LPA) has been shown to be a chemoattractant in in vitro studies. The present study was carried out to determine whether LPA enhances infiltration of inflammatory cells in in vivo studies with guinea pigs. LPA (1 – 10 μg/ml), when by guinea pigs for 5 min, substantially increased the numbers of eosinophils and neutrophils in the bronchoalveolar lavege fluid (BALF), which was recovered at over 4 h after the inhalation of LPA. Infiltration in BALF was significantly inhibited by inhalation of Y-27632, an inhibitor of Rho-associated protein kinase (ROCK). LPA also increased superoxide production of eosinophils and neutrophils. In contrast, Y-27632 inhibited superoxide production. These findings suggest that LPA may contribute to infiltration and activation of inflammatory cells in bronchial asthma; furthermore, the Rho/ROCK-mediated pathway may be involved.

***Keywords*:** lysophosphatidic acid, Rho-associated protein kinase, inflammatory cell, superoxide production

Introduction

Asthma is characterized by chronic pulmonary inflammation involving infiltration of inflammatory cells such as eosinophils and neutrophils, accompanying enhanced airway responsiveness (1). Lysophosphatidic acid (LPA) functions as a chemoattractant for human neutrophils in vitro (2) and also caused increases in binding of monocytes and neutrophils to human aortic endothelial cells (3). Therefore, it is reasonable to anticipate that LPA will induce the infiltration of such inflammatory cells into the pulmonary airway. However, these experiments have relied upon in vitro observations of chemoattractive activity; moreover, no conclusive proof regarding the involvement of LPA in vivo has been obtained.

Activated inflammatory cells including neutrophils, eosinophils, and macrophages produce various mediators. Major basic protein, an eosinophil-derived cationic granuloprotein, damages the bronchial epithelium and increases airway responsiveness (4). Superoxide radicals, which are also produced by these cells, exert various effects on pulmonary airway (5). Therefore, if LPA induces the activation of inflammatory cells as well as infiltration of these cells, it is suggested that LPA plays a role in the obstructive airway disease associated with airway inflammation, i.e., asthma.

Consequently, the ability of LPA to induce infiltration and activation effects of inflammatory cells was investigated in the guinea pig. Furthermore, the effects of Y-27632, an inhibitor of Rho-associated protein kinase (ROCK), are described.

Materials and Methods

Animals

Male Hartley guinea pigs weighing 250 – 700 g were obtained from Saitama Laboratory Animal, Inc. All experiments were conducted according to the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society.

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Materials

Monooleoyl phosphatidic acid monosodium (LPA) was obtained from Avanti Polar-Lipids, Inc. (Ontario, Canada). Y-27632 ((+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl)cyclohexanecarboxamide) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Additional reagents included the following: phorbol-12-myristate-13-acetate (PMA), superoxide dismutase (SOD), cytochrome c, polymyxin B sulfate, and Histopaque®-1077 (Sigma) and casein sodium (Wako Pure Chem., Osaka). All reagents were of the highest grade commercially available.

LPA was dissolved in deionized water and diluted with physiological saline. Y-27632 and polymyxin B were prepared with physiological saline. PMA was dissolved in dimethyl sulfoxide. Other reagents were dissolved in deionized water.

LPA inhalation

Guinea pigs were anesthetized by intraperitoneal injection of urethane (1.5 g/kg). An endotracheal cannula was inserted and connected to a respirator (SN-480-7; Shinano Seisakujyo, Tokyo) following anesthesia. Animals were ventilated at a tidal volume of 10 ml/kg with a frequency of 70 strokes/min. LPA was administered (1 or 10 μg/ml, for 5 min) using an ultrasonic nebulizer (NE-U12; Tateisi Electrics, Kyoto) linked to the respirator.

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed 2, 4, or 6 h following LPA inhalation. Under anesthesia, guinea pigs were exsanguinated from the femoral artery. The trachea was cannulated with a polyethylene tube through which the lungs were lavaged three times with 5 ml of physiological saline (15 ml total). Bronchoalveolar lavage fluid (BALF) was centrifuged at $150 \times g$ for 10 min. The pellet obtained was immediately suspended in physiological saline; total cell numbers in BALF were counted in duplicate via microscopic examination. Differential cell counts were made from centrifuged preparations stained with Wright-Giemsa; counts consisted of 500 or more cells in each animal.

Effect of Y-27632 on infiltration of inflammatory cells

To assess the relationship between the Rho/ROCK-mediated pathway and LPA-induced infiltration of inflammatory cells, the effect of Y-27632 on differential cell counts was determined. Y-27632 (1 mM) was inhaled for 5 min employing an ultrasonic nebulizer linked to a respirator prior to LPA inhalation. After 6 h, total cell numbers and differential cell counts were measured as described above.

Preparation of leukocytes fraction

Guinea pig peritoneal neutrophils were isolated from exudates 16 – 30 h after intraperitoneal injection of 3% casein sodium as described previously (6). Peritoneal exudate cells were washed twice with calcium- and magnesium-free Hank's balanced salt solution (HBSS). Following removal of contaminating erythrocytes via hypotonic treatment with 0.2% NaCl, the collected cells were suspended at 1×10^6 cells/ml in HBSS. The purity of neutrophils was approximately 90%. Eosinophils were isolated from the eosinophil-rich peritoneal cavity of polymyxin B-treated guinea pigs according to the method described by Pincus (7). Guinea pigs were injected intraperitoneally with 2 mg/animal polymyxin B weekly for more than 5 weeks. At 48 h following the final injection, the peritoneal cavity was lavaged after exsanguinations under anesthesia. Peritoneal exudate cells were washed twice with calcium- and magnesium-free HBSS. Following removal of contaminating erythrocytes via hypotonic treatment with 0.2% NaCl, cell suspension was overlaid on an equal volume of Histopaque®-1077. Tubes were centrifuged at $150 \times g$ for 20 min. Cells obtained from the bottom were washed twice and suspended at 1×10^6 cells/ml in HBSS. This preparation contained approximately 90% eosinophils. Viability of these leukocytes was examined by trypan blue stain, and cells with viability greater than 90% were utilized for the experiments.

Superoxide production

Production of superoxide from neutrophils or eosinophils was measured by a spectrophotometric assay of SOD-inhibitable cytochrome c reduction as described previously (8). The cell suspension was pre-warmed for 5 min at 37°C, challenged with LPA (final concentration: 10 μg/ml) or PMA (final concentration: 100 ng/ml) in the presence of 80 μM cytochrome c with or without 60 U/ml of SOD, and further incubated for 5, 20, or 60 min. The reaction was terminated by placement of the reaction tube in an ice-water bath. Following centrifugation at $450 \times g$ for 10 min, the absorbance of the supernatant was measured utilizing a spectrophotometer (U-2000; Hitachi, Tokyo).

Effect of Y-27632 on LPA-induced superoxide production

Effect of Y-27632 on superoxide production induced by LPA was investigated. The cell suspension was incubated with 1 or 10 μM Y-27632 at 37°C. LPA (10 μg/ml) was introduced to the same sample 10 min later and further incubated for 60 min.

Statistical analyses

Values are expressed as means ± S.E.M. Student's *t*-test or Dunnett's multiple comparison test was employed to calculate the statistical significance of differences between the means of the test and control groups. A *P*-value of less than 0.05 was considered significant.

Results

Infiltration of inflammatory cells induced by LPA

The results of BAL at 6 h following saline or LPA inhalation are shown in Fig. 1. The numbers of total cells, neutrophils, and eosinophils in the saline group were $2.38 \pm 0.34 \times 10^6$, $1.04 \pm 0.70 \times 10^5$, and $0.13 \pm 0.02 \times 10^5$, respectively. No significant changes were observed in comparison with the native group (total cells: $3.01 \pm 0.55 \times 10^6$, neutrophils: $0.48 \pm 0.16 \times 10^5$, eosinophils: $0.24 \pm 0.04 \times 10^5$). Consequently, infiltration of inflammatory cells was compared with the native group. Although 1 μg/ml LPA scarcely affected the cells, the numbers of total cells, neutrophils, and eosinophils increased by inhalation of 10 μg/ml LPA. Significant differences were detected between the group receiving 10 μg/ml LPA and the native group ($P<0.05$, at all cell counts measured).

The time course of BAL is displayed in Fig. 2.

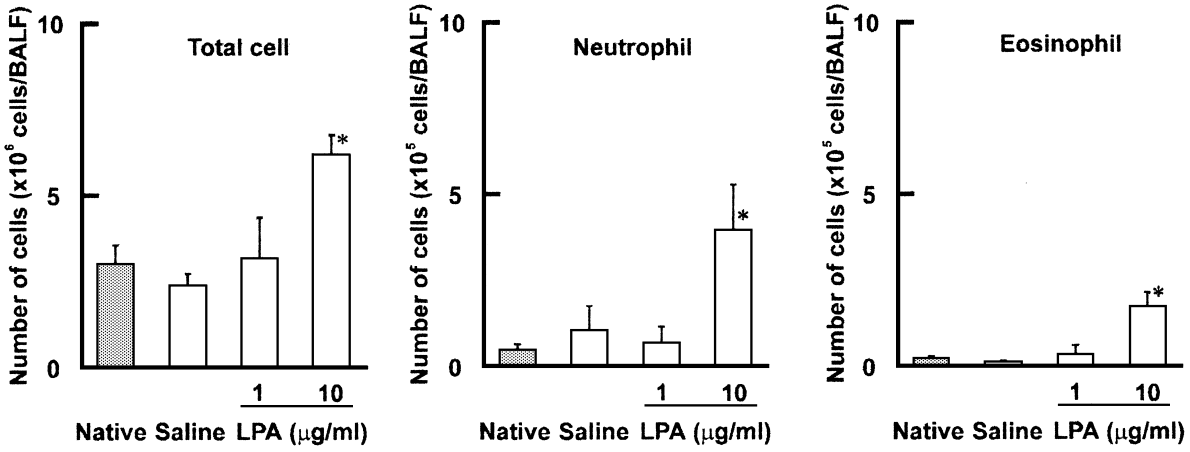

Fig. 1. Effect of LPA inhalation on differential cell counts recovered from bronchoalveolar lavage fluid in guinea pigs. LPA (1 or 10 μg/ml) was inhaled for 5 min employing an ultrasonic nebulizer. Bronchoalveolar lavage was performed 6 h after LPA or saline inhalation. Each column represents the mean ± S.E.M. of 4 – 5 animals. **P*<0.05: significantly different from the native group. (Dunnett's multiple comparison test).

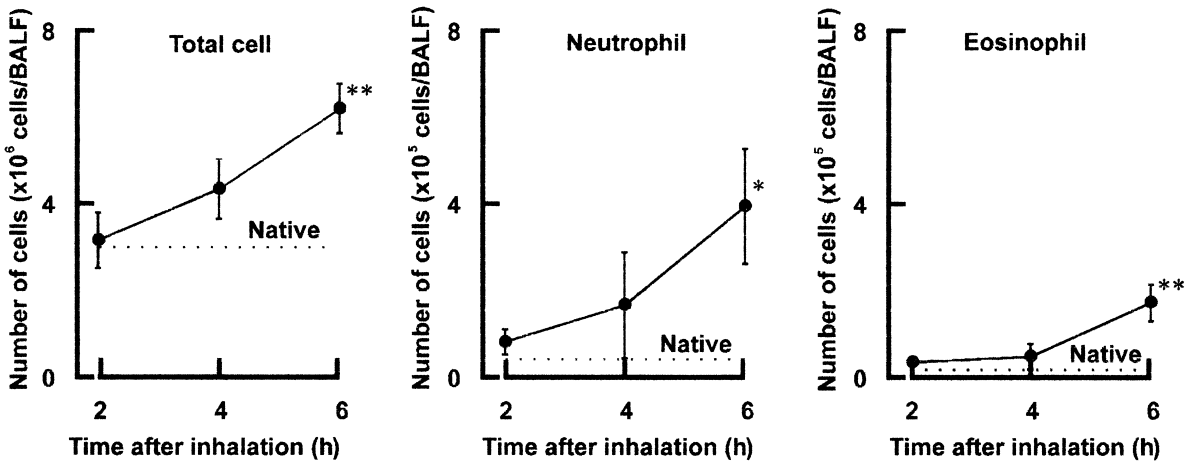

Fig. 2. Time course of cellular infiltration to the airway induced by LPA inhalation in guinea pigs. LPA (10 μg/ml) was inhaled for 5 min employing an ultrasonic nebulizer. Bronchoalveolar lavage was performed 2, 4, or 6 h after LPA inhalation. Each point represents the mean ± S.E.M. of 5 animals. **P*<0.05, ***P*<0.01: significantly different from the native group. (Dunnett's multiple comparison test).

The numbers of total cells, neutrophils, and eosinophils at 2 h after LPA inhalation were $3.15 \pm 0.64 \times 10^6$, $0.81 \pm 0.29 \times 10^5$, and $0.35 \pm 0.11 \times 10^5$, respectively. No significant differences were observed in comparison to the native group. In contrast, LPA increased the numbers of total cells ($4.33 \pm 0.69 \times 10^6$), neutrophils ($1.66 \pm 1.22 \times 10^5$), and eosinophils ($0.48 \pm 0.29 \times 10^5$) from 4 h after inhalation; moreover, significant differences were observed at 6 h after inhalation (total cells: $6.19 \pm 0.57 \times 10^6$; $P<0.01$, neutrophils: $3.95 \pm 1.33 \times 10^5$; $P<0.05$, eosinophils: $1.72 \pm 0.42 \times 10^5$; $P<0.01$).

Effect of Y-27632 on infiltration of inflammatory cells

Figure 3 illustrates the effect of Y-27632 on infiltration of inflammatory cells induced by LPA inhalation. LPA inhalation induced a significant increase in the total cells (native: $2.19 \pm 0.16 \times 10^6$, LPA: $4.53 \pm 0.47 \times 10^6$; $P<0.01$), neutrophils (native: $0.21 \pm 0.10 \times 10^5$, LPA: $1.56 \pm 0.47 \times 10^5$; $P<0.05$), and eosinophils (native: $0.08 \pm 0.03 \times 10^5$, LPA: $0.89 \pm 0.29 \times 10^5$; $P<0.05$). Y-27632 (1 mM) reduced these cell counts to levels observed in the native group. Significant differences were evident between the vehicle- and Y-27632-treated

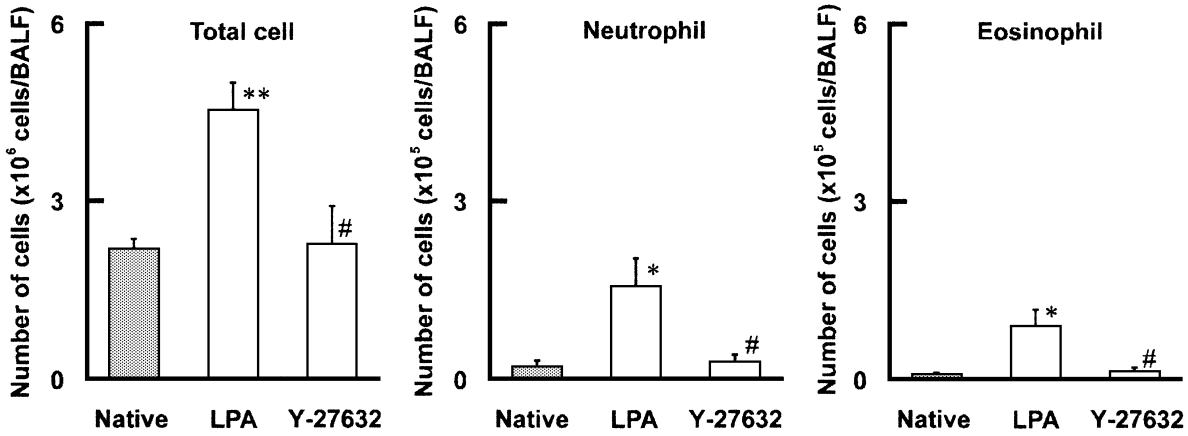

Fig. 3. Effect of Y-27632 on differential cell counts recovered from bronchoalveolar lavage fluid in guinea pigs following LPA inhalation. Y-27632 (1 mM) was inhaled for 5 min employing an ultrasonic nebulizer linked to a respirator prior to LPA inhalation. LPA (10 μg/ml) was inhaled for 5 min employing an ultrasonic nebulizer. Bronchoalveolar lavage was performed 6 h after LPA inhalation. Each column represents the mean ± S.E.M. of 4 – 5 animals. *$P<0.05$, **$P<0.01$: significantly different from the native group (Student's *t*-test). $^{\#}P<0.05$: significantly different from the vehicle-treated group (Student's *t*-test).

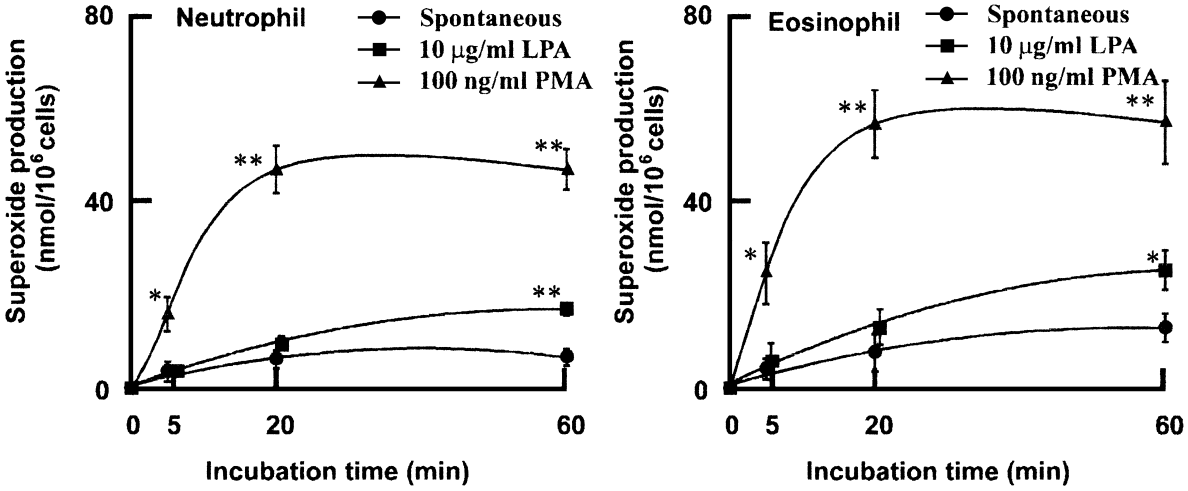

Fig. 4. Time course of superoxide production from guinea pig neutrophils and eosinophils after stimulation with LPA or PMA. The cell suspension was pre-warmed for 5 min at 37°C, challenged with LPA (final concentration: 10 μg/ml) or PMA (final concentration: 100 ng/ml) in the presence of 80 μM cytochrome c with or without 60 U/ml of SOD, and further incubated for 5, 20, or 60 min. Each point represents the mean ± S.E.M. of 3 – 4 experiments. *$P<0.05$, **$P<0.01$: significantly different from spontaneous production (Student's *t*-test).

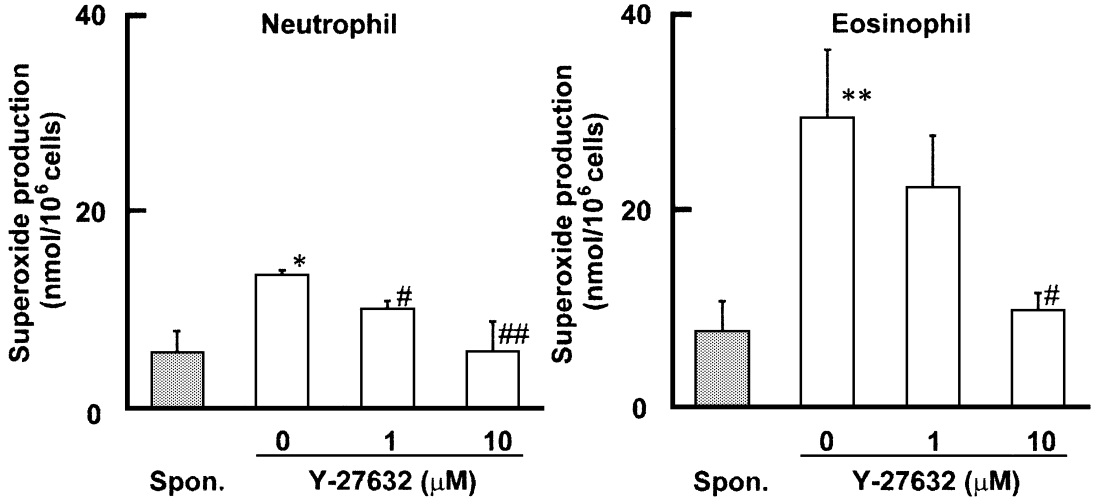

Fig. 5. Effect of Y-27632 on LPA-induced superoxide production from guinea pig neutrophils and eosinophils. The cell suspension pre-warmed for 5 min at 37°C. Following the addition of 1 or 10 μM Y-27632 and incubation for 10 min, the cell suspension was challenged with LPA (final concentration:10 μg/ml) for 60 min at 37°C. Each column represents the mean ± S.E.M. of 3 – 6 experiments. **P*<0.05, ***P*<0.01: significantly different from spontaneous production (Student's *t*-test). $^{\#}P$<0.05, $^{\#\#}P$<0.01: significantly different from the vehicle-treated group (Dunnett's multiple comparison test).

groups (P<0.05, at all the cell counts measured).

Superoxide production induced by LPA

Figure 4 illustrates superoxide production from neutrophils and eosinophils. Under the present conditions, spontaneous superoxide production from neutrophils was 3.73 – 6.75 nmol/10^6 cells. Incubation of cell suspension with 10 μg/ml LPA slightly but positively increased superoxide production, ranging from 120% to 250% in comparison with spontaneous production. Significant differences were observed between the LPA-treated and non-treated groups at 60 min (P<0.01). In contrast, 100 ng/ml PMA caused a significant increase in superoxide within 5 min; moreover, a maximum was reached at 20 min.

As in the case of neutrophils, superoxide production from eosinophils was observed in the treatment with 10 μg/ml LPA. Under the present conditions, superoxide production induced by LPA was 6.74 – 25.4 nmol/10^6 cells, which was 1.6 – 2.0-fold higher than that of spontaneous production. Statistically significant differences were apparent at 60 min (P<0.05).

Effect of Y-27632 on LPA-induced superoxide production

Y-27632 was pre-incubated with the cell suspension for 10 min prior to challenge. LPA-induced superoxide production from neutrophils was inhibited by Y-27632 at concentrations of 1 and 10 μM in a dose-dependent fashion (Fig. 5). Significant differences were evident between the Y-27632- and vehicle-treated groups at both concentrations (1 μM, P<0.05; 10 μM, P<0.01). Y-27632 (10 μM) also inhibited LPA-induced superoxide production from eosinophils. Statistically significant differences were observed between the 10 μM Y-27632-treated and vehicle-treated groups (P<0.05).

Discussion

LPA, a simple water-soluble phospholipid, is now recognized as an important intercellular messenger characterized by multiple biological activities (9). Previously, we reported that inhalation of LPA induced airway hyperresponsiveness to acetylcholine in guinea pigs (10); additionally, this airway hyperresponsiveness is attributable to activation of the Rho/ROCK-mediated pathway (11). Fischer et al. (2) described chemoattractive effects of LPA toward human neutrophils. LPA also caused an increase in binding of monocytes and neutrophils to human aortic endothelial cells (3). On the basis of these in vitro studies, we hypothesized that inhalation of LPA induces infiltration of inflammatory cells into the pulmonary airway in vivo. In the present studies, increased numbers of total cells, neutrophils, and eosinophils in the BALF were detected from 4 – 6 h following inhalation of LPA as many mediators, including LTB_4 (12), PAF (13), and eotaxin (14), induced infiltration of inflammatory cells in vivo. These data suggest that, at least in part, inflammatory cell accumulation in the asthmatic airway may be attributable to LPA.

Y-27632 (1 mM) inhibited the increase in cell counts in BALF 6 h after LPA inhalation. It has been document-

ed that eotaxin-induced eosinophil chemotaxis is significantly inhibited by Y-27632 (15). Niggli (16) also reported that chemotactic peptide led to a ROCK-dependent increase in phosphorylation of myosin light chain and that Y-27632 suppressed myosin light chain phosphorylation and chemotactic peptide-induced development of cell polarity and locomotion. The present findings, in conjunction with these reports, permit us to conclude that Rho/ROCK-mediated signaling pathways may be involved in infiltration of inflammatory cells induced by LPA similar to other chemoattractants; moreover, it appears to be a key signaling factor in the motile function of these cells.

Furthermore, it has been reported that LPA increases E-selectin and vascular cell adhesion molecule-1 (VCAM-1) cell surface expression in a dose-dependent manner (3). Therefore, LPA-induced infiltration of inflammatory cells may be attributable to endothelial cell-inflammatory cell interactions. Recently, we noted that LPA positively regulates fluid-induced local Ca^{2+} influx in bovine aortic endothelial cells, which indicates an important role for LPA as an endogeneous factor in fluid flow-induced endothelial function (17). On the basis of these observations, it may be considered that this Ca^{2+} mobilization and LPA-induced infiltration of inflammatory cells or endothelial cell-inflammatory cell interactions are correlated.

LPA slightly and positively increased the superoxide production from neutrophils and eosinophils. Fischer et al. (2) demonstrated that LPA acted as a chemoattractant and priming agent; however, it did not activate human neutrophils. In contrast, Chettibi et al. (18) noted that LPA was ineffective at concentrations below 2×10^{-5} M with respect to stimulation of the respiratory burst; however, at $1 - 2 \times 10^{-4}$ M, LPA exhibited a weak stimulatory effect. Although a clear explanation has not been formulated regarding the involvement of LPA in the activation of inflammatory cells, we hypothesize that LPA contributes to activation of inflammatory cells based on its efficacy in superoxide production from neutrophils and eosinophils.

LPA-induced superoxide production was inhibited by treatment with Y-27632. It has been reported that ROCK is involved in PMA-induced superoxide production in human polymorphonuclear leukocytes; additionally, this kinase may be located downstream of protein kinase C (19). Based on this data and the results from the present investigation, we theorize that LPA-induced superoxide production is also mediated, at least in part, by the Rho/ROCK-mediated signaling pathway.

There are very few reports that provide evidence for the role of LPA in the clinical asthmatic response. Also it should be noted that the source of LPA or amount of LPA in allergic conditions has not yet been studied in detail. However, secretory phospholipase A_2 ($sPLA_2$), which leads to biosynthesis of various lysophospholipids, is released during activation of inflammatory cells such as macrophage, neutrophils, and mast cells (20). Increased levels of $sPLA_2$ are also found in the BALF of sensitized guinea pig and of antigen-challenged allergic asthmaticus (21, 22). Thus, it appears likely that LPA may be involved in airway dysfunction such as bronchial asthma.

In summary, LPA induced a dose-dependent increase in eosinophils and neutrophils in vivo and activation in vitro. These findings indicate the potential role for LPA in pulmonary pathophysiology and contribution of pulmonary diseases such as bronchial asthma. The effect of LPA on the airway may be attributable to the activation of the Rho/ROCK-mediated pathway.

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