

Sensory neuropeptides are not directly involved in bronchial hyperresponsiveness induced by interleukin-8 in guinea-pigs *in vivo*

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Summary

Background Interleukin-8 (IL-8) has been shown to be a chemotactic factor for neutrophils, T-lymphocytes and eosinophils. Repeated intranasal administration of IL-8 enhances bronchial responsiveness to inhaled histamine in guinea-pigs. Neuropeptides which are released from C-fibre nerve-endings have been postulated to induce bronchial hyperresponsiveness through neurogenic inflammation.

Objective This study was conducted to examine whether sensory neuropeptides are involved in the IL-8-induced bronchial hyperresponsiveness.

Methods IL-8 at a dose of 5 µg/kg was administered intranasally to guinea-pigs twice a week for 3 weeks. One day after the last administration, animals were anesthetized and artificially ventilated through tracheal cannula, and lateral pressure at the tracheal cannula (Pao) was measured as an overall index of airway responses to increasing concentrations of inhaled histamine (25, 50, 100, and 200 µg/mL). A NK1 and NK2 dual antagonist FK224 (10 mg/kg), a selective NK1 antagonist FK888 (10 mg/kg) or vehicle was intravenously administered 10 min before measurement of bronchial responsiveness.

Results The IL-8 treatment significantly enhanced bronchial responsiveness to histamine (ANOVA $P < 0.01$). FK224 or FK888 did not alter the IL-8-induced bronchial hyperresponsiveness.

Conclusion We conclude that repeated intranasal administration of IL-8 causes bronchial hyperresponsiveness (BHR) and that neuropeptides such as neurokinin A and substance P do not directly contribute to the development of BHR induced by IL-8.

Keywords: bronchial hyperresponsiveness, guinea pigs, interleukin-8, NK1 receptors, NK2 receptors, sensory neuropeptides

Clinical and Experimental Allergy, Vol. 26, pp. 357–362. Submitted 6 April 1995; revised 16 June 1995; accepted 14 September 1995.

Bronchial hyperresponsiveness (BHR) and chronic airway inflammation have been proposed to be the fundamental feature of bronchial asthma. Airway sensory C-fibres provide an afferent limb for central neural reflex control

of airway function and also synthesize and store neuropeptides, such as substance P (SP), neurokinin A (NKA) and calcitonin gene-related peptide (CGRP), in granules found within their sensitive terminal varicosities. It has been shown that SP or NKA causes bronchoconstriction in animals [1] and in human asthmatic subjects [2–4] and potentiates bronchial responsiveness in animals [5–8].

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Interleukin-8 (IL-8) was purified from LPS-stimulated human peripheral blood mononuclear cell culture supernatants [9]. IL-8 is known as a potent chemoattractant and activating factor for neutrophils, and also a chemoattractant for T-lymphocytes [10,11]. However, recent studies have shown that IL-8 also triggers histamine and leukotriene release from basophils [12], induces contraction of airway smooth muscle [13] and causes eosinophil infiltration *in vivo* [14]. In addition, it has been demonstrated that bronchial epithelial cells and eosinophils stimulated with calcium ionophores can produce IL-8 *in vitro* [15,16], suggesting an important role for IL-8 in the genesis and persistence of bronchial inflammation in asthma. Our preliminary study showed the elevated level of IL-8 in sputum obtained from asthmatic patients, especially during asthma attacks (unpublished data).

Recently, we have shown that repeated intranasal administration of IL-8 (twice a week for 3 weeks) induced bronchial hyperresponsiveness in guinea-pigs *in vivo* [17]. This study was conducted to examine whether sensory neuropeptides are involved in the IL-8-induced bronchial hyperresponsiveness. We assessed the effect of a NK1 and NK2 dual antagonist FK224 [18] and a selective NK1 antagonist FK888 [19] on the development of BHR in the IL-8-treated guinea-pigs. We recently observed that 10 mg/kg of FK224 but not FK888 could inhibit bronchial hyperresponsiveness induced by both allergic bronchoconstriction and capsaicin inhalation when these compounds were intravenously administered 5 min before the inhalation of antigen and capsaicin (unpublished data).

Materials and methods

Animals

Male, albino, Hartley strain guinea-pigs weighing 350–400 g were obtained from Sankyo Laboratory Service (Toyama, Japan). After arrival at the Institute of Animal Experiments in our university, they were kept in conventional animal housing facilities for 1 week before the start of treatment with IL-8. They were allowed to drink and feed *ad libitum*.

Experimental protocols

Guinea-pigs underwent intranasal administration of 5 µg/kg of IL-8 ($n = 24$) or phosphate buffered saline (PBS) buffer (control; $n = 8$) twice a week for 3 weeks. Bronchial responsiveness was assessed 24 h after the last administration of IL-8. The 24 animals treated with IL-8 were divided into following three groups: treated with

10 mg/kg of FK224 ($n = 8$), 10 mg/kg of FK888 ($n = 8$) and vehicle (DMSO) ($n = 8$) intravenously 10 min before measurement of bronchial responsiveness. FK224 and FK888 were dissolved with DMSO to make concentration of 10 mg/mL.

Treatment with IL-8

IL-8 was dissolved in PBS at a concentration of 5 µg/mL. We administered 1 mL/kg of the IL-8 solution or PBS buffer alone intranasally twice a week for 3 weeks. Endotoxin content of the IL-8 solution was not detectable, being less than 50 pg/mg protein (Phrodick Limulus Test kit, Seikagaku Kogyo, Tokyo) [20].

Assessment of bronchial responsiveness

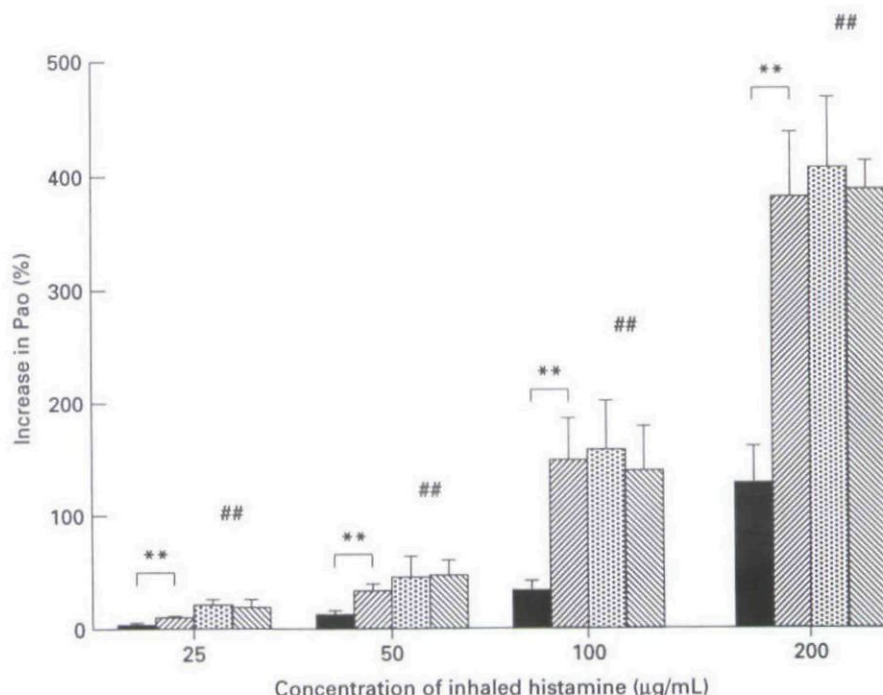
Preparation of animals One day after the last administration of IL-8, guinea-pigs were anesthetized with sodium pentobarbital (75 mg/kg ip). They were placed in the supine position. The trachea was cannulated with a polyethylene tube (outside diameter 2.5 mm, inside diameter 2.1 mm) and the left jugular vein was cannulated for administration of drugs.

After surgery the guinea-pig was artificially ventilated by a small animal respiratory pump (Model 1680, Harvard Apparatus Co., Inc., South Natick, MA, USA) adjusted to a tidal volume of 10 mL/kg at a rate of 60 strokes/min. The change in lung resistance to inflation, the lateral pressure of the tracheal tube (pressure at the airway opening; Pao) (cmH₂O), was measured by the modified method of Konzett and Rossler [21] as described by Jones *et al.* [22] (Model TP-603T, Nihon Koden Kogyo Co., Ltd, Tokyo, Japan). Since the change in Pao following inhalation of leukotriene C₄ (LTC₄) represented the average of the changes in pulmonary resistance (R_L) and reciprocal dynamic lung compliance ($1/C_{dyn}$) [23], we have used Pao as an overall index of bronchial response to bronchoactive agents.

When all procedures were completed, the animals received twice the tidal volume for two breaths by clamping the outlet port of the respirator to unify the volume history of the lung [23].

Histamine provocation After Pao had stabilized, increasing doses of histamine (25, 50, 100 and 200 µg/mL) were inhaled at 5 min intervals under continuous ventilation. The aerosol was generated during a 20 s period by an ultrasonic nebulizer developed for small animals at our institution. The amount of aerosol was 15.2 µL/min, and 46.4% of the aerosol was deposited in the lung as measured by radio-aerosol technique [24]. All of the histamine inhalation were completed within 60 min.

Fig. 1. Effects of a NK1 selective antagonist FK888 and a NK1 and NK2 dual antagonist FK224 on bronchial hyperresponsiveness to inhaled histamine induced by intranasal administration of 5 µg/kg of IL-8 twice a week for 3 weeks. FK888 and FK224 was intravenously administered in a dose of 10 mg/kg 10 min before starting histamine inhalation. # $P < 0.05$ and ## $P < 0.01$ among four groups with non-parametric ANOVA and ** $P < 0.01$ compared with PBS-treated animals using Mann-Whitney *U*-test. PBS-DMSO. Pretreatment with DMSO in animals treated with repeated intranasal administration of PBS (baseline control) (●). IL-8-DMSO. Pretreatment with DMSO in animals treated with repeated intranasal administration of IL-8 (control) (▨). IL-8-FK224. Pretreatment with 10 mg/kg of FK224 in animals treated with repeated intranasal administration of IL-8 (□), IL-8-FK888. Pretreatment with 10 mg/kg of FK888 in animals treated with repeated intranasal administration of IL-8 (▤). IL-8-FK888. Pretreatment with 10 mg/kg of FK888 in animals treated with repeated intranasal administration of 10 mg/kg of FK888 (▥).



Effects of FK224 and FK888 on bronchoconstriction induced by neurokinin A and substance P

To confirm the neuropeptide antagonist activity of FK224 and FK888 in this animal system *in vivo*, 10^{-6} M/kg of neurokinin A or 10^{-5} M/kg of substance P was intravenously injected 10 min after intravenous administration of 1 and 10 mg/kg of FK224, 1 and 10 mg/kg of FK888, or DMSO. Five animals were used for each group.

Statistical analysis

Dose-response curves for histamine-induced bronchoconstriction in the various treatment groups were compared by non-parametric ANOVA (Kruskal-Wallis test). Bronchoconstrictor responses to each individual concentration of histamine were compared using the Mann-Whitney *U*-test. Bronchoconstriction induced by neurokinin A or substance P was also compared between treatments with FK224 or FK888 and DMSO using the Mann-Whitney *U*-test. A *P*-value of 0.05 or less was considered significant. All results were expressed as mean $\pm$ standard error of the mean.

Chemicals

These following chemicals were used: sodium pentobarbital

(Abbot Laboratories, North Chicago, IL, USA), histamine (Wako Pure Chemical Ind., Osaka, Japan), DMSO (Wako Pure Chemical Ind., Osaka, Japan), FK888 (N2-[(4R)-4-hydroxy-1-(1-methyl-1H-indol-3-yl) carbonyl-L-prolyl]-N-methyl-N-phenylmethyl-3-(2-naphthyl)-L-alaninamide), FK224 (N-[N2-[N-[N-[N-[2,3-didehydro-N-methyl-N-[N-[3-(2-pentylphenyl)-propionyl]-L-threnonyl]tyrosyl]-L-leucynyl]-D-phenylanyl]-L-allo-threonyl]-L-asparaginyll]-L-serine-n-lactone) (Fujisawa Pharmaceutical Co., Ltd, Osaka, Japan). Human recombinant interleukin-8 (IL-8) was prepared as previously reported [20,25].

Results

The Pao values before inhalation of histamine were 10.2 ± 0.3 and 10.1 ± 0.3 cm H₂O in DMSO-administered animals treated with 5 µg/kg of IL-8 and PBS buffer, respectively. There was no significant difference between these groups. Dose-response curves of percentage increases in Pao by inhaled histamine in anesthetized and artificially ventilated guinea-pigs treated with IL-8 and PBS buffer are shown in Fig. 1. The IL-8 treatment significantly enhanced the airway responses to inhaled histamine when compared with PBS-treated control animals.

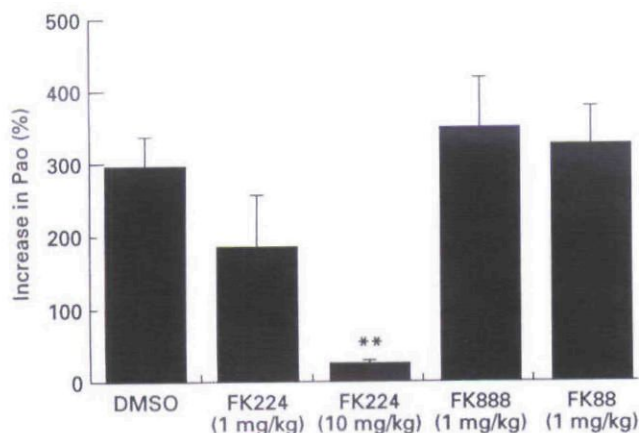


Fig. 2. Effect of intravenous administration of FK224 and FK888 on bronchoconstriction induced by intravenous administration of neurokinin A (10^{-6} M/kg). ** $P < 0.01$ compared with DMSO-treated animals using Mann-Whitney *U*-test.

The Pao values before histamine provocation were 10.3 ± 0.5 and 10.1 ± 0.4 cm H₂O in the IL-8-administered animals treated with 10 mg/kg of FK224 and 10 mg/kg of FK888, respectively. These values were not different from the value of the PBS-administered and DMSO-treated animals. Treatment with FK224 or FK888 did not significantly alter the increased bronchial responses to inhaled histamine compared with treatment with DMSO in animals treated with IL-8 (Fig. 1).

Percentage increase in Pao caused by NKA was

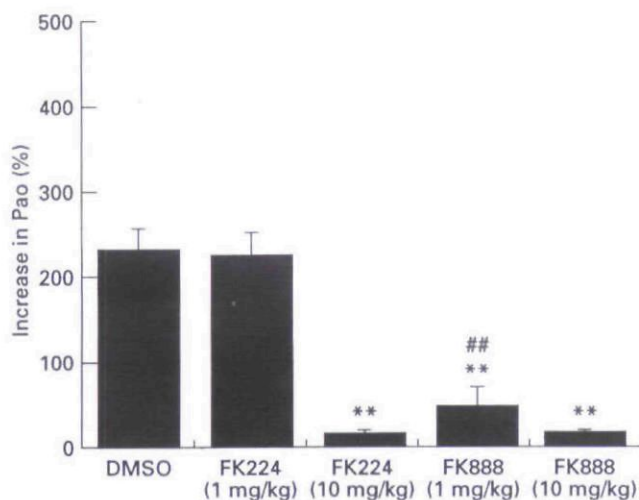


Fig. 3. Effect of intravenous administration of FK224 and FK888 on bronchoconstriction induced by intravenous administration of substance P (10^{-5} M/kg). ** $P < 0.01$ compared with DMSO-treated animals and ## $P < 0.02$ compared with 1 mg/kg of FK224-treated animals using Mann-Whitney *U*-test.

significantly inhibited by treatment with FK224 in a dose-dependent manner but not with FK888 (Fig. 2). On the other hand, percentage increase in Pao produced by SP was significantly reduced by treatment with FK888 and FK224 (Fig. 3). The value with 1 mg/kg of FK888 was significantly less than that with 1 mg/kg of FK224.

Discussion

In this study 3-week intranasal administration of IL-8 enhanced bronchial responsiveness to inhaled histamine and a NK1 and NK2 dual antagonist FK224 or a selective NK1 antagonist FK888 did not reduce the increased bronchial responsiveness induced by IL-8.

Although we did not examine airway inflammatory cells using bronchoalveolar lavage or histology in this study, we previously showed that the IL-8-induced bronchial hyperresponsiveness was accompanied by increased neutrophils, but not eosinophils, in both lavage fluid and bronchial tissues [17]. A significant correlation between neutrophil inflammation and bronchial hyperresponsiveness has been shown in animal studies [26–28], while Smith *et al.* [29] have reported that administration of IL-8 ($10 \mu\text{g}$) to guinea-pigs by the intraperitoneal route induced an increase in T-lymphocytes (maximal at 4 h) and eosinophils (maximal at 24 h) in bronchoalveolar lavage (BAL) fluid. They have also reported that a single inhalation of IL-8 ($50 \mu\text{g}$) caused an increase in the numbers of eosinophils, but not lymphocytes, in BAL fluid as compared with the control vehicle (0.25% BSA). Neither single intraperitoneal nor aerosolized administration of IL-8 induced BHR to intravenously administered histamine. Accordingly it has been controversial that IL-8 accumulates neutrophils into the airways and produced bronchial hyperresponsiveness. We do not know the reason why bronchial responsiveness was enhanced by IL-8 in our study but not in Smith *et al.*'s study [29]. Single administration of IL-8 in Smith *et al.*'s study and repeated administration in our study may account for the discrepancy. We administered IL-8 repeatedly because we hypothesized that repeated and/or continuous production of IL-8 in the airways might contribute to the BHR seen in stable asthmatics.

FK224 is an antagonist for NK1 and NK2 receptors [18] and FK888 is a selective antagonist for NK1 receptors [19]. Fujii *et al.* [30] have shown that FK888 preferentially inhibits SP (10^{-8} M)-induced contraction with an IC₅₀ value of 3.2×10^{-8} M compared with a value of 3.8×10^{-6} M for NKA (10^{-9} M)-induced contraction of guinea-pig isolated trachea. They [31] have also shown that FK888 and FK224 displace ³H-SP (10^{-9} M) binding to guinea-pig lung membrane preparation with IC₅₀ values of 2.7×10^{-9} M and 1×10^{-7} M,

respectively. FK888 inhibits SP-induced contraction of guinea-pig ileum with an IC_{50} value of 5.4×10^{-10} M but the value of FK224 is 3.7×10^{-8} M. On the other hand, the IC_{50} values of FK888 and FK224 against contraction of rat vas deferens by NKA are 1.1×10^{-5} M and 7.2×10^{-8} M. These data shows that FK888 is a very potent and selective inhibitor of the NK1 receptors and FK224 is an equipotent inhibitor of NK1 and NK2 receptors *in vitro*. In *in vivo* experiments, intravenous administration of FK224 and FK888 inhibits SP (13.5 µg/kg, iv)-, NKA (1.1 µg/kg, iv)- and capsaicin (3.1 µg/kg, iv)-induced plasma extravasation of guinea-pig airways with ED_{50} values of 0.14 and 0.011 mg/kg, 0.29 and 0.0063 mg/kg and 0.30 and 0.01 mg/kg, respectively [30]. Hirayama *et al.* [32] have also reported that FK888 is only 5–10 times more potent than FK224 in inhibiting SP-induced plasma exudation in guinea-pigs *in vivo*. We assessed activities of FK224 and FK888 as the neuropeptide antagonist in our animal system. FK888 strongly and dose-dependently inhibited bronchoconstriction induced by NKA but not by SP. On the other hand, FK224 inhibited both NKA- and SP-induced bronchoconstriction in a dose-dependent manner. These findings ensure that FK224 and FK888 is a NK1 and NK2 dual antagonist and a selective NK1 antagonist, respectively, and indicate that the single dose (10 mg/kg) of FK224 and FK888 used in this study is appropriate and enough to examine the involvement of neuropeptides in IL-8 induced bronchial hyperresponsiveness. Although we did not measure heart rate or blood pressure in this study, it has been shown that 10 mg/kg of FK224 has no cardiovascular effects [33] and 10 mg/kg of FK888 has transient hypotensive effect within 5 min when these are intravenously administered to guinea-pigs (pers. comm. from Karino M, Department of Pharmacology, Fujisawa Pharmaceutical Co., Ltd, Osaka, Japan).

In this study neither FK224 nor FK888 in a dose of 10 mg/kg inhibited the development of BHR induced by IL-8. It has been shown that substance P or NKA causes bronchoconstriction in animals [1] and in human asthmatic subjects [2–4] and potentiates bronchial responsiveness in animals [5–8]. Tamura *et al.* [6] reported that bronchoconstrictor responses to inhaled methacholine were augmented in Japanese monkeys for at least 4 weeks after a single 2 min exposure to NKA aerosol, while no such change was evident after similar exposure to SP. Sheppard and Scypinsky [7] demonstrated that tachykinins are involved in the development of bronchial hyperresponsiveness following toluene diisocyanate exposure in guinea-pigs. Omini *et al.* [8] showed that SP potentiates acetylcholine-induced bronchoconstriction in guinea-pigs and this effect is abolished by

vagotomy. We also investigated that inhaled capsaicin and antigen-induced bronchial hyperresponsiveness was significantly inhibited by pretreatment with 0.1 and 1.0 mg/kg of FK224 in a dose-dependent manner, but not with 1.0 mg/kg of FK888 in guinea-pigs (unpublished data). Together, we think that if neuropeptides released from bronchial C-fibres potentiate bronchial responsiveness NKA might be a major contributor to the development of bronchial hyperresponsiveness. However, our results that sufficient dose of FK224 or FK888 did not reduce bronchial hyperresponsiveness induced by repeated intranasal administration of IL-8 indicate no direct involvement of neuropeptides in the IL-8-induced bronchial hyperresponsiveness in guinea-pigs. To clarify the involvement of neuropeptides in development of bronchial hyperresponsiveness in human asthmatics further studies are needed in which effects of selective antagonists for SP or NKA such as FK224 and FK888 on bronchial hyperresponsiveness in asthmatics should be examined.

Acknowledgment

This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture (04807055) by the Japanese Government. We wish to thank Fujisawa Pharmaceutical Co., Ltd, Osaka, Japan, for kindly supplying FK224 or FK888.

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