

Effects of Theophylline on Chronic Inflammatory Lung Injury Induced by LPS Exposure in Guinea Pigs

Yoshio Kaneko¹, Kazumi Takashima¹, Naomi Suzuki¹ and Kenjiro Yamana¹

ABSTRACT

Background: Pathogenesis of COPD is, at least in part, attributable to the chronic accumulation of neutrophils in the airways, and morphological changes such as hyperplasia of goblet cells in the airways are often observed in this disease. These structural changes were induced in guinea pigs by repetitive inhalations of LPS, and the effects of theophylline and dexamethasone were examined.

Methods: Male Hartley Guinea pigs weighing about 300 g were exposed to a nebulized solution of LPS (30 µg/mL) for 1 hour. Exposure to LPS was performed 15 times at 48-hour intervals. Histological analysis was performed, and infiltration of leukocytes in BALF, airway hyperreactivity and hydroxyproline content of the lung were measured 24 or 48 hours after the final exposure of LPS. Drugs were administered every day until 30 minutes before the final exposure.

Results: Repetitive exposure to LPS induced an influx of inflammatory cells into the BALF. Histological changes such as accumulation of inflammatory cells in the lung parenchyma, enlargement of alveoli, swelling of the alveolar walls and goblet cell hyperplasia in the airways were observed. Airway hyperreactivity and increased lung hydroxyproline content were also found in this model of chronic inflammatory lung injury. Some of these changes induced by repetitive LPS exposure were improved by treatment with theophylline or dexamethasone.

Conclusions: Theophylline improved airway injury as well as airway hyperreactivity induced by repetitive exposure of the guinea pigs to LPS. These results suggest that theophylline treatment has ameliorative effects on airway disease with chronic inflammation.

KEY WORDS

airway hyperreactivity, COPD, goblet cell hyperplasia, lipopolysaccharide exposure, theophylline

INTRODUCTION

Chronic airway diseases including chronic obstructive pulmonary disease (COPD) are becoming important therapeutic targets, as mortality from these diseases rises. Many methods have been developed for animal studies of chronic airway diseases such as allergic inflammation, bronchitis, airway remodeling, emphysema or fibrosis that were induced by antigen or ovalbumin (OVA),^{1,3} lipopolysaccharide (LPS),⁴⁻¹⁰ cigarette smoke,^{11,12} diesel exhaust,¹³ bleomycin,^{14,15} elastase^{16,17} or silica.¹⁸

LPS is an endotoxin from gram-negative bacteria that induces circulatory shock, coagulopathy and

multiorgan dysfunction as well as a strong response in the immune system. Administration of LPS causes influx of inflammatory cells such as macrophages, neutrophils and lymphocytes, with increasing release of pro-inflammatory cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-1 β and interferon- γ .^{19,20} These inflammatory reactions induced by a single dose of LPS to the airway via inhalation or intratracheal administration have been used in animal models of acute lung injury (ALI)^{21,22} or acute respiratory distress syndrome (ARDS).^{23,24} On the other hand, repetitive exposure of LPS induces prolonged inflammation characterized by neutrophil accumulation in the airway of mice and guinea pigs.⁴⁻¹⁰ During

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chronic inflammation by repeated LPS exposure, some pathological changes similar to those observed in COPD patients have been reported. For example, goblet cell hyperplasia in the airways^{25,26} and chronic neutrophilia in the bronchi, bronchioles and alveoli²⁷ showed some of the pathological features of chronic bronchitis, and swelling of the alveolar walls and alveolar enlargement²⁸ represent aspects of emphysema.

Theophylline, a nonselective phosphodiesterase (PDE) inhibitor, has been used in the treatment of respiratory diseases such as bronchial asthma for many years. The beneficial effects of theophylline have usually been attributed to both bronchodilatory and anti-inflammatory activities based on their activity in the inhibition of PDE enzymes, especially PDE isoenzyme type IV. Furthermore, theophylline has been reported to show many mechanisms in inflammatory reactions, for example, adenosine receptor antagonism, increased IL-10 release, mediator inhibition, inhibition of inflammatory cytokines, and increased histone deacetylase activity (HDAC).²⁹⁻³¹ These actions of theophylline may be efficacious for the treatment of chronic airway disease. In international guidelines for the treatment of COPD, theophylline has been categorized as a bronchodilator together with inhaled anticholinergics and beta-agonists.³² However, anti-inflammatory effect theophylline may be reflected on its clinical effects in addition to bronchodilatory effects.

Theophylline has been reported to have a strong ameliorative effect on chronic airway inflammation in animal models, but little is known about its effects on histological changes after prolonged inflammation. In the present study, the lung injury characterized by chronic neutrophilic inflammation was induced by repetitive exposure of the guinea pigs to LPS, and the effects of theophylline on inflammation and histological changes in the airway were examined.

METHODS

ANIMALS

Male Hartley Guinea pigs weighing about 300 g were purchased from Charles River Japan (Yokohama, Japan). The animals were housed in an air-conditioned room (23 ± 2°C, 50 ± 10% humidity) lit from 8:00–20:00, and were allowed food and water *ad libitum* throughout the experiments. The experiments were undertaken according to the “Guidelines for the care and use of experimental animals” provided by the Japanese Association for Laboratory Animal Science (1987).

DRUGS AND MATERIALS

LPS (*Escherichia coli*, serotype O55: B5) and dexamethasone were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Theophylline was purchased from Wako Pure Chemical Industries, Ltd., (Osaka,

Japan). LPS was dissolved in phosphate-buffered saline (PBS). Dexamethasone and theophylline were suspended in a 0.5% solution of carboxymethylcellulose sodium (Wako Pure Chemical Industries, Ltd.). Pentobarbital sodium was purchased from Dainippon Sumitomo Pharma Co., Ltd. Trypan Blue and Diff Quick were purchased from GIBCO-BRL (Gaithersburg, MD, USA) and International Reagent Corp. (Kobe, Japan). Acetyl-β-methylcholine bromide (methacholine) and hydroxyproline were purchased from Sigma-Aldrich.

EXPERIMENTAL PROTOCOL FOR LPS EXPOSURES

Guinea pigs were exposed to an aerosolized solution of LPS (30 µg/mL dissolved in physiological saline, 60 mL) for 1 hour. Exposure was performed 15 times at 48-hour intervals using an ultrasonic nebulizer (NE-U12, Omron, Kyoto, Japan). Twenty-four hours after the final exposure, bronchoalveolar lavage fluid (BALF) was collected and the lungs were removed for histological analysis. In other groups of guinea pigs methacholine-induced airway hyper-reactivity (AHR) was measured 48 hours after the final exposure, and their lungs were removed to measure the hydroxyproline content. Test drugs and vehicle were administered every day during the course of the experiment until 30 minutes before the final exposure to LPS. Animals in the sham-exposure group were not exposed to LPS. These experimental procedures are shown in Figure 1.

LEUKOCYTE COUNT IN BALF

The leukocytes in BALF were counted at 4, 24 and 47 hours after the first exposure and at 24 hours after the 5th, 10th and 15th exposures to LPS. Guinea pigs were deeply anesthetized with pentobarbital sodium and sacrificed by exsanguination. In the time course study of cell accumulation (Fig. 2A), five times, 5 mL of physiological saline was injected into, and fluid was collected from, the whole lung via a tracheal cannula (total 25 mL). In another study of cell accumulation (Fig. 3A), 2.5 mL of physiological saline was injected five times into, and fluid was collected from, the left lobe of the lung via a tracheal cannula (total 12.5 mL), while the right bronchus was ligatured. BALF was centrifuged at 500 g, 4°C for 10 minutes, and the pellet was resuspended in 5 mL of PBS after brief hemolytic treatment with hypotonic solution, and the cells were washed twice with 5 mL of PBS. The total and differential cell numbers were counted by light microscopy after staining with trypan blue and Diff Quick, respectively.

HISTOLOGICAL ANALYSIS

Immediately after BAL, the lobes of the right lung were removed, rinsed with PBS, and fixed by 2.5 mL of 10% phosphate-buffered formalin (pH 7.4) via tra-

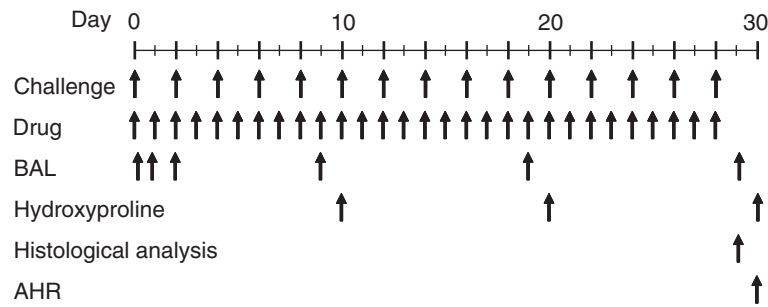


Fig. 1 Schedule of the study. LPS exposure was performed for 60 minutes every 2 days for a maximum of 15 times. BALF was collected at 4 hours, 24 hours and 47 hours after the first exposure to LPS, and 24 hours after the 5th, 10th and 15th exposure to LPS. Lung sampling for hydroxyproline assay was carried out at 48 hours after the 1st, 5th, 10th and 15th exposure to LPS. Lungs for histological analysis were sampled at 24 hours, and AHR was measured at 48 hours after the final exposure to LPS. Test drugs were administered every day, usually 30 minutes before exposure on days of LPS challenge.

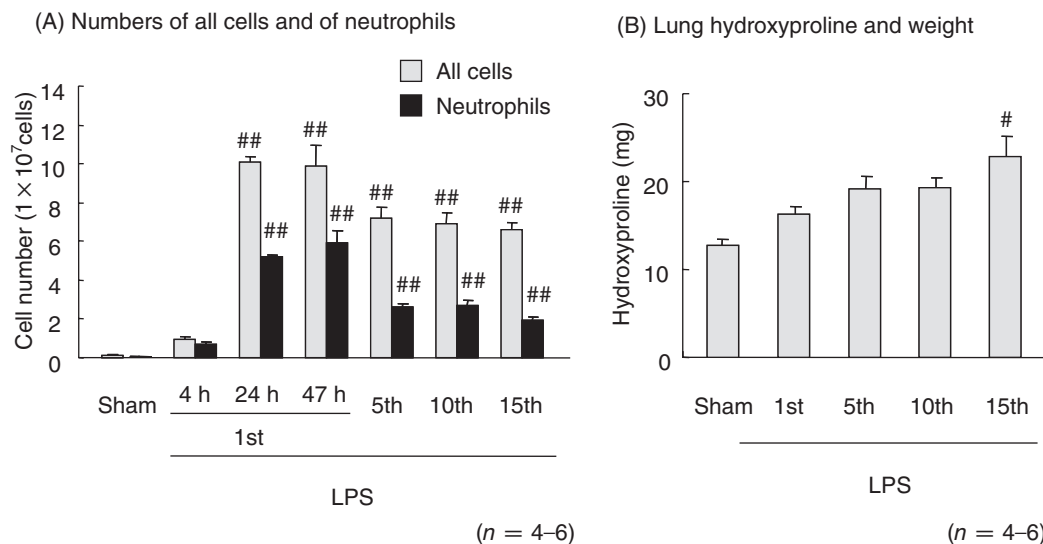


Fig. 2 Time course of the changes of inflammatory cell accumulation in the BALF and lung hydroxyproline content induced by repetitive exposure to LPS. LPS was inhaled at the 1st, 5th, 10th and 15th exposure. **(A)** The number of total cells and neutrophils in the BALF was counted at 4, 24 and 47 hours after the 1st exposure and at 24 hours after the 5th, 10th and 15th exposure to LPS. **(B)** The lung hydroxyproline content was measured at 24 hours after the 1st, 5th, 10th and 15th exposure to LPS. Each column represents means $\pm$ S.E.M. (n = 4–6). #: $p < 0.05$, ##: $p < 0.01$ v.s. sham group.

cheal cannula attached to the right bronchus. The lung was preserved in 10% phosphate-buffered formalin for one more day. After fixation, portions of the center of the middle lobe were removed so that the large bronchi appeared at the surface, and embedded in paraffin. Sections 10 μ m thick were cut, deparaffinized, and stained with hematoxylin and eosin (H&E), Alcian blue-periodic acid Schiff (AB-PAS) or Elastica-Masson's trichrome (EM). In order to esti-

mate the lung parenchymal inflammation quantitatively, cells that had accumulated in the parenchyma were counted in 10 randomly selected fields of 500 μ m squares in an H&E stained section using light microscopy. Quantification of alveolar enlargement was determined by mean linear intercepts measured from 10 randomly selected lines passing through the lung in the H&E stained sections. Changes in appearance of the goblet cells were also quantitatively analyzed

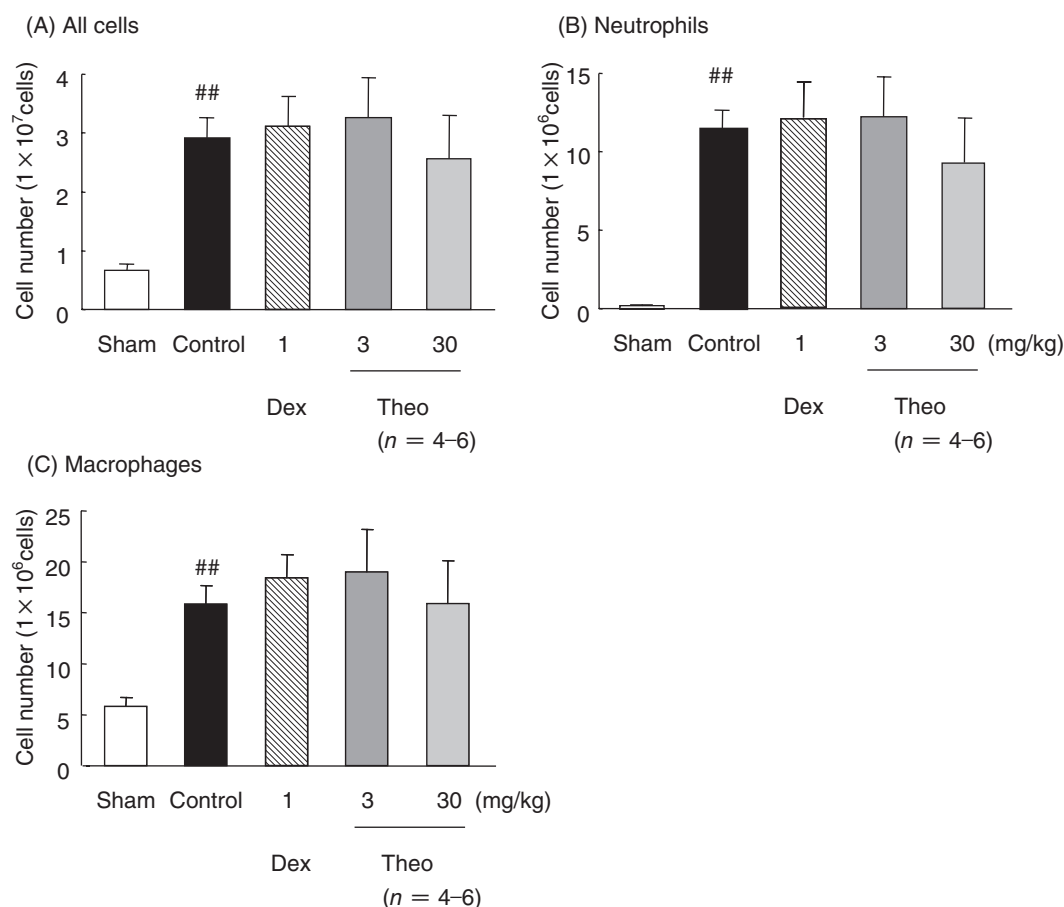


Fig. 3 The number of total cells, neutrophils and macrophages in the BALF was measured 24 hours after the 15th exposure to LPS. Total cell counts and differential cell counts (neutrophils) were carried out with light microscopy after staining with Trypan Blue and Diff Quick, respectively. Each column represents means $\pm$ S.E.M. ($n = 4-6$). Dex: Dexamethasone, Theo: Theophylline. #: $p < 0.05$, ##: $p < 0.01$ v.s. sham group.

with in AB-PAS-stained sections by light microscopy and imaging software (Scion Image software, Scion Corporation; National Institutes of Health, Frederick, MD). The total area stained with AB-PAS at the airway surface was expressed in percentages of the internal area of the airway epithelium including airway lumen.

MEASUREMENT OF AIRWAY HYPER-REACTIVITY

Airway hyper-reactivity was measured at 48 hours after the final exposure of LPS. Measurement of airway hyper-reactivity was carried out using the Konzett-Rössler method³³ with some modification. In brief, Guinea pigs were anesthetized with intraperitoneal injection of 30 mg/kg pentobarbital sodium, and polyethylene catheters were inserted into the trachea and jugular vein. After the arrest of spontaneous breathing by intravenous injection of 5 mg/kg succinylcholine chloride, guinea pigs were ventilated using

a respirator (model 7025, Ugo-basile Varese, Italy). The lung was inflated by a fixed volume of air under 5 cm H₂O pressure at a rate of 60 breaths/minute, and ventilation overflow was measured by a differential pressure transducer (PUL123-DP45F, M.I.P.S., Japan). Respiratory overflow was amplified by bioelectric amplifier (AP-621 G, Nihon Kohden, Tokyo, Japan) and recorded (WR 8500, Graphtec, Yokohama, Japan). Escalating concentrations of methacholine of 0, 1.875, 3.75, 7.5, 15, and 30 μ g/mL/kg were injected intravenously via a catheter in the jugular vein, with dose intervals of 1 minute. Percentages of bronchoconstriction after injection of methacholine at each concentration were calculated from the following formula: Percentage of bronchoconstriction (%) = (Overflow after methacholine injection - Overflow before methacholine injection) / (Overflow at complete occlusion - Overflow before methacholine injection) $\times$ 100.

Airway hyper-reactivity of each group was ex-

pressed as PC50, where the dose of methacholine that induced 50% bronchoconstriction was calculated from logarithmic regression of the dose-response curves.

MEASUREMENT OF LUNG HYDROXYPROLINE CONTENT

After measurement of AHR, the lung was removed and weighed. Care was taken to remove adhered tissue such as bronchia and vessels. The lungs were then homogenized in 10 mL PBS. An aliquot of 0.5 mL of homogenate was hydrolyzed by 0.5 mL of 12N HCl at 120°C for 24 hours. After hydrolysis, 5 µL of each sample was placed into a 96-well plate and mixed with 5 µL citrate/acetate solution (238 mM citric acid, 1.2% glacial acetic acid, 532 mM sodium acetate, and 85 mM sodium hydroxide), and 100 µL of chloramine-T solution (0.282 g chloramine-T added to 16 mL citrate/acetate solution, 2.0 mL of n-propanol, and 2.0 mL distilled water) and incubated for 30 minutes at room temperature. Ehrlich's reagent (100 µL; 2.5 g p-dimethylaminobenzaldehyde added to 9.3 mL of n-propanol and 3.9 mL of 70% perchloric acid) was added, and incubated at 65°C for 30 minutes. The absorbance of each sample was then measured at 550 nm with a microplate reader (Bio-Rad, benchmark 170-6850, Tokyo, Japan).

STATISTICAL ANALYSIS

The data was represented as the mean $\pm$ S.E.M. Statistical significance was assessed by StatLight (Microsoft Windows 3.1 or 95/Excel: Yukms Tokyo, Japan). For comparison between the sham group and the control group, the *F*-test and Student's *t*-test or Wilcoxon rank sum test were used. Multiple comparisons of the control group with the other treated groups were evaluated by the Bartlett test, 1-way ANOVA and Dunnett's multiple test. A *p*-value of less than 0.05 was considered to indicate a significant difference.

RESULTS

TIME COURSE OF CELL ACCUMULATION IN BALF AND LUNG HYDROXYPROLINE CONTENT

We examined the changes in the number of BALF cells and lung hydroxyproline content after the beginning of LPS exposure. The number of total cells and of neutrophils in BALF were found at maximum after 24 hours and 47 hours of 1st exposure of LPS (Fig. 2A). Significant increases of the number of BALF cells were also observed throughout the course of this study, but repetition of LPS exposure (5th, 10th and 15th exposure) did not induce any further increase of the number of BALF cells. On the other hand the lung hydroxyproline content increased with repeated LPS exposures, and a significant increase was found after the 15th exposure to LPS (Fig. 2B). It was noted that lung weight was also

increased after the beginning of LPS exposure, with a similar pattern of change to hydroxyproline content.

MEASUREMENT OF THE NUMBER OF LEUKOCYTES IN BRONCHOALVEOLAR LAVAGE FLUID

The effects of either treatment with dexamethasone or theophylline on the number of BALF cells are shown in Figure 3. After the 15th exposure to LPS, the total leukocyte count in BALF in the control group was significantly increased ($2.92 \pm 0.35 \times 10^7$ cells, $p < 0.01$), compared with cells in the sham group ($0.66 \pm 0.11 \times 10^7$ cells) (Fig. 3A). Differential cell counts revealed that the number of neutrophils was also significantly increased in the control group ($11.27 \pm 1.49 \times 10^6$ cells, $p < 0.01$) compared with that in the sham group ($0.08 \pm 0.01 \times 10^6$ cells) (Fig. 3B), and the number of macrophages was also significantly increased in the control group ($15.89 \pm 1.79 \times 10^6$ cells, $p < 0.01$) compared with that in the sham group ($5.8 \pm 0.91 \times 10^6$ cells) (Fig. 3C). However, the number of eosinophils and lymphocytes did not change significantly (data not shown). Dexamethasone or theophylline treatment did not effect any increase in the total numbers of cells, neutrophils and macrophages in BALF induced by LPS (Figs. 3A, 3B, 3C).

HISTOLOGICAL ANALYSIS

Accumulation of inflammatory cells on H&E stained parenchyma of the lung after the 15th exposure to LPS is shown in Figure 4. A large number of inflammatory cells such as macrophages and neutrophils were found in the control group at higher magnification (Fig. 8). In contrast, only a limited number of inflammatory cells could be found in the dexamethasone- and theophylline-treated groups. The average number of inflammatory cells in ten randomly chosen fields (500 µm squares) counted with light microscopy is shown in Figure 5A. The number of cells counted in the control group was significantly increased (47.1 ± 5.17 cells/field, $p < 0.01$), compared with the cells in the sham group (14.1 ± 0.60 cells/field). The increase in the number of cells induced by LPS was significantly inhibited by theophylline (3 mg/kg group, 34.7 ± 2.76 cells/field; 30 mg/kg group, 23.1 ± 3.05 cells/field; $p < 0.01$) or dexamethasone (29.1 ± 1.07 cells/field; $p < 0.05$). Furthermore, the mean linear intercept in the control group was significantly increased (79.4 ± 7.99 µm, $p < 0.01$), compared with the sham group (38.7 ± 2.25 µm). The mean linear intercept of the theophylline-treated group (3 mg/kg group, 71.7 ± 9.20 µm, $p = 0.54$ and 30 mg/kg group, 50.1 ± 5.48 µm; $p < 0.05$) was reduced significantly compared with that of the control group. A reduced mean linear intercept was also found in the dexamethasone-treated groups (62.4 ± 8.49 µm, $p = 0.18$), but this change was not significant.

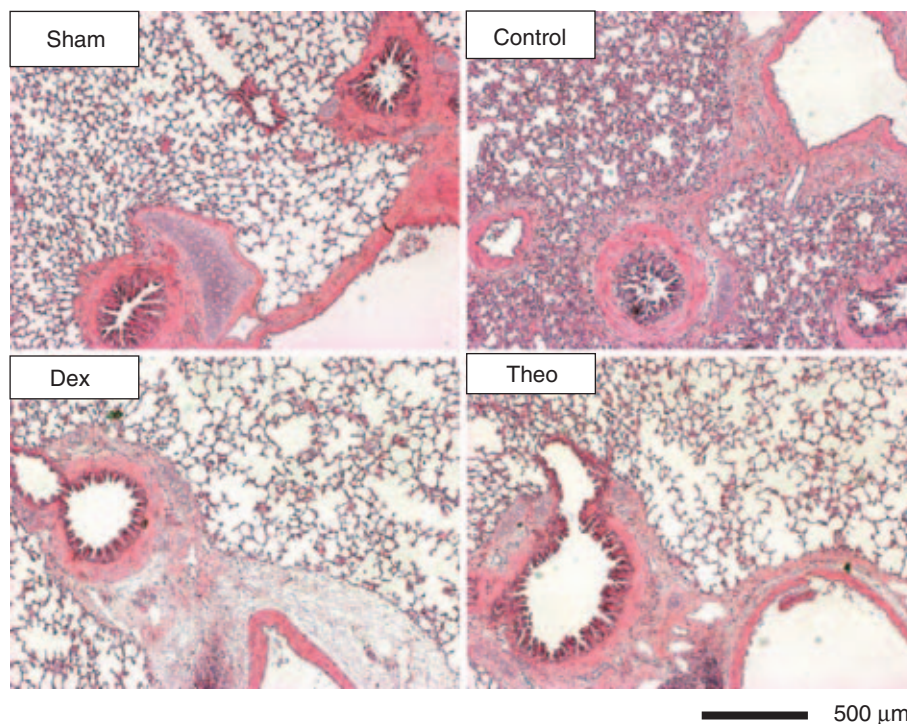


Fig. 4 Lung section stained with H&E at 24 hours after the 15th exposure to LPS. The photos show the sham group, the control group, the group treated with 1 mg/kg of dexamethasone (Dex) and the group treated with 30 mg/kg of theophylline (Theo). Bar = 500 μ m.

cant. Figure 6 shows large airway stained with AB-PAS. The epithelial cells stained purple-magenta with AB-PAS seemed to be typical goblet cells, and AB-PAS-positive cells showed an increase in the control group. These changes—such as goblet cell hyperplasia—were conspicuously reduced in the theophylline-treated group and the dexamethasone-treated group. Quantitative analysis of the AB-PAS stained area is shown in Figure 7. The AB-PAS stained area in the control group was significantly increased ($8.06 \pm 2.17\%$, $p < 0.05$), compared with that in the sham group ($3.43 \pm 0.94\%$). The AB-PAS stained area of the theophylline-treated group (3 mg/kg group; $2.34 \pm 0.40\%$, 30 mg/kg group; $1.86 \pm 0.64\%$) was significantly ($p < 0.05$) reduced from the corresponding area in the control group. The AB-PAS stained area of the dexamethasone-treated group ($4.81 \pm 0.83\%$) was found to be reduced, but the difference in AB-PAS-positive areas between the dexamethasone-treated group and the control group was not significant. Swelling of the alveolar wall, and increases of collagen, elastic fiber and cell infiltration were found stained with EM in the control group (Fig. 8). These changes were less prominent in the dexamethasone-treated group, and the theophylline-treated group showed an almost normal structure similar to that found in the sham group.

MEASUREMENT OF LUNG HYDROXYPROLINE CONTENT

After the 15th exposure to LPS, lung collagen content was measured quantitatively using hydroxyproline analysis, and the results are shown in Figure 9A. Lung hydroxyproline contents were 17.0 ± 2.88 mg/lung in the control group, 10.1 ± 1.36 mg/lung in the sham group, 11.5 ± 1.21 mg/lung in the theophylline treated group at a dose of 3 mg/kg, 11.8 ± 1.11 mg/lung in the theophylline treated group at a dose of 30 mg/kg, and 13.5 ± 2.11 mg/lung in the dexamethasone treated group, respectively. Lung hydroxyproline contents in the control group were significantly increased ($p < 0.05$), compared with those in the sham group. Lung hydroxyproline contents in the theophylline treated group or dexamethasone treated groups were found to be inhibited, but the differences in these groups were not significant. Lung weight was also increased (control group; 4.07 ± 0.21 g, $p < 0.05$, *v.s.* sham group; 3.04 ± 0.10 g) after the 15th exposure to LPS, but this increase in lung weight was not influenced by treatment with theophylline or dexamethasone (Fig. 9B).

MEASUREMENT OF AIRWAY HYPER-REACTIVITY

Airway hyperreactivity was determined 48 hours after the 15th exposure to LPS. Responses of each group to

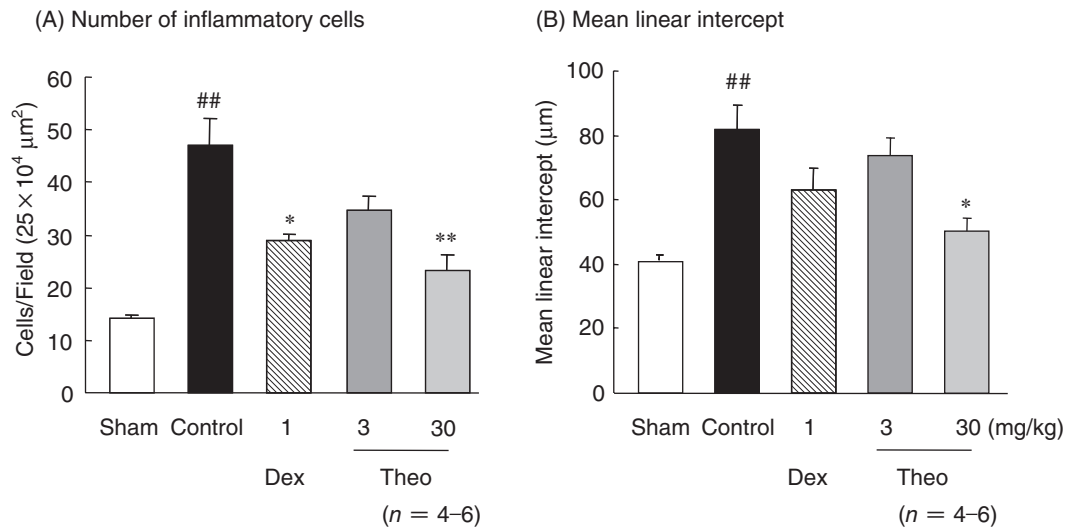


Fig. 5 Average number of inflammatory cells in ten randomly chosen fields (500 μm squares) and mean linear intercept measured from 10 randomly selected lines passing through the lung in the H&E stained sections. Each column represents means $\pm$ S.E.M. ($n = 4-6$). Dex: Dexamethasone, Theo: Theophylline. ^{##}: $p < 0.01$ v.s. sham group, and ^{*}: $p < 0.05$, ^{**}: $p < 0.01$ v.s. control group.

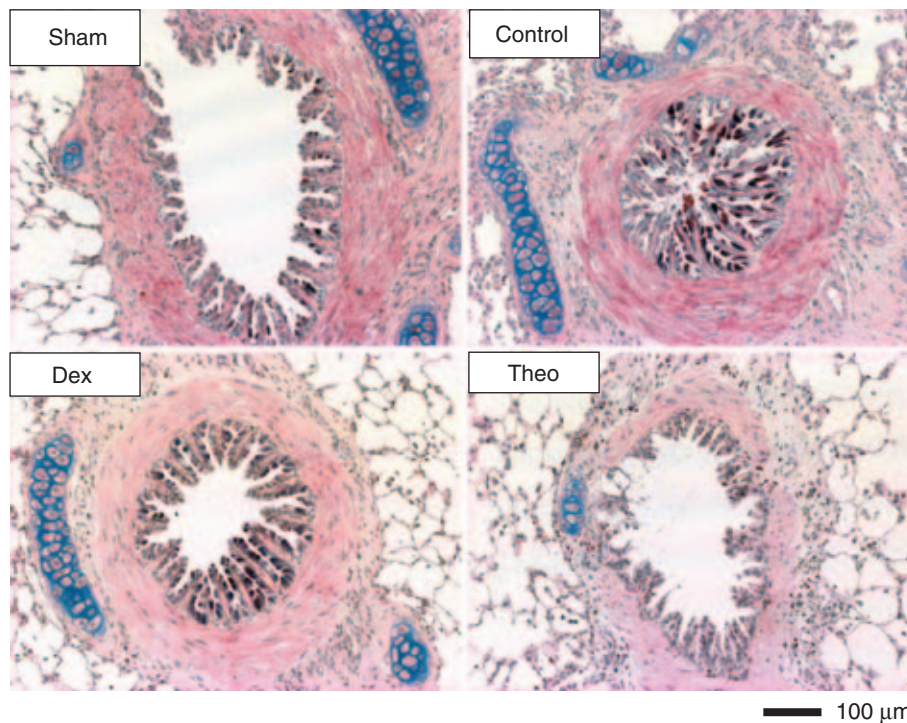


Fig. 6 Typical changes of airway epithelium stained with AB-PAS after the 15th exposure to LPS. The photos show the sham group, the control group, the group treated with 1 mg/kg of dexamethasone (Dex) and the group treated with 30 mg/kg of theophylline (Theo). The epithelial cells which stained purple-magenta with AB-PAS were determined as goblet cells. Bar = 100 μm .

the escalating dose of methacholine are shown in Figure 10A. The control group showed the most sensitive response to methacholine among all groups stud-

ied. The airway hyper-reactivity of each group is expressed as PC₅₀ (Fig. 10B). The PC₅₀ value of the control group was significantly decreased (2.57 ± 0.70

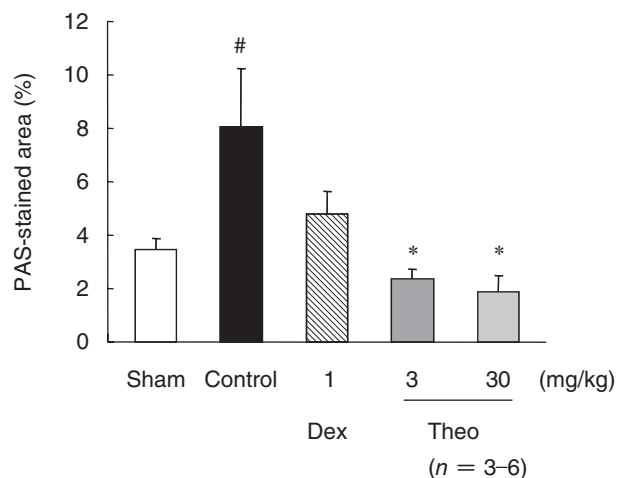


Fig. 7 Goblet cell hyperplasia was quantitatively analysed and expressed as a percentage of the AB-PAS positive area at the airway lumen by image software. Each column represents means $\pm$ S.E.M. ($n = 3-6$). Dex: Dexamethasone, Theo: Theophylline. #: $p < 0.05$ v.s. sham group, and *: $p < 0.05$ v.s. control group.

$\mu\text{g/kg}$, $p < 0.01$), compared with that of the sham group ($6.06 \pm 1.08 \text{ g/kg}$). The theophylline-treated group at a dose of 30 mg/kg showed the highest PC50 value ($9.73 \pm 3.81 \text{ }\mu\text{g/kg}$, $p < 0.05$). The PC50 value of the dexamethasone-treated group was also high, but this change was not significant ($8.36 \pm 3.49 \text{ }\mu\text{g/kg}$, $p = 0.19$).

DISCUSSION

In the present study, infiltration of inflammatory cells in BALF was observed after first exposure to LPS, and maintained at higher levels during repetitive exposure to LPS. In contrast, lung hydroxyproline content increased with repetition of LPS exposures, and significant increases were found after the 15th exposure to LPS (Fig. 2). Repetitive exposure to LPS induced several pathological changes such as inflammatory cell accumulation in the lung parenchyma, hyperplasia of goblet cells, hypersecretion of mucus, enlargement of alveoli, swelling of the alveolar wall, and increase of collagenic and elastic structures in the alveolus (Figs. 4, 6, 8). These pathological changes were not evident until the 15th exposure to LPS (30 days after the start of the first exposure to LPS). These results may suggest that chronic accumulation of inflammatory cells was primarily induced by LPS, and other pathological changes were induced secondarily. The pathological changes observed in this study were consistent with those in previous reports,^{9,10} and some of these changes have been known in human lung injury such as COPD³² and ARDS.²³

The accumulation of inflammatory cells in the lung parenchyma after the 15th exposure to LPS was inhibited

by treatment with either theophylline or dexamethasone, but the increased number of inflammatory cells in the BALF was not changed by either treatment. This discrepancy might be explained in part by the different strength of LPS exposure, *i.e.* the proximal bronchus was exposed to too much LPS to prove the response to drugs used, and the lung parenchyma was exposed to the minimal quantity of LPS that could cause pathological changes observed by the present study condition of LPS at $30 \text{ }\mu\text{g/mL}$. As shown in Figures 4, 8, inflammatory cell accumulation in the alveolus was clearly inhibited by the treatment of both theophylline and dexamethasone, and such anti-inflammatory effects of both drugs in the lung parenchyma may contribute to reduce further destruction of the lung and airway epithelium.

Anti-inflammatory effects of dexamethasone on LPS-induced cell accumulation in the BALF could not be found in the present study. However, some reports showed inhibition of cell infiltration in the BALF after LPS exposure. Toward *et al.* reported that dexamethasone reduced neutrophil influx at a dose of 20 mg/kg p.o. in a guinea pig model of pulmonary edema induced by chronic LPS exposure.¹⁰ Another report showed inhibition of neutrophilia in guinea pigs after single exposure to LPS with treatment of 5 mg/kg dexamethasone.³⁴ Thus it might be suggested that larger doses of dexamethasone would be required in the present study to inhibit cell infiltration in the BALF. On the other hand, the effect of theophylline on LPS-induced cell accumulation in the BALF has been examined, and theophylline at a dose of 50 mg/kg p.o. could not inhibit inflammatory cell influx in the BALF of guinea pig³⁵ and rat³⁶ models of pulmonary edema induced by single LPS exposure. Escofier *et al.* reported that theophylline at a dose of 100 mg/kg inhibited neutrophilia induced by single exposure to LPS in rats,³⁷ but a dose of 100 mg/kg theophylline seemed to be inadequate for repeated administration because of severe adverse effects. These reports suggest that the anti-inflammatory effect of theophylline might not be sufficient for preventing inflammatory cell influx in the BALF, whereas theophylline showed improvement of inflammatory cell accumulation in the lung parenchyma.

Hypersecretion of mucus and hyperplasia of goblet cells in the airway is recognized as an important cause of airflow limitation in patients with COPD. Similar histological changes of the airway have been observed in some animal models including LPS exposure.^{9,38} Hyperplasia of goblet cells was also found in the present study (Fig. 6), and such change induced by repetitive exposure to LPS was prevented by treatment with either theophylline or dexamethasone. One report using chronic LPS exposure showed that dexamethasone and rolipram, a selective inhibitor of PDE IV, inhibited the increase of goblet cells in the airway of guinea pigs dose dependently.⁹ The protec-

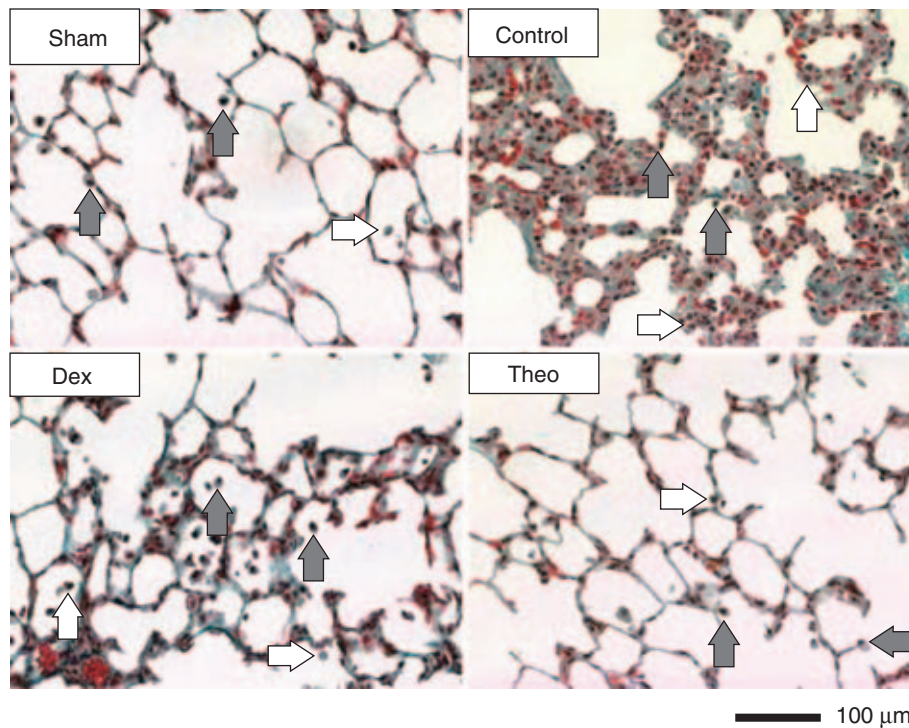


Fig. 8 Representative sections of the lung were stained with EM after the 15th exposure to LPS. The photos show the sham group, the control group, the group treated with 1 mg/kg of dexamethasone (Dex) and the group treated with 30 mg/kg of theophylline (Theo). The alveolar walls which stained green-blue and dark blue with EM were found to be collagenic fibers and elastic fibers, respectively. The inflammatory cells in the alveolar sections are indicated by gray arrows (macrophages) and white arrows (neutrophils). Bar = 100 μm.

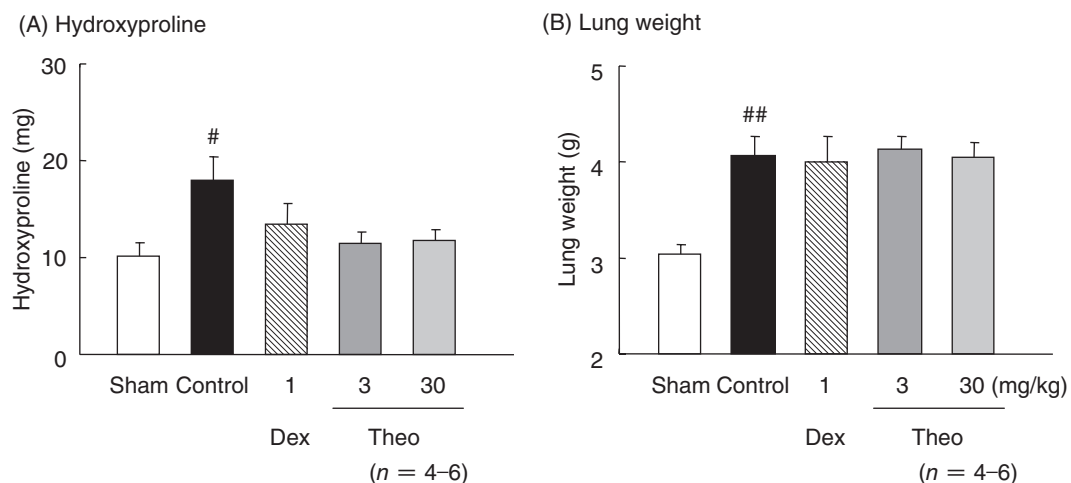


Fig. 9 After the 15th exposure to LPS, lung collagen content was measured quantitatively using hydroxyproline analysis. Lung weight (A) and hydroxyproline content (B) were measured 48 hours after the 15th exposure to LPS. The lungs were removed and weighed, then hydroxyproline of the lung was measured using Ehrlich's reagent. Each column represents means $\pm$ S.E.M. ($n = 4-6$). Dex: Dexamethasone, Theo: Theophylline. #: $p < 0.05$, ##: $p < 0.01$ v.s. sham group.

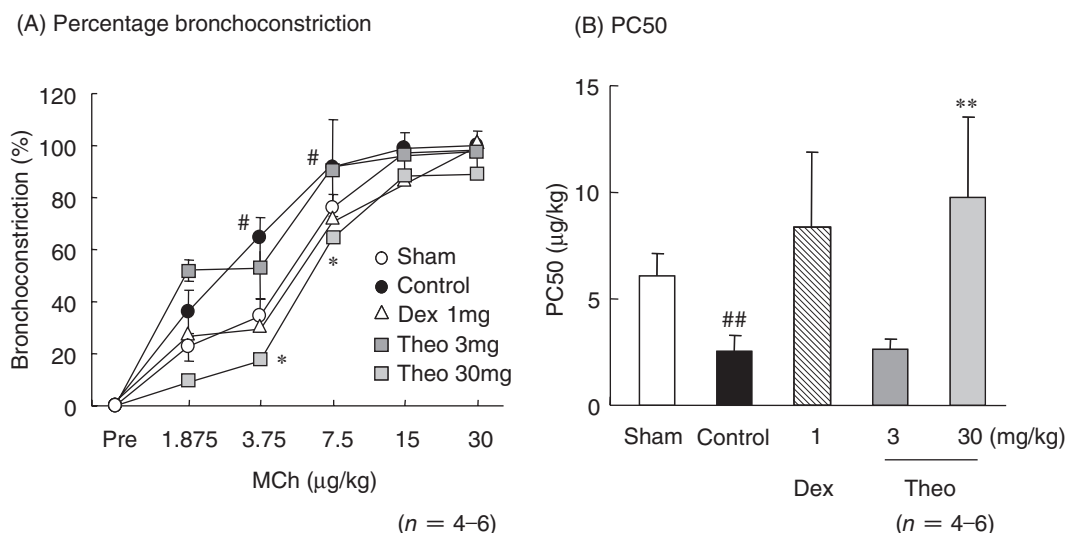


Fig. 10 Airway hyperreactivity was examined after the 15th exposure to LPS with increasing concentration of methacholine **(A)**. Airway response to intravenous injection of methacholine was expressed as relative bronchoconstriction where mechanical occlusion of tracheal cannula was determined as 100%. Each point represents means $\pm$ S.E.M. ($n = 4-6$). Dex; Dexamethasone, Theo; Theophylline. #: $p < 0.05$ v.s. sham group, and *: $p < 0.05$ v.s. control group. **(B)** Airway hyper-reactivity of each group was expressed as PC50, indicating the methacholine dose (mg/kg) at which 50% bronchoconstriction was induced. Each column shows means $\pm$ S.E.M. ($n = 4-6$). Dex; Dexamethasone, Theo: Theophylline. ##: $p < 0.01$ v.s. sham group, and **: $p < 0.01$ v.s. control group.

tive effects on goblet cell hyperplasia induced by OVA were also reported; for example, Kumar *et al.* reported ameliorative effects of roflumilast, a selective PDE IV inhibitor, on goblet cell hyperplasia using a remodeling model of mice induced by OVA.³⁹ Furthermore, Sun *et al.* reported a positive relation between the extent of goblet cell hyperplasia and increase of cAMP-PDE activity.⁴⁰ In consideration of the results of these reports, the ameliorative effects of theophylline on goblet cell hyperplasia were dependent on its inhibition of cAMP-PDE.

Swelling and destruction of the alveolar wall are considered to be other causes of symptoms in COPD patients. Irreversible destruction of the alveolar wall and airspace enlargement lead to decreased lung elastic recoil, and then cause airflow limitation and dynamic airway compression.²⁸ In the present study, enlargement of alveoli and swelling of the alveolar wall was noted after the 15th exposure to LPS (Figs. 5B, 8). These pathological changes might be related to the increase in lung hydroxyproline content during the course of this experiment (Fig. 9), suggesting fibrotic aspects of the pathophysiology of this guinea pig model induced by the present experimental condition. Theophylline improved such pathological changes, but the mechanisms of action were not obvious. In the previous report using a chronic LPS exposure model in mice, swelling of the alveolar wall was observed together with large increases in transforming growth factor (TGF)- β 1 concentration in BALF

and Penh, an index of airflow limitation.⁷ TGF- β has been recognized as the most important factor of fibrotic disease such as emphysema, pulmonary fibrosis and airway remodeling. Recent reports have shown that induction of COL1 (type I collagen) mRNA and α -smooth muscle actin protein by TGF- β stimulus is inhibited by theophylline in *in vitro* experiments with lung fibroblast.⁴¹ Taking these reports into account, it may be suggested that the observed effects of theophylline on improvement of swelling of the alveolar wall and increases in lung hydroxyproline content appeared via inhibition of the signal transduction system of TGF- β .

At last, AHR was examined as a functional endpoint, and it was found that higher doses of theophylline inhibited the airway response to methacholine (Fig. 10). Uno *et al.* reported that theophylline (50 mg/kg) could inhibit acetylcholine induced AHR without inhibiting influx of inflammatory cells in the BALF,³⁵ and our results were found to be consistent with this report. The effects of theophylline on airway disease are usually believed to depend on its anti-inflammatory action and bronchodilatory action. Improved airway hyperreactivity observed in the theophylline-treated group in the present study, however, depends mainly on its anti-inflammatory actions because airway hyperreactivity was measured at 48 hours after final administration of theophylline.

Anti-inflammatory effects of theophylline might play important roles for the prevention of lung de-

struction and goblet cell hyperplasia, and airway reactivity might be maintained at normal levels with preserved lung structure. Furthermore, the anti-inflammatory effects of theophylline might depend on its inhibition of PDE. In addition, other mechanisms of action including adenosine receptor antagonism,⁴³ inhibition interleukin release (IL-1 β , IL-4 and IL-5),^{30,44} increased IL-10 release,⁴⁴ mediator inhibition (leukotriene (LT) B₄, LTC₄, prostaglandins, TNF- α),^{42,44} inhibition of intracellular calcium release,⁴⁵ inhibition of NF-kappa B,⁴⁶ and increased histone deacetylase activity³¹ have been proposed for the clinical effects of theophylline. These mechanisms of action might contribute to the ameliorative effects of theophylline observed in the present study.

In summary, theophylline improved some aspects of the pathological changes induced by repetitive exposure to LPS. These include accumulation of inflammatory cells in the lung parenchyma, hyperplasia of goblet cells, and swelling of the alveolar wall. Airway hyperreactivity was also improved by treatment with theophylline. These effects of theophylline observed in the present study may contribute to improvement of some symptoms, pathological changes as well as airflow limitations in patients with chronic airway disease.

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