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Original Article

Thromboxane A_2 mediates cation-induced airway hyperresponsiveness through the bradykinin B_2 receptor in guinea pigs

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Abstract

To elucidate the mechanisms of cationic protein-induced airway hyperresponsiveness (AHR), the airway response to methacholine, and the concentrations of thromboxane B_2 (TXB_2) and 6-keto-PG$F_1\alpha$ in the broncho-alveolar lavage fluid (BALF) of guinea-pigs were measured after inhalation of poly-L-lysine (P-L-L). The airway responsiveness (AR) was evaluated by specific airway resistance. The inhalation of P-L-L significantly enhanced AR to methacholine, and increased TXB_2 and 6-keto-PG$F_1\alpha$ in the BALF. The enhanced AR and the increase of TXB_2 and 6-keto-PG$F_1\alpha$ were both significantly inhibited by pretreatment with low molecular weight heparin (LMWH; anionic protein; 10 mg/mL for 6 min inhalation). Furthermore, thromboxane (TX) synthase inhibitor (ozagrel), thromboxane A_2 (TXA_2) receptor antagonist (ONO-3708), and bradykinin B_2 receptor antagonist (BBRA; Nα-adamantaneacetyl-D-Arg [Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin) inhibited the cation-induced AHR. BBRA significantly inhibited the increase of those mediators in the BALF. The data suggest that TXA_2, which is newly generated by the stimulation of the bradykinin B_2 receptor after inhalation of cationic proteins, plays an important role in cationic protein-induced AHR in guinea pigs.

Key words: airway hyperresponsiveness, bradykinin, cationic protein, low molecular weight heparin, poly-L-lysine, 6-keto-PG$F_1\alpha$, thromboxane A_2.

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Introduction

Bronchial asthma is considered to be a form of eosinophilic desquamative bronchitis[1] and eosinophil-derived granuloproteins play an important role in airway hyperresponsiveness (AHR).[2] It has been demonstrated that major basic protein (MBP), one of the eosinophil-derived cationic granuloproteins, damages the bronchial epithelium of guinea-pigs *in vitro,*[3,4] and increases airway responsiveness in guinea-pigs *in vitro,*[5] in primates *in vivo,*[6] and in rats *in vivo.*[7] These investigators[3–7] suggested that epithelium desquamation is the most important phenomenon in MBP-induced AHR.

Barker *et al*.[8] demonstrated that AHR induced by MBP was suppressed by pretreatment with polyanionic proteins, suggesting that cationic charges derived from MBP enhance the airway responsiveness. Synthetic cationic proteins also induce AHR, which was similarly inhibited by polyanionic proteins such as heparin and poly-L-glutamine in rats *in vivo*[9,10] and in guinea-pigs *in vitro.*[10,11] However, epithelial desquamation was not observed in cation-induced AHR, which suggested that the cation-induced AHR may have been the result of epithelial dysfunction and not epithelial desquamation.

In epithelial dysfunction induced by cationic proteins, the release of neuropeptides[12] and the increased generation of bradykinin[13] have been shown. Arachidonic acid (AA) metabolites may also contribute to the mechanism of cation-induced AHR,[14] because cationic proteins stimulate the release of prostaglandins in guinea-pig epithelial cells[15,16] and in cultured mouse fibroblasts.[17]

In order to elucidate the mechanism of the AHR induced by cationic proteins in guinea-pigs, we investigated the effects of an H_1 antagonist, a thromboxane (TX) synthase inhibitor, a thromboxane A_2 (TXA_2) receptor antagonist, a neurokinin receptor antagonist, and a

bradykinin receptor antagonist on the AHR induced in guinea pigs by poly-L-lysine (P-L-L), which has potent strong cationic charges.

In addition, in order to investigate the participation of the products from the cyclo-oxygenase pathway in the cation-induced AHR, we measured the airway resistance and the concentrations of thromboxane B_2 (TXB_2), the stable metabolite of TXA_2) and 6-keto-$PGF_1\alpha$ (the stable metabolite of PGI_2) in the guinea-pig's bronchoalveolar lavage fluid (BALF).

Materials and Methods

Animals

Male Hartley specific pathogen-free guinea-pigs (Japan SLC Co., Hamamatsu, Japan) weighing 350–500 g were used for this study. The guinea-pigs were housed four to a cage in standard cages and were fed with standard lab chow (CG-7, Japan Kurea Co., Osaka, Japan). They were kept in a room maintained at 23 ± 2°C and at 55 ± 5% humidity. All animal experiments were conducted according to the 'Guidelines for animal experimentation at the Kobe University School of Medicine' (permission number: P931012).

Drugs

Poly-L-lysine, pyrilamine, low molecular weight heparin (LMWH) and bradykinin B_2 receptor antagonist ((BBRA) Nα-adamantaneacetyl-D-Arg-[Hyp^3,Thi^{5,8} D-Phe^7]-bradykinin) were obtained from Sigma Chemical Co. (St Louis, MO, USA). FK-888 was donated by the Fujisawa Pharmaceutical Co. (Tokyo, Japan). Ozagrel and ONO-3708 were donated by Ono Pharmaceutical Co. (Osaka, Japan). Dimethyl sulfoxide (DMSO) and methacholine were obtained from Wako Pure Chemical Industries (Osaka, Japan). Thiopental sodium was from Tanabe Chemical Co. (Osaka, Japan) and saline was from Otsuka Chemical Co. (Tokyo, Japan).

Experimental protocol

Poly-L-lysine was dissolved in sterile physiological saline and prepared at the final concentration of 2.5 mg/mL. The guinea-pig was placed in a chamber box and kept awake. It breathed spontaneously. Aerosolized P-L-L was delivered into the chamber box from an ultrasonic nebulizer (Model NE-U12, Omron Co., Tokyo, Japan) for 6 min. One hour later, the animal was transferred to another box for the measurement of airway response to methacholine. In the negative control group, saline in place of P-L-L was inhaled.

The effects of LMWH, an anionic substance, on the P-L-L-induced AHR were examined in two groups: the LMWH group, with an inhalation of LMWH for 6 min (10 mg/mL) 1 h before P-L-L inhalation; and the control group, whereby saline in place of LMWH was inhaled.

FK-888, neurokinin NK_1 receptor antagonist, dissolved in DMSO was injected intraperitoneally at a dose of 1 mg/kg or 10 mg/kg of FK-888 1 h before P-L-L inhalation. In the negative control group, DMSO in place of FK-888 was injected.

The effects of pyrilamine (H_1 antagonist), ozagrel (TX synthase inhibitor), and ONO-3708 (TXA_2 receptor antagonist) on the P-L-L-induced AHR were examined. These substances were each dissolved in sterile physiological saline and injected separately into the intraperitoneal cavity of guinea-pigs 1 h before P-L-L inhalation. The administered doses were 10 mg/kg of pyrilamine, 20 mg/kg and 100 mg/kg of ozagrel, and 10 mg/kg of ONO-3708. In the negative control group, saline was injected in place of these substances.

Bradykinin B_2 receptor antagonist was dissolved in the sterile physiological saline and injected into the intraperitoneal cavity of guinea pigs 15 min before P-L-L inhalation. The final concentrations were 100 nmol/kg and 1000 nmol/kg. In the negative control group, saline in place of BBRA was injected into the intraperitoneal cavity.

We performed the assessment of airway responsiveness and of BALF using the different guinea-pig groups. The experimental protocol is summarized in Figure 1.

Assessment of the airway responsiveness

The airway responsiveness to methacholine was assessed 1 h after P-L-L inhalation while the animals were awake and breathing spontaneously. Specific airway resistance (SRaw) was measured using a double-flow plethysmograph (Model PLYUN2P, Buxco, Sharon, CT, USA), with the method of Pennock *et al*.[18] Each animal was placed in the two-compartment plethysmograph with its head protruding from the front of the chest box and into the head box through a hole covered by a rubber faceplate. Changes in the thoracic gas volume and in the gas volume at the mouth were measured with a differential pressure transducer (Model DP45–14, Buxco), and SRaw was calculated using the respiratory analyzer (Model

PMUA + SAR, Buxco). After stabilization of the baseline SRaw, aerosolized 0.9% saline and doubled concentrations of methacholine (0.03–4 mg/mL) with the ultrasonic nebulizer were introduced into the head box via bias flow. Each dose was inhaled for 1 min and peak SRaw was determined in the following 2 min. Increased concentrations of methacholine were delivered until the SRaw reached more than twice the baseline value. The dose of methacholine required to increase SRaw to twice its baseline value was used to determine the PC100. The SRaw values were transformed to their normal logarithms prior to statistical analyses because they showed logarithmic normal distribution.

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed using other guinea-pigs 1 h after saline inhalation or 1 h after P-L-L inhalation.

Guinea-pigs were killed by an overdose, by intraperitoneal injection, of 2 mL thiopental sodium, 25 mg/mL. The trachea was carefully exposed after incision. A 21-gauge cannula was inserted through an incision in the upper trachea and tied in place with surface thread. Lavage was performed by introducing 3 mL of sterile physiological saline into the lungs via tracheal cannula and carefully withdrawing the fluid. This procedure was repeated three times. The BALF was centrifuged at 800 r.p.m. for 3 min, and the cell pellet was resuspended in 0.5 mL physiological saline. An air-dried slide preparation was then made at 800 r.p.m. for 3 min in a cytospin. This was stained with Diff-Quik, and a differential cell count of at least 400 cells was made according to standard morphological criteria. The numbers of cells recovered per guinea-pig was calculated and expressed as the mean and standard error of mean (SEM) for each treatment group.

Measurement of TXB_2 and 6-keto-$PGF_{1\alpha}$

The supernatant of the BALF was used for measuring TXB_2 and 6-keto-$PGF_1\alpha$. Both mediators were analyzed by an enzyme-linked immunosorbent assay (ELISA) method at the Laboratory of Ono Pharmaceutical Co., Ltd.

To investigate the effect of LMWH and BBRA on the production of TXB_2 and 6-keto-$PGF_1\alpha$, we recovered BALF 1 h after P-L-L inhalation in a control group and in a group pretreated with LMWH or with BBRA.

Statistics

Data are expressed as the geometric means ± geometric standard error of the means (SEM). Statistical analysis of PC100 was performed on logarithmically transformed data, because it showed logarithmic normal distribution. Comparisons between two groups were performed using Student's *t*-test. Comparisons among more than two groups were performed using two-tailed analysis of variance (ANOVA) and post-comparison was performed using Fisher's PLSD. All *P*-values were two-tailed, and a *P*-value < 0.05 was considered to be significant.

RESULTS

Airway responsiveness

The effects of P-L-L on airway response and the effects of LMWH on cation-induced AHR are summarized in Table 1. The inhalation of P-L-L significantly increased the airway

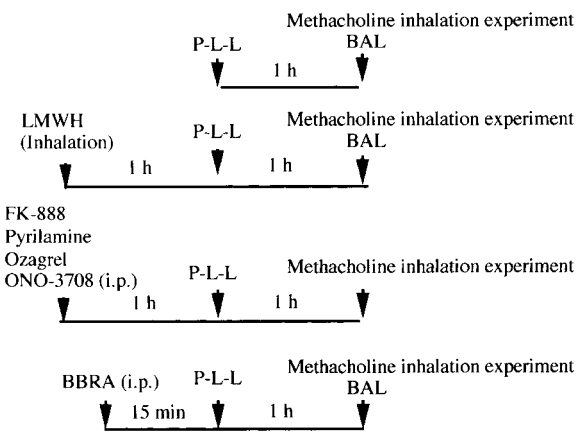
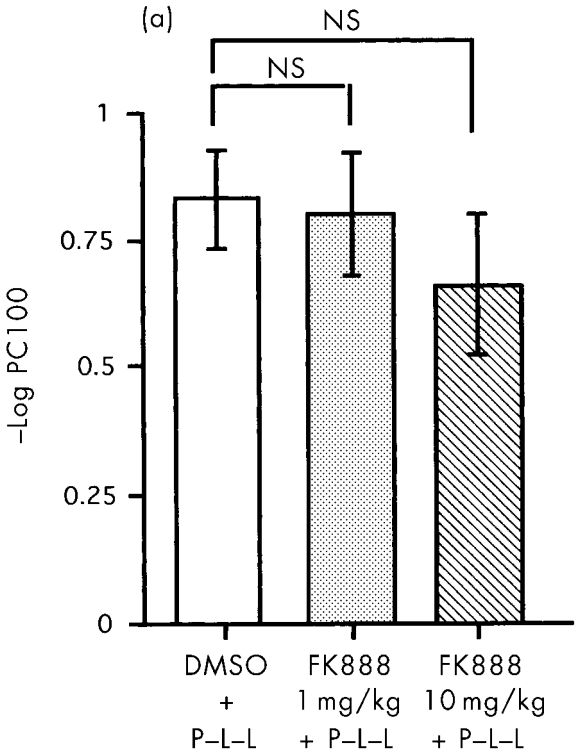


Fig. 1 Experimental protocol for assessment of airway responsiveness and of bronchoalveolar lavage fluid (BALF). P-L-L, poly-L-lysine; LMWH, low molecular weight heparin; i.p.

Table 1. Effects of poly-L-lysine and low molecular weight heparin (LMWH) on airway response

Pretreatment	Inhalation	Number of animals	-Log PC100
Saline		8	0.366 ± 0.104
Saline	P-L-L	8	0.817 ± 0.199*
LMWH	P-L-L	8	0.410 ± 0.072**

Results are expressed as the mean ± SEM. *P < 0.01 compared with saline inhalation; **P < 0.05 compared with saline pretreatment with P-L-L inhalation; P-L-L, poly-L-lysine.



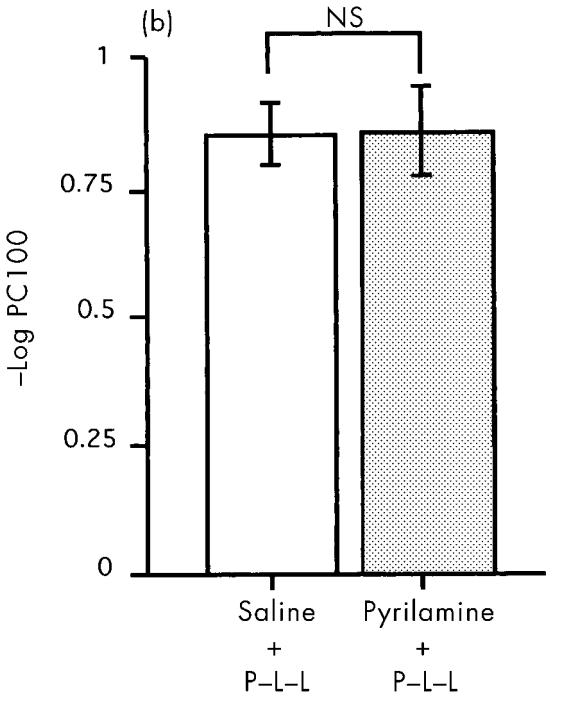
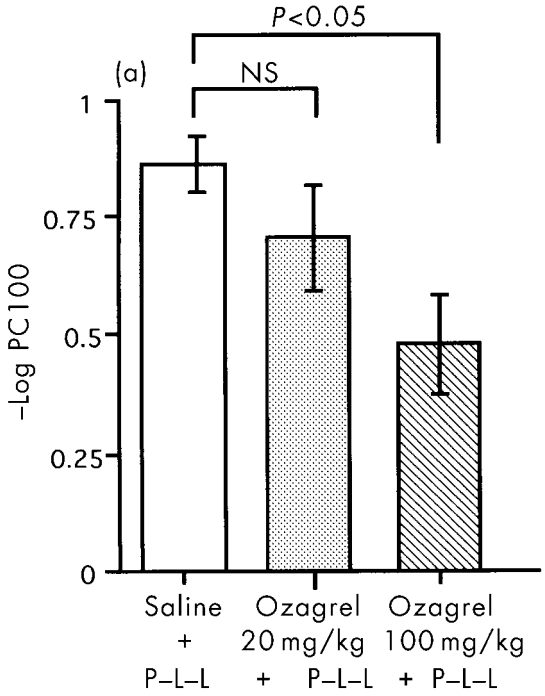


Fig. 2 The effects of pretreatment of FK-888 or pyrilamine on the airway hyperresponsiveness induced by poly-L-lysine (P-L-L). (a) Pretreatment of dimethyl sulfoxide (□), FK-888 1 mg/kg (▨) or 10 mg/kg (▧). (b) Pretreatment of saline (□) or pyrilamine 10 mg/kg (▨). Results are expressed as the mean ± SEM of -PC100 for eight animals; NS, not significant.



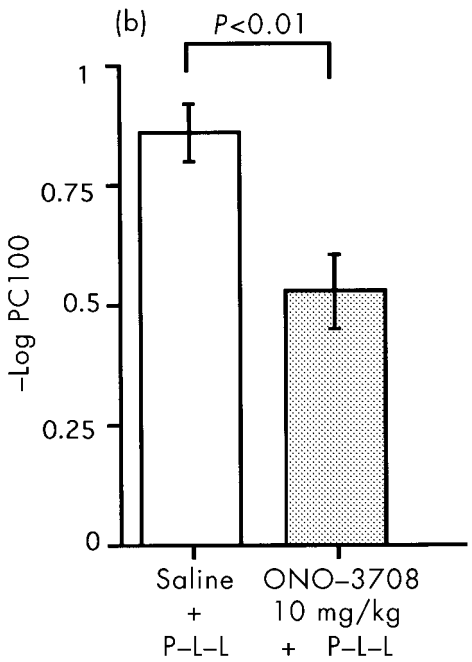


Fig. 3 The effects of ozagrel and ONO-3708 on the airway hyperresponsiveness induced by P-L-L. (a) Pretreatment of saline (□), ozagrel 20 mg/kg (▨) or ozagrel 100 mg/kg (▧). (b) Pretreatment of saline (□) or ONO-3708 10 mg/kg (▨). Results are expressed as the mean ± SEM of -PC100 for eight animals; NS, not significant.

responsiveness to methacholine compared with saline inhalation. Pretreatment of LMWH significantly inhibited the AHR.

Figure 2 shows the effects of FK-888 and pyrilamine on P-L-L-induced AHR. Pretreatment of FK-888 and of pyrilamine did not inhibit P-L-L-induced AHR.

The effects of ozagrel and ONO-3708 on P-L-L-induced AHR are exhibited in Fig. 3. Although the pretreatment with low-dose ozagrel (20 mg/kg) did not have a significant inhibitory effect on P-L-L-induced AHR, the high-dose ozagrel (100 mg/kg) significantly inhibited the P-L-L-induced AHR (Fig. 3a). Further, 10 mg/kg of ONO-3708 significantly inhibited P-L-L-induced AHR (Fig. 3b).

The effects of BBRA on P-L-L-induced AHR are shown in Fig. 4. The pretreatment of low-dose BBRA (100 nmol/kg) did not show a significant inhibitory effect on P-L-L-induced AHR, but high-dose BBRA (1000 nmol/kg) inhibited it significantly.

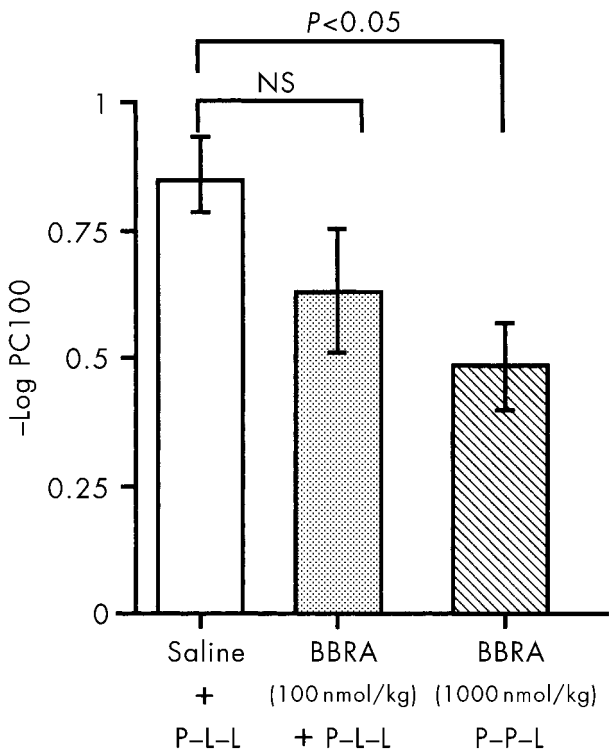


Fig. 4 The effect of bradykinin B_2 receptor antagonist (BBRA; Nα-adamantaneacetyl-D-Arg-[Hyp^3,Thi^{5,8}, D-Phe^7]-bradykinin) on the airway hyperresponsiveness induced by P-L-L. Pretreatment of saline (□), BBRA 100 nmol/kg (▨) or BBRA 1000 nmol/kg (▧). Results are expressed as the mean ± SEM of -PC100 for eight animals; NS, not significant.

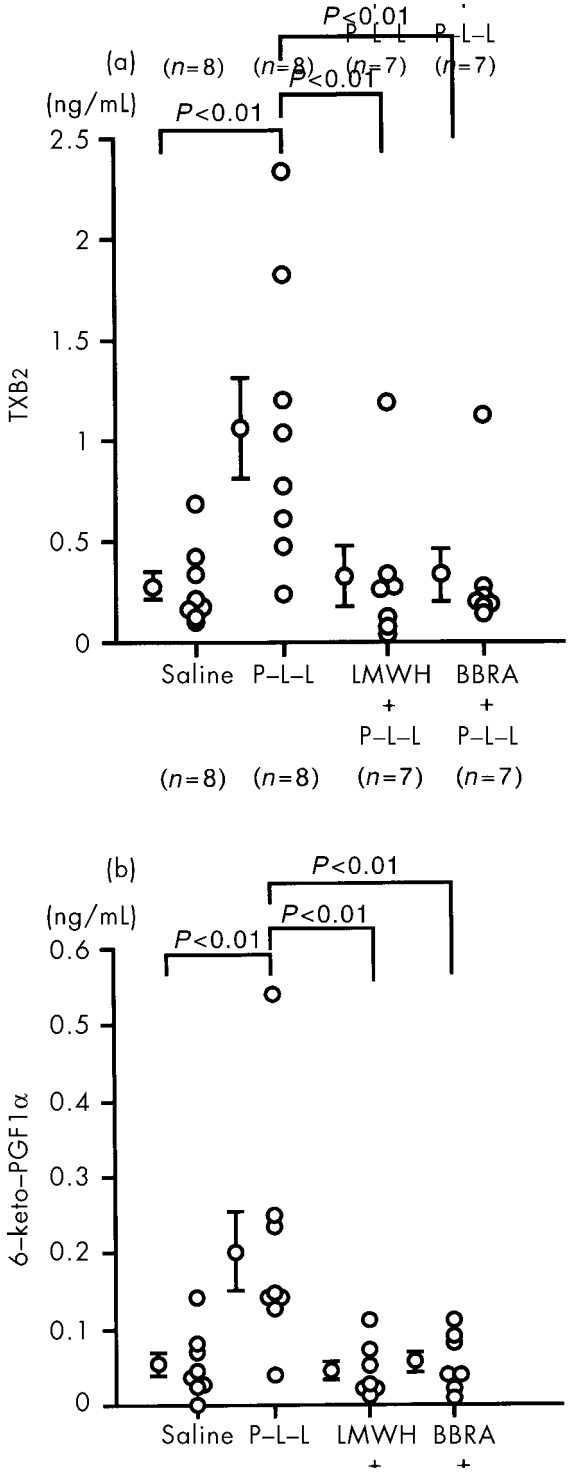


Fig. 5 The effect of the pretreatment with low molecular weight heparin (LMWH) and bradykinin B_2 receptor antagonist (BBRA) on (a) TXB_2 and (b) 6-keto-$PGF_{1\alpha}$ concentrations in the BALF to inhalation of poly-L-lysine (P-L-L).

Differential cell counts in bronchoalveolar lavage fluid

The inhalation of P-L-L did not affect the differential cell count in the BALF. Epithelial cells in BALF had a tendency to increase by P-L-L inhalation.

Concentration of TXB_2 and 6-keto-PG$F_{1\alpha}$ in bronchoalveolar lavage fluid

P-L-L inhalation significantly increased cyclo-oxygenase products, TXB_2 and 6-keto-PG$F_{1\alpha}$, in the BALF compared with saline inhalation.

The pretreatments of LMWH and BBRA inhibited the increase of the cyclo-oxygenase products in the BALF induced by P-L-L (Fig. 5a,b).

DISCUSSION

The results showed that synthetic cationic proteins induced airway hyperresponsiveness in guinea pigs, and that this AHR resulted from the stimulation of the cyclo-oxygenase pathway mediating the bradykinin B_2 receptor.

Various chemical mediators and neuropeptides contribute to the mechanism of cation-induced AHR.[12,13,19] Santana *et al*.[19] have reported that inflammatory changes induced by poly-L-arginine, another type of polycation, are dependent on the mast cell activation and the electrostatic interactions in rats. The pretreatment of pyrilamine (an H_1 antagonist) in the present study did not inhibit the P-L-L-induced AHR, suggesting that the H1 receptor did not participate in the P-L-L-induced AHR in guinea-pigs. Therefore, the electrostatic interaction might play an important role in the P-L-L-induced AHR. In addition, substance *P*, which was released from sensory nerves, played an important role in the cationic protein-induced AHR in rats through stimulating the neurokinin NK_1 receptor.[12] However, FK-888, which is a potent, competitive and selective antagonist for NK_1 receptor,[20] did not inhibit the P-L-L-induced AHR in the present study. We also suggest that the neurokinin NK_1 receptor does not participate in the P-L-L-induced AHR in guinea-pigs.

Arachidonic acid metabolites may contribute to the mechanism of cation-induced AHR because major basic protein and synthetic polycations in the guinea-pig epithelium produce bronchoconstrictor prostanoids,[15,16] and cationic proteins stimulate the release of prostaglandins in cultured mouse fibroblasts.[17] Coincubation of major basic proteins in the presence of AA with guinea-pig epithelial cells shows the decrease of prostaglandin E_2 and the increase of thromboxane B_2. The mechanism of the production of prostanoids stimulated with major basic protein and P-L-L is not related to cytotoxic effects but to charge interactions.[15] Of the prostanoids, TXA_2, which is a potent mediator of airway inflammation, has been involved in AHR.[21] In contrast, PGI_2, which is a relaxant mediator,[22] acts against the AHR.[23] Therefore, we examined the effect of a TX synthase inhibitor (ozagrel) and a TXA_2 receptor antagonist (ONO-3708), and measured TXB_2 and 6-keto-PG$F_{1\alpha}$ in BALF after P-L-L inhalation.

It has been reported that the concentrations used in the present study of ozagrel and ONO-3707 are indeed effective in inhibiting the bronchoconstriction.[24,25] Our results showed that the concentrations of TXB_2 and 6-keto-PG$F_{1\alpha}$ were both increased in BALF after P-L-L inhalation, and that both ozagrel and ONO-3708 inhibited AHR. The increased concentration of TXB_2 and 6-keto-PG$F_{1\alpha}$ after inhalation of cationic material was induced by the activation of cyclo-oxygenase pathway. In our results, the concentration of TXB_2 was approximately five-fold higher than that of 6-keto-PG$F_{1\alpha}$ in BALF and cation-induced AHR was inhibited by ozagrel and ONO-3708, suggested that TXA_2 plays an important role in AHR induced by P-L-L inhalation.

It has been shown that bradykinin induces the release of TXB_2 into the BALF of guinea-pigs,[26] and that TX synthase inhibitor and TXA_2 receptor antagonist reduce the bradykinin-induced airflow obstruction in guinea-pigs.[24,27] TXA_2 receptor antagonist inhibits the bradykinin-induced airway contraction in humans,[28,29] suggesting that bradykinin contributes to the modulation of airway response through TXA_2. Bradykinin is an important inflammatory mediator that is generated from kininogens by the actions of plasma and tissue kallikreins.[30] Coyle *et al*.[13] have shown that cation-induced AHR is also associated with bradykinin generation. Experimental study has shown that incubation of guinea-pig epithelium with major basic protein or P-L-L augments the production of prostanoids to the subsequent response to bradykinin.[15] In the present study, BBRA inhibited both the P-L-L-induced AHR and the production of TXA_2 and PGI_2 induced by P-L-L inhalation.

The bradykinin B2 receptor antagonist used in the present study has a potent inhibitory effect on bradykinin B_2 receptor.[31] Bradykinin B_2 receptors are present in peripheral airways, including epithelial cells and smooth muscle cells.[32] Epithelial desquamation was not significant in BALF and airway histology was not significantly changed in gross appearance in our preliminary study after P-L-L inhalation (T Shirotani *et al*., unpubl. data,

1995). It is possible that P-L-L-induced AHR is provoked by prostaglandin formation, especially TXA_2, through BBRA. We did not address the question of what kinds of airway cells contributed to the production of bradykinin after inhalation of polycation.

We concluded that poly-L-lysine-induced AHR is due to the increase of newly generated TXA_2 by the stimulation of the cyclooxygenase pathway mediating bradykinin B_2 receptors in airway cells in guinea pigs, without macromorphological derangement of bronchial epithelium. An orally active, non-peptide BBRA has been discovered recently,[33] and how BBRA contributes to the pathophysiology of the airway hyperreactivity in bronchial asthma will be further investigated.

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