Participation of Leukotriene D₄ and Tumor Necrosis Factor on Lipopolysaccharide-Induced Airway Hyperresponsiveness in Guinea Pigs

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In guinea pigs, a marked increase in airway responsiveness to acetylcholine (Ach) was observed at 2h after lipopolysaccharide (LPS) inhalation. To examine the mediators responsible for the airway hyperresponsiveness, the changes of peptide-leukotrienes (LTs), tumor necrosis factor (TNF), interleukin-1 (IL-1), histamine, and 5-hydroxytryptamine (5-HT) levels in bronchoalveolar lavage fluid (BALF) were measured. Airway responsiveness to Ach reached a peak 2h after LPS inhalation. The influx of neutrophils into BALF increased gradually and reached a peak 24h after LPS inhalation. After the inhalation of LPS, LTD₄ and TNF contents in BALF increased within the first 2h after LPS inhalation. However, other mediators were not detected or increased 6h after LPS inhalation. Aeroinhalation of LTD₄ and murine recombinant TNF-α caused airway hyperresponsiveness in guinea pigs. In addition, a LTD₄ antagonist, BAY x7195, and an inhibitor of TNF, pentoxifylline, inhibited the LPS-induced airway hyperresponsiveness. These results suggest that LTs and/or TNF play an important role in the onset of airway hyperresponsiveness in guinea pigs.

Key words lipopolysaccharide; airway hyperresponsiveness; leukotriene; tumor necrosis factor

Airway hyperresponsiveness is one of the characteristic features of bronchial asthma and other respiratory diseases such as adult respiratory distress syndrome (ARDS). In these diseases, lung injury induced by infiltrated inflammatory cells is considered to be the major cause of airway hyperresponsiveness because it is closely related to airway inflammation. We recently reported that lipopolysaccharide (LPS) exposure resulted in an airway hyperresponsiveness to acetylcholine (Ach), with a late onset of airway inflammation in guinea pigs. In this model, the increase in airway responsiveness to Ach was observed before the accumulation of polymorphonuclear leucocytes into the epithelium, and the degree of hyperresponsiveness was not correlated to the severity of airway inflammation.

Our previous studies also indicate that acute airway hyperresponsiveness by LPS was inhibited by some anti-inflammatory drugs such as tranilast and ketotifen and some other anti-inflammatory agents. Additionally, it is demonstrated that increased permeability in pulmonary capillaries is an important incident in the induction of LPS-induced airway hyperresponsiveness. These results suggest that factors other than the influx of inflammatory cells may be involved in the induction of airway hyperresponsiveness.

LPS acts mainly on macrophages, and stimulates the production of monokines; tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) and lipid mediators; leukotrienes (LTs) and platelet activating factor (PAF). Recently, some of these macrophage-derived mediators have been reported to cause airway hyperresponsiveness in animals and human.

Kips et al. reported that aeroinhalation of TNF caused airway hyperresponsiveness in rats. Arm et al. reported that leukotriene E₄ (LTE₄) inhalation resulted in an increase of airway responsiveness in human.

The present study, therefore, was conducted to investigate what kind of chemical mediator is responsible for the induction of airway hyperresponsiveness induced by LPS inhalation in guinea pigs.

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MATERIALS AND METHODS

Animals Male Hartley guinea pigs and female DA rats were purchased from Japan SLC (Hamamatsu, Japan). Animals were housed in an air-conditioned room (22 °C, 60% humidity) and given a standard laboratory diet and tap water ad libitum before use. Experiments were undertaken following guidelines for the care and use of experimental animals set by the Japanese Association for Laboratory Animal Science in 1987.

Drugs and Materials LPS (Escherichia coli Serotype 055:B5, Sigma, MO, U.S.A.), metoprine (Sigma, MO, U.S.A.), Ach (Wako Pure Chemical Industries, Ltd., Osaka, Japan), LTD₄ (Cascade Biochem Ltd., Berkshire, UK) and actinomycin D (Life Technologies, Inc., NY, U.S.A.) were purchased commercially. Murine recombinant TNF-α (mTNF-α) was kindly donated by Dr. N. Tsurukawa (Suntory Co., Ltd., Kyoto, Japan). Murine IL-1β (mIL-1β) was purchased from Genzyme (Boston, MA, U.S.A.). BAY x7195 was kindly donated by Bayer Yakuhin (Osaka, Japan). Pentoxifylline was purchased from Sigma (MO, U.S.A.).

Experimental Protocol for Measurement of Airway Hyperresponsiveness Caused by LPS The experiment was carried out according to the method previously described. In brief, guinea pigs weighing 300—420g were exposed to aerosolized LPS (0.01% dissolved in physiological saline) for 30 min. The aerosol was generated by an ultrasonic nebulizer (TUR-3200, Nihon Kohden, Tokyo, Japan). Metoprine (50 mg/kg), an inhibitor of 11β hydroxylase involved in endogenous glucocorticoid synthesis, was injected intravenously into the guinea pigs immediately before the exposure to LPS. Metoprine was used in this study because it had previously been shown to accelerate the onset and development of LPS-induced airway obstruction. To measure the Ach-induced bronchoconstriction, surgery was performed on guinea pigs under urethane anesthesia (1.0 g/kg, i.p.). Urethane (1.0 g/kg, i.p.) was also injected into the animals at the
end of the operation to arrest spontaneous breathing. Animals were then immediately ventilated with a rodent ventilator (New England Medical Instruments, MA, U.S.A.) at 60 strokes/min, with a tidal volume of 3 ml. Bronchoconstriction was measured according to the overflow method described by Konzett and Rössler using a volume recorder (Ugo Basil, Milano, Italy) connected to the tracheal cannula. Airway responsiveness to Ach is represented as the PD_{50} value that is a provocative dose of Ach to induce 50% bronchoconstriction. Bronchoalveolar lavage fluid (BALF) was obtained after the measurement of airway responsiveness to Ach, according to the method previously described.

Measurement of LTs BALF was obtained at various times (0—2 h) after LPS exposure and centrifuged at 800 rpm for 10 min at 4°C. One ml of the supernatant was diluted with 4 ml of ethanol to prevent the enzymatic destruction of LTs. The supernatant was then centrifuged at 3000 rpm for 10 min at 4°C. The pellet was resuspended in 1 ml of 80% ethanol and the same centrifugation procedure was repeated. The supernatants were mixed and then evaporated to dryness under reduced pressure. The residue was dissolved in an enzyme immunoassay (EIA) buffer and the LTs were measured by EIA using a commercial kit (Leukotriene C_4/D_4/E_4 EIA system, Amersham, Buckinghamshire, UK).

Measurement of TNF TNF in a supernatant of BALF was measured by cytolytic assay using actinomycin D-treated murine L929 cells, as described by Aggarwal et al.

Measurement of IL-1 IL-1 in a supernatant of BALF was measured using the D10 G4.1 cell line. Cells were cultured in complete Clicht’s medium, and seeded in a 96-well microplate at a cell concentration of 4 x 10^4/200 μl/well with serially diluted standard (mL-1/β) and BALF samples. After 72 h incubation at 37°C in 5% CO_2, an Alamar Blue solution (Alamar Biosciences, Sacramento, CA, U.S.A.) in a volume of 20 μl was added to each well and incubated for 12 h. The fluorescence intensity of each well was measured using a Millipore CytoFluor plate reader (Millipore-Waters, Ontario, Canada) at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. IL-1 activity in BALF was calculated from a standard curve of mL-1/β.

Measurement of 5-Hydroxytryptamine (5-HT) 5-HT was measured by bioassay using rat stomach strips. Rats weighing 300—450 g were used. Under anesthesia with diethyl ether, the whole stomach was isolated. Then, the fundus was isolated (approximately 20 mm in length and 2 mm in width) and suspended in an organ bath containing 10 ml of Tyrode solution (mM: NaCl 136.9, KCl 2.7, CaCl_2 1.8, MgCl_2 1.0, NaHCO_3 11.9, glucose 5.6) maintained at 37°C and gassed with air. The contractile response was recorded isotonically. The bioassay was carried out in the presence of a muscarinic antagonist (atropine, 10 μM), histamine H_2 antagonist (diphenhydramine, 10 μM), LTD_4 antagonist (BAY x7195, 10 μM) and thromboxane A_2 antagonist (BAY 3405, 10 μM).

Measurement of Histamine The amount of histamine in BALF was measured fluorometrically by a post-column derivation method on an automated histamine analyzing system (Tooh Co., Ltd., Tokyo, Japan).

Airway Responsiveness after mTNF-α Exposure in Guinea Pigs Guinea pigs were anesthetized with urethane (1.0 g/kg, i.p.) and a tracheal cannula was inserted into the trachea. Aerosolized mTNF-α (5 x 10^4 U/ml dissolved in physiological saline) was inhaled through the tracheal cannula. Aerosolized mTNF-α was generated by a nebulizer (Devilbiss Pulmo Aide, PA, U.S.A.) for 30 min. Metopirone (50 mg/kg) was injected intravenously into the guinea pigs prior to the exposure of mTNF-α. Immediately after the inhalation of mTNF-α, airway responsiveness to Ach was measured as described above.

Airway Responsiveness after LTD_4 Exposure in Guinea Pigs Guinea pigs were inhaled with aerosolized LTD_4 (10^-6 M dissolved in physiological saline). Aerosolized LTD_4 was generated by an ultrasonic nebulizer (TUR-3200, Nihon Kohden, Tokyo, Japan) for 30 min. Metopirone (50 mg/kg) was injected intravenously into guinea pigs prior to the exposure of LTD_4. One hour after the inhalation of LTD_4, airway responsiveness to Ach was measured as described above.

Effects of BAY x7195 and Pentoxifylline on LPS-Induced Airway Hyperresponsiveness A LTD_4 antagonist, BAY x7195, and an inhibitor of TNF production, pentoxifylline, were orally administered 1 h before LPS inhalation. Two hours after LPS inhalation, airway hyperresponsiveness to Ach was measured as described above.

Statistics Data were expressed as the mean ± S.E.M. Statistical analyses of the results were performed using Student’s t-test or Dunn’s multiple comparison test. A p value of less than 0.05 was regarded as statistically significant.

RESULTS

Airway Responsiveness to Ach after LPS Exposure Airway responsiveness to Ach was examined 1, 2, 6 and 24 h after LPS exposure. A significant increase in airway responsiveness to Ach was observed throughout the experiment. The peak response was observed 2 h after LPS exposure (Fig. 1).

Inflammatory Cell Infiltration in BALF The number of neutrophils in the BALF increased significantly 2 h after exposure and then gradually increased (Fig. 2). No significant change in the number of lymphocytes, eosinophils or macrophages was observed at 1, 2 and 6 h after LPS inhalation. However, the number of macrophages and lymphocytes increased 24 h after LPS exposure. In the saline inhalation group, no significant change in the number of inflammatory cells was observed at any of the time points.

Amounts of LTD_4, TNF, IL-1, 5-HT and Histamine in BALF The concentration of LTD_4 in BALF increased transiently but significantly 1 h after LPS inhalation (Fig. 3). TNF activity in BALF gradually increased, with a peak at 2 h, and then decreased (Fig. 4). Twenty-four hours after the LPS exposure, TNF activity in BALF was not detected. Histamine content in BALF did not change until 2 h after LPS exposure, and then increased from 6 h after LPS exposure (Fig. 5). IL-1 and 5-HT activities in BALF
Fig. 1. Time Course Study of Airway Responsiveness to Ach after LPS Exposure in Guinea Pigs

Each symbol and vertical bar represents the mean and S.E.M. of 6 animals, respectively. Animals inhaled aerosolized saline and 0.01% LPS for 30 min. Airway responsiveness to Ach (1–25 μg/kg, i.v.) was measured at 1, 2, 6 and 24 h after saline or LPS exposure. Animals were treated with metoprine (50 mg/kg, i.v.) prior to exposure. * ** Significantly different from the saline exposure at the corresponding time at p<0.05 and p<0.01, respectively.

Fig. 2. Time Course Study of the Number of Leukocytes in BALF after LPS Exposure in Guinea Pigs

Each column and vertical bar represents the mean and S.E.M. of 6 animals, respectively. Animals inhaled aerosolized saline and 0.01% LPS for 30 min. Bronchoalveolar lavage (BAL) was performed immediately after the measurement of airway responsiveness to Ach 1, 2, 6 and 24 h after saline or LPS exposure. Animals were treated with metoprine (50 mg/kg, i.v.) prior to exposure. * ** Significantly different from the saline exposure at the corresponding time at p<0.05 and p<0.01, respectively.

Airway Responsiveness to Ach after mTNF-α Exposure

Immediately after the inhalation of mTNF-α (5 x 10^4 U/ml), a significant increase in the airway responsiveness to Ach was observed in guinea pigs (Fig. 6).

Airway Responsiveness to Ach after LTD₄ Exposure

One hour after the inhalation of LTD₄ (10^-6 M), a significant increase in the airway responsiveness to Ach was observed (Fig. 7).

Effects of BAY x 7195 and Pentoxifylline on LPS-Induced Airway Hyperresponsiveness

As shown in Table 1, BAY x 7195 at a dose of 10 mg/kg, p.o., significantly inhibited the LPS-induced airway hyperresponsiveness. However, this compound showed no influence on neutrophil accumulation in BALF. Pentoxifylline at a dose of 100 mg/kg, p.o., also inhibited the LPS-induced airway hyperresponsiveness. Pentoxifylline did not inhibit the increase in the number of neutrophils in BALF, but significantly inhibited the elevation of TNF activity in BALF (Fig. 8).
DISCUSSION

The present study was carried out to determine the mediators responsible for the acute airway hyperresponsiveness induced by LPS exposure in guinea pig. The results suggest that LTs and/or TNF might be responsible for the induction of the airway hyperresponsiveness.

In the present study, the time course of airway responsiveness by LPS was apparently different from that of leukocyte infiltration into the airways. The increase in airway responsiveness reached a maximum 2 h after the LPS exposure, whereas neutrophil infiltration into BALF gradually increased until 24 h. These results suggest that factors other than the influx of inflammatory cells may be involved in the modulation of airway responsiveness. The present data confirm our previous data\(^1\) and that of others.\(^{15}\) Pauwels et al.\(^{15}\) demonstrated that the peak response of LPS-induced hyperresponsiveness to 5-HT in rats occurred earlier than the peak response of exudative inflammation.

In the present study, LTs, TNF and histamine contents in BALF significantly increased after LPS inhalation. However, IL-1 and 5-HT were not detected in the supernatant of BALF at any time after LPS exposure. LTs significantly increased 1 h after LPS exposure. TNF activity in BALF increased rapidly, reached a peak at 2 h, and then decreased. On the other hand, histamine gradually increased from 6 h after the inhalation. These results indicate that LTs and TNF play an important role in the LPS-induced airway hyperresponsiveness in guinea pig.

The major source of TNF and LTs is thought to be macrophages, but infiltration of macrophages into airways was very weak when these mediators increased in BALF. This result suggests that alveolar macrophages stimulated with LPS rapidly release these mediators, then migrate into airway lumen. In contrast, the cells responsible for histamine production remains unclear, but recent evidence has demonstrated that mast cells, epithelial cells and macrophages release histamine or activate histidine decarboxylase, the enzyme-forming histamine, after the LPS stimulation,\(^{16,17}\) suggesting that these cells play an important role in the production of histamine.

To confirm the role of LTs and TNF-\(\alpha\), we have investigated the effects of inhalation of LTD\(_4\) and TNF-\(\alpha\) and the effect of each inhibitor on LPS-induced airway hyperresponsiveness in guinea pigs. The aero-inhalation of LTD\(_4\) and TNF-\(\alpha\) resulted in an airway hyperresponsiveness to Ach within 1 h, without the infiltration of inflammatory cells in airway lumen. The facts that LTs and TNF caused airway hyperresponsiveness in rats and human have already been reported by others.\(^{7-10}\) However, our present finding is that LTD\(_4\) and TNF-\(\alpha\) cause an airway hyperresponsiveness within 1 h in guinea pig.
Table 1. Effects of BAYx7195 and Pentoxifylline (PTX) on LPS-Induced Airway Hyperresponsiveness in Guinea Pigs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Bronchial reactivity (µg/kg, i.v.)</th>
<th>Cell number in BALF (×10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PD_{50} of Ach</td>
<td>Lymphocyte</td>
</tr>
<tr>
<td>Saline</td>
<td>7</td>
<td>18.37 ± 3.07**</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>LPS</td>
<td>5</td>
<td>9.43 ± 1.03</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>LPS + BAYx7195 10 mg/kg, p.o.</td>
<td>5</td>
<td>18.49 ± 2.13**</td>
<td>0.01 ± 0.00</td>
</tr>
<tr>
<td>Saline</td>
<td>6</td>
<td>18.86 ± 1.97**</td>
<td>0.06 ± 0.03</td>
</tr>
<tr>
<td>LPS</td>
<td>6</td>
<td>7.08 ± 1.21</td>
<td>0.12 ± 0.03</td>
</tr>
<tr>
<td>LPS + PTX 100 mg/kg, p.o.</td>
<td>6</td>
<td>24.44 ± 1.75**</td>
<td>0.08 ± 0.02</td>
</tr>
</tbody>
</table>

Results represent the mean ± S.E.M. of 5—7 animals. Animals inhaled saline and 0.01% LPS for 30 min. Airway responsiveness to Ach (1—25 mg/kg, i.v.) was measured 2 h after saline or LPS exposure. Animals were treated with metopirone (50 mg/kg, i.v.) prior to exposure. PD_{50} is the dose of Ach required to induce 50% bronchoconstriction. BAYx7195 (10 mg/kg) and PTX (100 mg/kg) were orally administered 1 h before LPS exposure. * * , * * * Significantly different from the LPS exposure at p < 0.01 and p < 0.05, respectively.

Fig. 8. Effect of Pentoxifylline (PTX) on the Elevation of TNF Activity in BALF after LPS Exposure

Each column and vertical bar represent the mean and S.E.M. of 6 animals, respectively. Animals inhaled aerosolized saline and 0.01% LPS for 30 min. BALF was obtained 2 h after saline or LPS exposure. Animals were treated with metopirone (50 mg/kg, i.v.) prior to exposure. TNF activity was measured by cytolytic assay using actinomycin D-treated murine L929 cells. PTX (100 mg/kg) was orally administered 1 h before LPS exposure. * Significantly different from the LPS exposure at p < 0.05.

Our findings suggest that LTs and TNF contribute to the induction of airway hyperresponsiveness at the exudative phase because our previous data indicated that LPS induced marked exudation in guinea pigs within 1 h of LPS inhalation.11

The contribution of LTs and TNF was also supported by the examination using BAYx7195 and pentoxifylline. BAYx7195 is a potent and selective LTD4 antagonist,18 and completely inhibits LTD4-induced bronchoconstriction at a dose of 10 mg/kg, p.o.19 Pentoxifylline is a non-selective phosphodiesterase (PDE) inhibitor and is reported to suppress TNF production in vitro20 and in vivo.21 In the present study, BAYx7195 at a dose of 10 mg/kg, p.o. inhibited airway hyperresponsiveness. Moreover, pentoxifylline at a dose of 100 mg/kg, p.o. also inhibited the reaction and TNF production. These results support the hypothesis that LTs and TNF are responsible for the induction of the airway hyperresponsiveness by LPS. Further investigation, however, will be needed to clarify the participation of TNF because in the present study we could not rule out the possibility that pentoxifylline will suppress the Ach-induced bronchoconstriction due to the inhibition of PDE in airway smooth muscle.

There are some reports indicating the importance of other chemical mediators on the pathogenesis of airway hyperresponsiveness by LPS. For example, Arimura et al.22 postulated the role of thromboxane A2. Since other mediators might play an important role in the induction of airway hyperresponsiveness by LPS, further experiments will be needed to clarify the contribution of other mediators and the correlation among them.

In conclusion, LPS-induced airway hyperresponsiveness might be at least mediated by LTs and/or TNF in guinea pigs.

REFERENCES


