

IL-1 β -Induced Transcriptional Up-Regulation of Bradykinin B₁ and B₂ Receptors in Murine Airways

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Hyperresponsiveness to bronchoconstrictor stimuli is a major pathophysiologic feature of asthma, but the molecular mechanisms behind this are not fully understood. The release of TNF- α and IL-1 β during the inflammatory process is believed to play an important role in airway hyperresponsiveness. We have previously demonstrated, using a murine *in vitro* model of chronic airway inflammation, that TNF- α up-regulated bradykinin B₁ and B₂ receptors in the airway smooth muscle. By using the same model, the present study was designed to investigate the effects of IL-1 β and its interaction with TNF- α on the expression of bradykinin B₁ and B₂ receptors in mouse tracheal smooth muscle. IL-1 β up-regulated bradykinin B₁ and B₂ receptor expression and increased contractile response to bradykinin B₁ and B₂ receptor agonists (des-Arg⁹-bradykinin and bradykinin, respectively) in the tracheal smooth muscle. Transcriptional inhibitor actinomycin D, c-Jun N-terminal kinase (JNK) inhibitors SP600125 and TAT-TI-JIP₁₅₃₋₁₆₃, but not extracellular signal-regulated kinase 1 and 2 (ERK 1/2) inhibitor PD98059, significantly attenuated this up-regulation, indicating that a transcriptional mechanism and intracellular JNK signal transduction pathway were involved. In addition, IL-1 β did not affect bradykinin B₁ and B₂ receptor mRNA stability. Remicade, an anti-TNF- α antibody, markedly suppressed IL-1 β -induced up-regulation of bradykinin B₁ and B₂ receptors, suggesting that TNF- α was involved in the up-regulation, which is further supported by the fact that IL-1 β enhanced TNF- α mRNA expression in the tracheae. Intracellular JNK pathway and TNF- α might provide key links between inflammatory mediators like IL-1 β and airway hyperresponsiveness to bradykinin.

Keywords: IL-1 β ; Remicade; bradykinin; receptor; JNK

Asthma is characterized by airway hyperresponsiveness. The pathologic process is believed to be closely linked to chronic airway inflammation (1). Proinflammatory mediator IL-1 β released from monocytes, macrophages, and airway epithelial cells has been shown to affect airway smooth muscle response to various contractile agonists like bradykinin (2, 3). Studies have demonstrated that in bronchoalveolar lavage fluids from patients with asthma, the level of IL-1 β was elevated in parallel with an increased expression of IL-1 β mRNA in macrophages (4, 5). Administration of IL-1 β increased the amount of neutrophils in bronchoalveolar lavage fluid and airway responsiveness to bradykinin in rat *in vivo* and in a human bronchial preparation

CLINICAL RELEVANCE

This article demonstrates how IL-1 β that appears in bronchoalveolar lavage from individuals with asthma, induces airway hyperresponsiveness via up-regulation of bradykinin B₁ and B₂ receptors. This process involves TNF- α and JNK, which might be of importance for the development of asthma.

in vitro (2, 6). Taking all together, these studies indicate that IL-1 β plays an important role in airway inflammation and the development of airway hyperresponsiveness.

Bradykinin is an important airway constrictor, formed from the kininogen precursor after proteolytic cleavage by kallikrein, which is further converted to des-Arg⁹-bradykinin by carboxypeptidases (7). The effects of different kinins are mediated by two G protein-coupled receptors termed bradykinin B₁ and B₂. Bradykinin B₂ receptors show a high affinity for bradykinin and kallidin, whereas bradykinin B₁ receptors show a high affinity for their metabolites, des-Arg⁹-bradykinin and des-Arg¹⁰-kallidin (8). Bradykinin is a weak constrictor of isolated human bronchi, but a potent bronchoconstrictor *in vivo*, in patients with asthma (9). The bradykinin B₂ receptor is constitutively expressed, whereas the bradykinin B₁ receptor is inducible (10, 11).

IL-1 β and TNF- α have close relationships during inflammation. Both of them are agonists for intracellular mitogen-activated protein kinase (MAPK) pathways that consist of three major intracellular signal pathways: c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase 1 and 2 (ERK 1/2), and p38 (12, 13). We have recently demonstrated, using a murine *in vitro* model of chronic airway inflammation, that TNF- α could up-regulate bradykinin B₁ and B₂ receptors in the tracheal smooth muscle via activation of the intracellular JNK and ERK 1/2 pathways (14). By using the same model, the present study was designed to investigate effects of IL-1 β on bradykinin B₁ and B₂ receptor expression in the airway smooth muscle, to study the underlying intracellular MAPK signal transduction mechanisms and to evaluate roles of TNF- α in this process.

MATERIAL AND METHODS

In Vitro Pharmacology

Tracheae from 9- to 10-wk-old male BALB/c J mice (MB A/S, Ry, Denmark) was dissected and cut into three to four segments and placed individually into wells of a 96-well plate (Ultra-low attachment; Sigma, St Louis, MO) with 300 μ l serum-free Dulbecco's Modified Eagle's Medium (DMEM) supplemented with penicillin (100 U/ml) and streptomycin (100 μ g/ml) (15). The segments were incubated for 1, 2, 4, or 8 d at 37°C in humidified 5% CO₂ in air in the absence and presence of murine IL-1 β (IL-1 β), murine TNF- α (mTNF- α), or human TNF- α (hTNF- α), with and without inhibitors (Remicade, actinomycin D, SP600125, TAT-TI-JIP₁₅₃₋₁₆₃, or PD98059). The segments were moved

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into new wells containing fresh medium with the supplements of the cytokines and inhibitors every day.

The cultured segments were immersed in temperature-controlled (37°C) myographs containing 5 ml Krebs-Henseleit buffer-solution continuously equilibrated with 5% CO₂ in O₂ to result in a stable pH of 7.4 for recording of isometric tension. A pre-tension of 0.8 mN was applied to each segment, which has been demonstrated to be optimal (15). The segments were incubated with 3 μ M indomethacin 30 min before administration of des-Arg⁹-bradykinin or bradykinin.

All data were expressed as mean values $\pm$ SEM. Each agonist concentration–effect curve was fitted to the Hill equation using an iterative, least square method (GraphPad Prism, San Diego, CA), to provide estimates of maximal contraction ($E_{\max}$) and pEC₅₀ values (negative logarithm of the agonist concentration that produces 50% of the maximal effect). Unpaired Student's *t* test or two-way ANOVA with Bonferroni post-test were used when two sets of data were compared, and one-way ANOVA with Dunnett's post-test was used for comparisons of more than two sets of data. $P \leq 0.05$ was considered to be statistically significant (n = number of experiments performed).

Chemicals

Recombinant murine IL-1 β , normal control IgG, murine and human TNF- α (R&D Systems, Abingdon, UK), bradykinin and des-Arg⁹-bradykinin (Neosystem S.A., Strasbourg, France), PD98059 (2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one), N-monomethyl-L-arginine (L-NMMA), actinomycin D, indomethacin, DMEM and Krebs-Henseleit buffer (Sigma), Remicade (Centocor B.V., Leiden, The Netherlands), SP600125 (anthrax(1,9-cd)pyrazol-6(2H)-one), and TAT-TI-JIP_{153–163} (Calbiochem, Bad Soden, Germany) were used. The vehicle for SP600125, TAT-TI-JIP_{153–163}, and PD98059 was dimethyl sulfoxide (DMSO).

mRNA Study

Tracheal smooth muscle strips or whole tracheae were homogenized and total RNA was extracted using RNeasy Mini-kit (QIAGEN GmbH, Hilden, Germany). Reverse transcription of total RNA (absorption ratio 1.6–2.0) to cDNA was performed using Omniscript reverse-transcriptase-kit (QIAGEN) in 20 μ l volume reaction at 37°C for 1 h.

Real-time polymerase chain reaction (real-time PCR) was performed with QuantiTect SYBR Green PCR-kit (QIAGEN) in 25- μ l reaction volumes and performed with heating 95°C for 15 min followed by touch down PCR—that is, denaturing at 94°C for 30 s and annealing at 66°C for the first PCR cycle; thereafter, a decrease of 2°C for the annealing temperature in every cycle until down to 56°C. Finally, 40 thermal cycles with 94°C for 30 s and 55°C for 1 min were performed (Smart Cycler II system; Cepheid, Sunnyvale, CA). The data were analyzed with the threshold cycle (Cr) method, and the specificity of the PCR products were checked by the dissociation curves.

Specific primers were synthesized by DNA Technology A/S (Aarhus, Denmark). The sequences are as follows. Bradykinin B₁ receptor: forward, 5'-CCA TAG CAG AAA TCT ACC TGG CTA AC-3'; reverse, 5'-GCC AGT TGA AAC GGT TCC-3'. Bradykinin B₂ receptor: forward, 5'-ATG TTC AAC GTC ACC ACA CAA GTC-3'; reverse, 5'-TGG ATG GCA TTG AGC CAA C-3'. TNF- α : forward, 5'-GAC TCA AAT GGG CTT TCC GA-3'; reverse, 5'-TCC AGC CTC ATT CTG AGA CAG AG-3'. β -actin: forward, 5'-TGG GTC AGA AGG ACT CCT ATG TG-3'; reverse, 5'-CGT CCC AGT TGG TAA CAA TGC-3'.

The relative amount of mRNA was obtained by the Cr values of mRNA for bradykinin B₁ or B₂ receptor or TNF- α in relation to the Cr values of mRNA for the housekeeping gene β -actin in the same sample. Data are presented as mean $\pm$ SEM. Statistical analyses were performed with unpaired Student's *t* test, and $P \leq 0.05$ was considered to be significant.

Western Blot

After the organ culture, the tracheae were homogenized in SDS sample buffer (0.5 M Tris pH 6.8, glycerol, Coomassie blue, 20% SDS, 0.05 M DTT, 0.1 M PSMF). After the homogenization, the samples were heated at 95–100°C for 5 min and centrifuged at 10,000 rpm for 10 min. The total protein was measured by Advanced Protein Assay Kit (Sigma-Aldrich Fluka, Buchs, Switzerland). Equal amounts of the protein were loaded onto NuPAGE Bis-Tris 4–12% gel (Invitrogen, Carlsbad, CA) and separated by electrophoresis (Mini vertical gel system; Thermo EC, Waltham, MA).

The blot was transferred to Immobilon-P PVDF membranes (Millipore, Billerica, MA) and blocked in Tris-buffered saline (10 mM Tris-HCl pH 7.4, 0.9% NaCl) containing 5% milk for 1 h at room temperature, then incubated with primary antibody against mouse bradykinin B₂ receptor (1: 2,000; Alexis Biochemicals, Lausen, Switzerland) overnight at 4°C, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody (20 ng/ml) for 2 h at room temperature in Tris-buffered saline containing 5% milk. The immunoreactive signals were visualized by using SuperSignal West Pico chemiluminescent substrate (Pierce Biotechnology, Rockford, IL) and detected by using MAN-X X-ray system (Fuji-film Science Imaging Systems, Tokyo, Japan).

RESULTS

IL-1 β Increased des-Arg⁹-Bradykinin- and Bradykinin-Induced Contraction and Up-Regulated Bradykinin B₁ and B₂ Receptor Expression

The tracheal segments were cultured for 4 d with different concentrations of IL-1 β (0.1, 1, 10, or 100 ng/ml). An increase of the contractile responses to des-Arg⁹-bradykinin and bradykinin was observed. This increase was concentration-dependent on IL-1 β (Table 1). The maximal contractile response for both agonists was reached with 10 ng/ml IL-1 β . The potency for the agonist-induced contraction also increased with IL-1 β concentrations, and reached statistical significance at 10 ng/ml for both agonists.

To assess time-course of IL-1 β , tracheal segments were cultured for 1, 2, 4, or 8 d in the absence and presence of IL-1 β (10 ng/ml). At Day 1, a significant increase of the response induced by des-Arg⁹-bradykinin and bradykinin in presence of IL-1 β was seen. The maximal contractions for des-Arg⁹-bradykinin were increased at the similar level as after 2, 4, and 8 d of culture (Figures 1A, 1C, 1E, and G), whereas bradykinin-induced contraction reached the maximum at 4 d of culture and then, after 8 d, returned toward the same level as Day 1 (Figures 1B, 1D, 1F, and 1H). A general increase in potency was also seen during the culture periods, reaching statistical significance at 1 and 2 d for des-Arg⁹-bradykinin (Figures 1A, 1C, 1E, and 1G) and at 2 and 4 d for bradykinin (Figures 1B, 1D, 1F, and 1H).

The relative amount of mRNA for bradykinin B₁ and B₂ receptors was quantified after 4 d organ culture by real-time PCR. Compared with the control (organ culture without IL-1 β), mRNA levels of bradykinin B₁ and B₂ receptors were increased in the smooth muscle isolated from the tracheae treated by IL-1 β (Figures 2A and 2B, $P < 0.05$).

Protein expression for bradykinin B₂ receptors was examined by Western blot. There was a marked increase of bradykinin B₂ receptor protein expression in the trachea cultured for 4 d in

TABLE 1. CONCENTRATION EFFECTS OF IL-1 β ON DES-ARG⁹-BRADYKININ- AND BRADYKININ-INDUCED CONTRACTION

IL-1 β (ng/ml)	<i>n</i>	des-Arg ⁹ -Bradykinin		Bradykinin	
		$E_{\max}$ (mN)	pEC ₅₀	$E_{\max}$ (mN)	pEC ₅₀
0 (control)	18	0.89 $\pm$ 0.16	6.67 $\pm$ 0.17	13 1.93 $\pm$ 0.35	5.78 $\pm$ 0.19
0.1	7	0.79 $\pm$ 0.19	7.14 $\pm$ 0.32	6 2.78 $\pm$ 0.93	6.12 $\pm$ 0.30
1	9	2.01 $\pm$ 0.59*	6.81 $\pm$ 0.29	6 2.88 $\pm$ 0.54	6.38 $\pm$ 0.32
10	8	2.04 $\pm$ 0.22*	7.38 $\pm$ 0.11	12 4.37 $\pm$ 0.41*	7.43 $\pm$ 0.26*
100	5	1.60 $\pm$ 0.22	7.09 $\pm$ 0.35	6 4.92 $\pm$ 0.79*	7.38 $\pm$ 0.31

Definition of abbreviations: $E_{\max}$, maximal contraction; pEC₅₀, negative logarithm of the agonist concentration that produces 50% of the maximal effect.

des-Arg⁹-bradykinin- and bradykinin-induced $E_{\max}$ and pEC₅₀ values are presented. The tracheal segments were cultured for 4 d in the absence (control) and presence of different concentrations of IL-1 β (0.1, 1, 10, or 100 ng/ml). Data are expressed as mean $\pm$ SEM. Statistical analysis was performed by one-way ANOVA with Dunnett's post test.

* Control versus IL-1 β , $P < 0.05$ was considered to be significant (n = number of experiments performed).

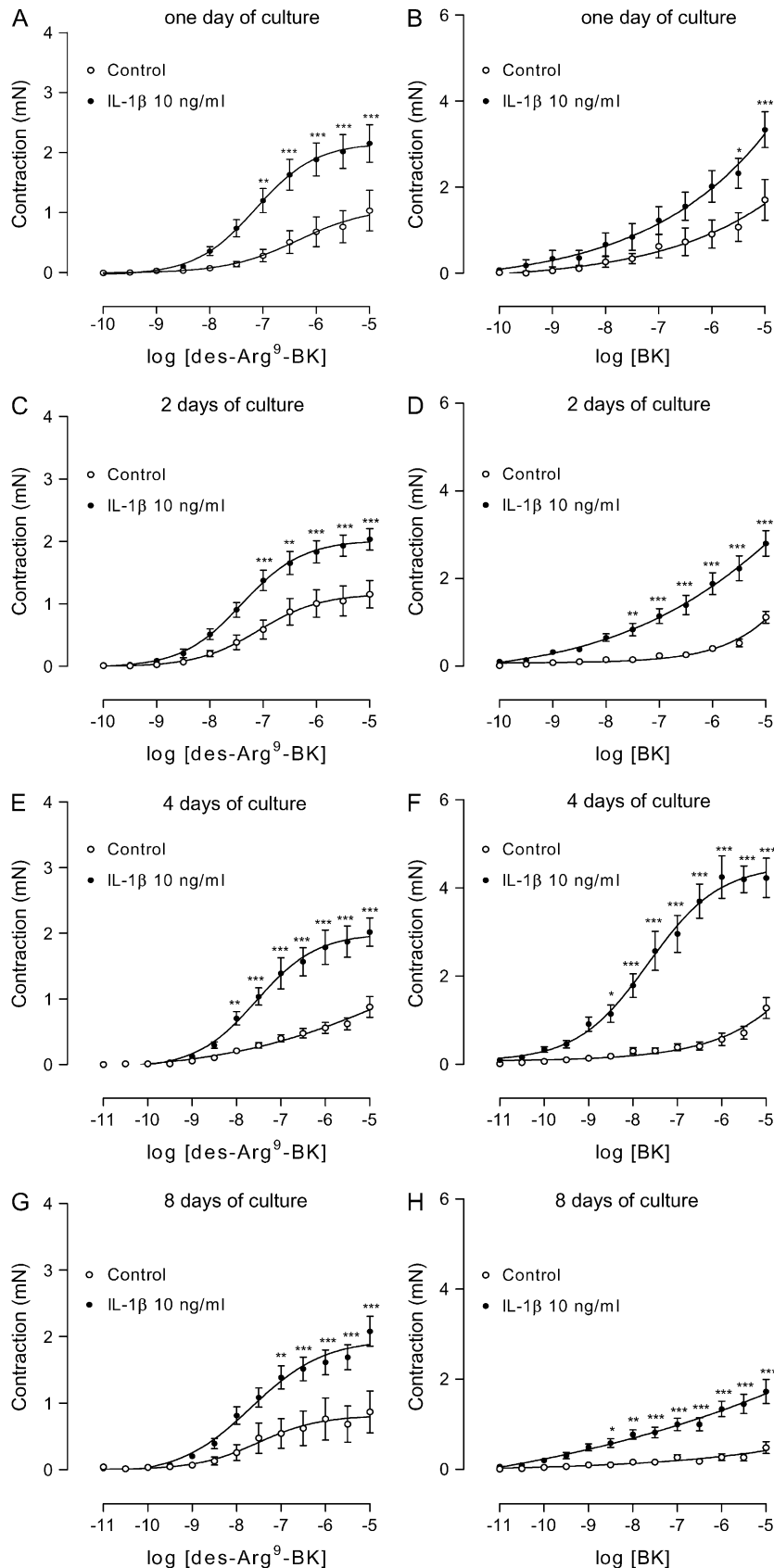


Figure 1. Time-course of IL-1 β on the contractile response to des-Arg⁹-bradykinin (A, C, E, G) and bradykinin (B, D, F, H). The tracheal segments were cultured for 1 d (A, B), 2 d (C, D), 4 d (E, F) and 8 d (G, H) in the absence (control) and presence of 10 ng/ml IL-1 β . Each data point is derived from 8–18 experiments and presented as mean $\pm$ SEM. Statistical analysis was performed by two-way ANOVA with Bonferroni post-test. Asterisks indicate control versus IL-1 β : * P < 0.05, ** P < 0.01, *** P < 0.001.

the presence of IL-1 β , compared with the control (without IL-1 β treatment) (Figure 2C).

IL-1 β (10 ng/ml) did not affect carbachol-induced the maximal contraction in the segments cultured for 1 d (control

$E_{\max} = 7.9 \pm 0.7$ versus IL-1 β $E_{\max} = 7.1 \pm 1.0$ mN, $n = 6$, $P > 0.05$), 2 d (control $E_{\max} = 6.5 \pm 0.7$ versus IL-1 β $E_{\max} = 6.6 \pm 0.7$ mN, $n = 7$, $P > 0.05$), 4 d (control $E_{\max} = 6.1 \pm 0.4$ versus IL-1 β $E_{\max} = 6.3 \pm 1.0$ mN, $n = 6$, $P > 0.05$), and 8 d (control

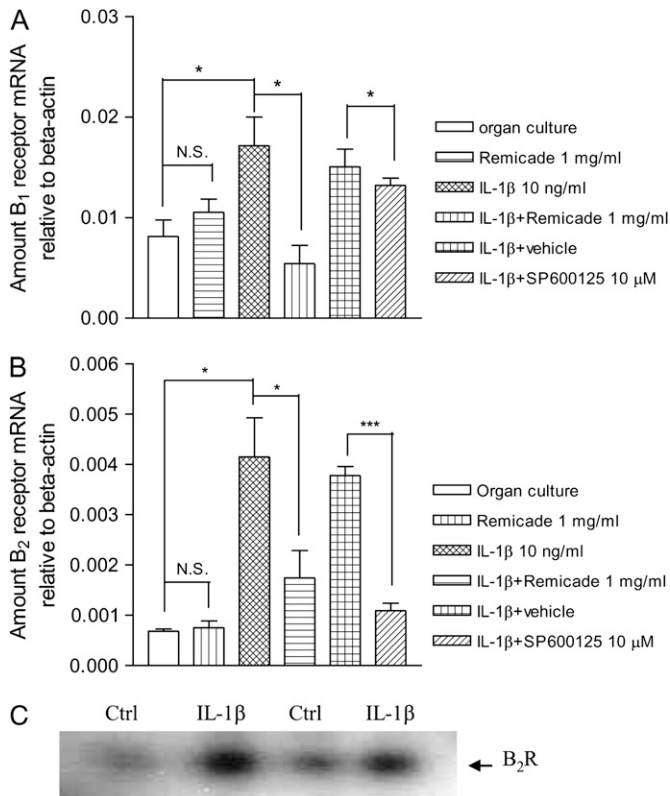


Figure 2. Bradykinin B₁ (A) and B₂ receptor (B) mRNA expression in the tracheal smooth muscle. The tracheae were cultured for 4 d in the absence (organ culture) and presence of 10 ng/ml IL-1β, with and without 1 mg/ml Remicade or 10 μM SP600125 or vehicle (DMSO). Each data point is derived from three to four experiments and presented as mean ± SEM. Unpaired Student's *t* test was used. Organ culture versus Remicade or IL-1β; IL-1β versus IL-1β + Remicade; IL-1β + vehicle versus IL-1β + SP600125, N.S. = not significant; **P* < 0.05 and ****P* < 0.001. (C) Western blot demonstrated an increase in bradykinin B₂ receptor protein expression. The tracheae were cultured for 4 d in the absence (control) and presence of IL-1β (10 ng/ml). Three identical experiments were performed. Each sample was derived from two mice. Lanes 1 and 3, control (Ctrl); lanes 2 and 4, IL-1β (10 ng/ml).

$E_{\max} = 3.6 \pm 0.5$ versus IL-1β $E_{\max} = 3.5 \pm 0.5$ mN, $n = 12$, $P > 0.05$).

IL-1β Enhanced Transcription of Bradykinin B₁ and B₂ Receptors

To investigate transcriptional mechanisms, actinomycin D (a general transcriptional inhibitor) was used. The results showed that actinomycin D almost completely abolished IL-1β-enhanced contractile responses to des-Arg⁹-bradykinin and bradykinin (Figures 3A and 3B, $P < 0.001$). Accordingly, the mRNA levels were decreased after treatment with actinomycin D, but the receptor mRNA expression for either bradykinin B₁ or B₂ receptors mRNA was not altered by IL-1β (Figures 3C and 3D). Thus, the up-regulation of bradykinin B₁ and B₂ receptor mRNA expression by IL-1β was not mediated through an increase of receptor mRNA stability.

IL-1β Up-Regulated Bradykinin B₁ and B₂ Receptor Expression via JNK Pathway

Inhibitors for intracellular JNK (SP600125) and ERK 1/2 (PD98059) were used to investigate if intracellular JNK and

ERK 1/2 signal transduction pathways were required for the up-regulation of bradykinin B₁ and B₂ receptors by IL-1β. Tracheal segments were cultured for 4 d with IL-1β (10 ng/ml) together with either SP600125 (1, 10 μM), PD98059 (100 μM), or vehicle (DMSO). SP600125 at 10 μM almost completely abolished IL-1β-enhanced contraction induced by des-Arg⁹-bradykinin and bradykinin (Figures 4A and 4B, $P < 0.001$), while PD98059 had no effects (Figures 4A and 4B, $P > 0.05$). In addition, IL-1β-enhanced mRNA expression for bradykinin B₁ and B₂ receptors in the tracheal smooth muscle was significantly attenuated by SP600125 (Figures 2A and 2B, $P < 0.05$).

Since SP600125 is relatively specific to JNK pathway (16), verification of the results was assessed by using another highly specific JNK inhibitor TAT-TI-JIP₁₅₃₋₁₆₃, a small peptide and structurally unrelated to SP600125 (17). TAT-TI-JIP₁₅₃₋₁₆₃ significantly inhibited IL-1β-enhanced contractile response to des-Arg⁹-bradykinin and bradykinin (Figures 4A and 4B, $P < 0.05$), which agrees well with SP600125 and that the JNK pathway at least in part was involved.

Remicade Attenuated IL-1β-Enhanced the Expression of Bradykinin B₁ and B₂ Receptors

To ascertain if TNF-α was involved in IL-1β-enhanced the expression of bradykinin B₁ and B₂ receptors, the tracheal segments were cultured for 4 d with IL-1β (10 ng/ml) in the absence and presence of Remicade (0.1 and 1 mg/ml), a chimeric monoclonal anti-TNF-α antibody. Remicade inhibited IL-1β-enhanced bradykinin B₁ and B₂ receptor-mediated contraction (Figures 5A and 5B). The inhibition was more marked in des-Arg⁹-bradykinin-induced contraction (~90% reduction) than in bradykinin-induced contraction (~60% reduction). In the control experiment, normal control IgG did not affect the contractile responses to des-Arg⁹-bradykinin and bradykinin in the tracheal segments cultured for 4 d in the presence of IL-1β (Figures 5A and 5B).

Furthermore, Remicade (1 mg/ml) abolished IL-1β-enhanced bradykinin B₁ receptor mRNA expression by 100% and B₂ receptor mRNA expression by 70% in the tracheal smooth muscle (Figures 2A and 2B, $P < 0.05$).

However, in the tracheal segments without IL-1β treatment, Remicade (1 mg/ml) had no inhibitory effects on des-Arg⁹-bradykinin-induced contraction (control $E_{\max} = 1.03 \pm 0.23$ versus Remicade $E_{\max} = 1.83 \pm 0.36$ mN, $n = 5$, $P > 0.05$) and bradykinin-induced contraction (control $E_{\max} = 1.93 \pm 0.35$ versus Remicade $E_{\max} = 2.28 \pm 0.49$ mN, $n = 5$, $P > 0.05$). In the same way, Remicade did not inhibit bradykinin B₁ and B₂ receptor mRNA expression (Figures 2A and 2B).

Remicade Attenuated both mTNF-α- and hTNF-α-Enhanced Bradykinin B₁ and B₂ Receptor-Mediated Contraction and the Receptor mRNA Expression

Remicade (0.1, 1 mg/ml) concentration-dependently decreased bradykinin B₁ and B₂ receptor-mediated contraction in the segments cultured for 4 d in the presence of mTNF-α (100 ng/ml) (Figure 5C-D). To confirm the results from mTNF-α, effects of Remicade on hTNF-α were tested. Remicade (1 mg/ml) almost totally revoked the hTNF-α-enhanced bradykinin B₁ and B₂ receptor-mediated contraction (Figure 5E-F). The inhibitory effect of Remicade was stronger in the hTNF-α than in the mTNF-α group, indicating that Remicade may have stronger affinity for hTNF-α than for mTNF-α.

In addition, Remicade 1 mg/ml significantly suppressed both mTNF-α- and hTNF-α-enhanced bradykinin B₁ and B₂ receptor mRNA expression in the tracheal smooth muscle (Figures 6A and 6B).

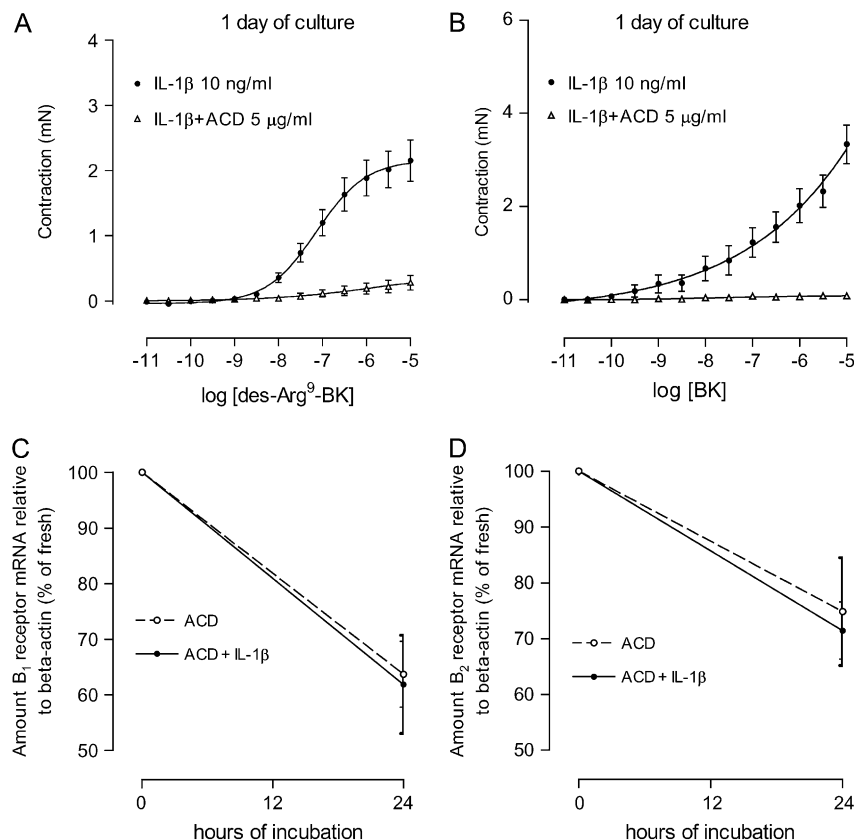


Figure 3. Effects of actinomycin D on des-Arg⁹-bradykinin (A)- and bradykinin (B)-induced contraction, and of IL-1β on bradykinin B₁ (C) and B₂ (D) receptor mRNA stability. The tracheae were cultured in the absence and presence of 10 ng/ml IL-1β with 5 μg/ml actinomycin D (ACD) for 24 h. Actinomycin D was added to the culture 30 min before IL-1β administration. Each data point is derived from 5–11 (for contraction) and 3–4 (for mRNA stability) experiments and presented as mean ± SEM.

IL-1β Increased TNF-α mRNA Expression

To further investigate interactions between IL-1β and TNF-α, TNF-α mRNA expression was quantified by real-time PCR. The results showed that IL-1β (10 ng/ml) increased TNF-α mRNA expression in the whole tracheae with epithelium (Figure 7). Similarly, IL-1β (10 ng/ml) increased TNF-α mRNA expression in the tracheal smooth muscle; however, the level of TNF-α mRNA expression in the tracheal smooth muscle was much lower than in the whole tracheae with epithelium (Figure 7).

L-NMMA Did Not Affect IL-1β-Enhanced Bradykinin B₁ and B₂ Receptor-Mediated Contraction

To test if nitric oxide (NO) production was involved in our system, the NOS inhibitor L-NMMA (100 μM) was added to

the tissue bath 30 min before the administration of des-Arg⁹-bradykinin and bradykinin. There were no significant difference between L-NMMA and control groups in the segments cultured for 4 d with IL-1β (10 ng/ml), for des-Arg⁹-bradykinin (control group E_{max} 2.05 ± 0.55 and pEC_{50} 7.83 ± 0.19 versus L-NMMA group E_{max} 3.01 ± 0.33 and pEC_{50} 7.73 ± 0.25, n = 5, P > 0.05) and for bradykinin (control group E_{max} 4.59 ± 0.39 and pEC_{50} 7.45 ± 0.19 versus L-NMMA group E_{max} 4.97 ± 0.44 and pEC_{50} 7.51 ± 0.09, n = 5, P > 0.05).

DISCUSSION

The inflammatory mediator TNF-α, IL-1β, and bradykinin play critical roles in asthmatic inflammation, but the interactions

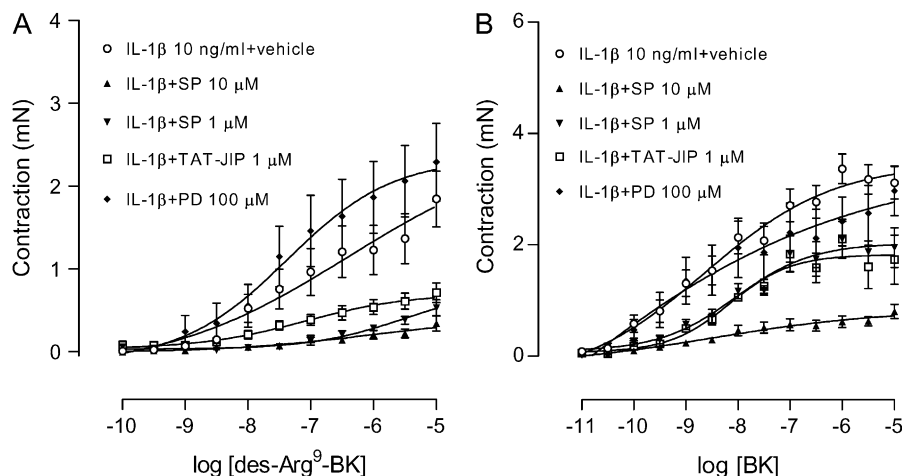


Figure 4. Effects of SP600125, TAT-TI-JIP₁₅₃₋₁₆₃ and PD98059 on IL-1β-enhanced contractile responses to des-Arg⁹-bradykinin (A) and bradykinin (B). The tracheal segments were cultured for 4 d in the presence of 10 ng/ml IL-1β with and without 10 μM SP600125 (SP), 1 μM TAT-TI-JIP₁₅₃₋₁₆₃ (TAT-JIP), 100 μM PD98059 (PD), or vehicle (DMSO). Each data point is derived from 6–14 experiments and presented as mean ± SEM.

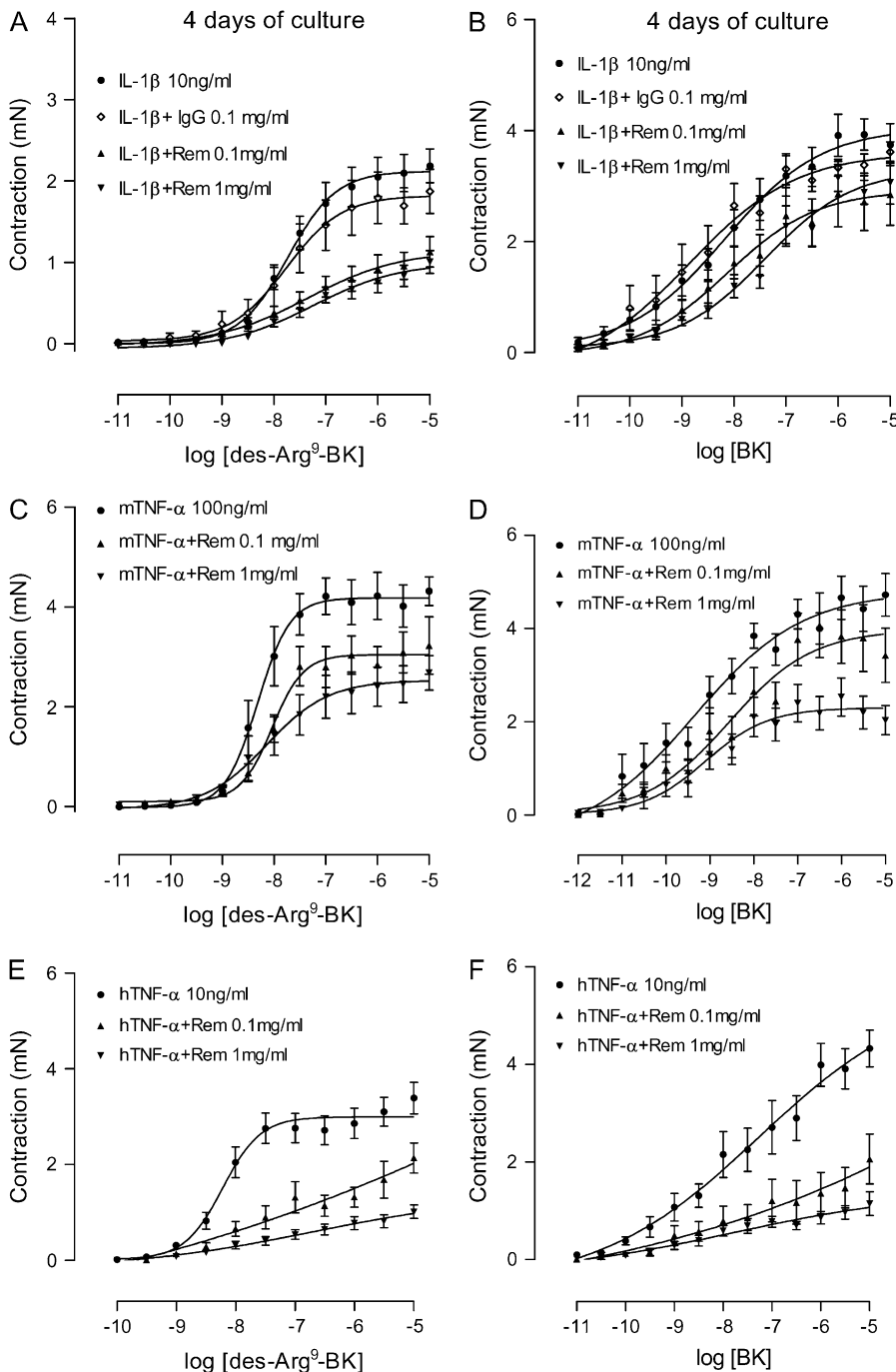


Figure 5. Effects of Remicade on IL-1 β (A, B)-, mTNF- α (C, D)-, and hTNF- α (E, F)-enhanced contractile response to des-Arg⁹-bradykinin (A, C, E) and bradykinin (B, D, F). The tracheal segments were cultured for 4 d in the presence of 10 ng/ml IL-1 β , 100 ng/ml mTNF- α , or 10 ng/ml hTNF- α , with and without Remicade (0.1, 1 mg/ml) (Rem) or normal control IgG (0.1 mg/ml) (IgG). Each data point is derived from 7–18 experiments and presented as mean $\pm$ SEM.

between these mediators are far from known (1). We have developed an *in vitro* model of airway inflammation, and demonstrated that TNF- α up-regulated the expression of bradykinin B₁ and B₂ receptors in the airway smooth muscle and that intracellular JNK and ERK 1/2 pathways were involved in this process (14). The present study revealed a similar phenomenon that IL-1 β -up-regulated bradykinin B₁ and B₂ receptor-mediated contraction as well as the receptor expression in mouse tracheal smooth muscle. JNK inhibitor SP600125 and TAT-TI-JIP_{153–163}, but not ERK 1/2 inhibitor PD98059 inhibited this up-regulation, suggesting that intracellular JNK signal transduction pathway was involved. A general transcriptional inhibitor, actinomycin D, completely abolished the IL-1 β -enhanced contraction-induced by des-Arg⁹-bradykinin and bradykinin, indicating a transcriptional mechanism that was responsible and further sup-

ported by IL-1 β did not affect bradykinin B₁ and B₂ receptor mRNA stability. Remicade, a chimeric monoclonal anti-TNF- α antibody, markedly inhibited IL-1 β -up-regulated bradykinin B₁ and B₂ receptor-mediated contraction and the receptor mRNA expression in the airway smooth muscle. This reveals that the effect of IL-1 β was at least partly mediated by TNF- α production. In addition, IL-1 β increased the expression of TNF- α mRNA in the airway.

It has been reported that IL-1 β acted on the Rho/Rho-kinase pathway and up-regulated contractile protein expression in coronary artery smooth muscle *in vivo* in the porcine model (18, 19). However, we have previously demonstrated that IL-1 β induced a down-regulation of ET_B, while it did not affect ET_A receptor-mediated contraction in the tracheal smooth muscle in an *in vitro* model (20). By using the same methods, the present study

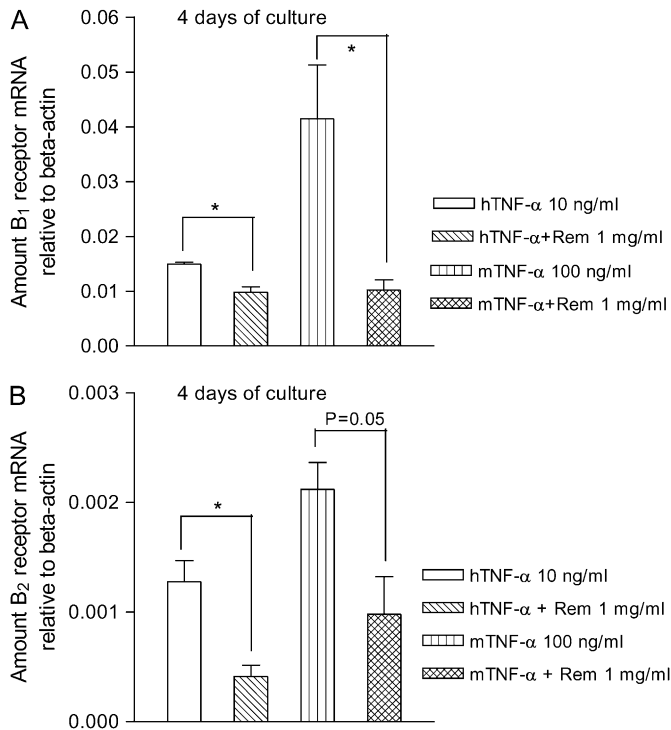


Figure 6. Bradykinin B₁ (A) and B₂ receptor (B) mRNA expression in the tracheal smooth muscle. The tracheae were cultured for 4 d in the presence of 100 ng/ml mTNF-α or 10 ng/ml hTNF-α, with and without 1 mg/ml Remicade (Rem). Each data point is derived from three to four experiments and presented as mean ± SEM. Unpaired Student's *t* test was used. TNF-α versus TNF-α + Rem; **P* < 0.05.

has revealed that IL-1β up-regulated bradykinin B₁ and B₂ receptors; however, it did not alter carbachol-induced contraction. Taking all the results from our *in vitro* model together, the data suggest that IL-1β induces a receptor alteration, rather than the general hyperresponsiveness (i.e., increase in contractile protein expression). This discrepancy is most likely due to the differences between the airway and the artery smooth muscle cells, animals, experimental assays, *in vitro* and *in vivo* models.

In mice, bradykinin and des-Arg⁹-bradykinin are commonly used to activate bradykinin B₂ and B₁ receptors, respectively. The potency for bradykinin B₂ receptor is Lys-bradykinin = bradykinin > >> Lys-des-Arg⁹-bradykinin = des-Arg⁹-bradykinin; for the B₁ receptor it is Lys-des-Arg⁹-bradykinin > des-Arg⁹-bradykinin ≥ Lys-bradykinin > bradykinin (21). We have revealed that simultaneous use of bradykinin B₁ and B₂ receptor antagonists, [Des-Arg¹⁰]HOE140 (100 nM) and HOE140 (100 nM), caused a further rightward shift and depressed maximal contraction of bradykinin concentration–effect curves, suggesting that bradykinin at high concentrations might also activate bradykinin B₁ receptors (14). Bradykinin B₁ and B₂ receptors display low sequence homology and they exhibit different expression patterns. Bradykinin B₁ receptor expression is inducible (11). The expression level of contractile bradykinin B₁ receptors is generally low during normal circumstances, but is up-regulated or synthesized *de novo* during inflammatory conditions (22). Our studies revealed that in fresh tracheal segments from mouse, there is only negligible response to des-Arg⁹-bradykinin (14), while the segments after culture in presence of IL-1β have strong contractile response to des-Arg⁹-bradykinin, and this is in parallel with an increase in bradykinin B₁ receptor mRNA expression, suggesting that bradykinin B₁ receptors are induced.

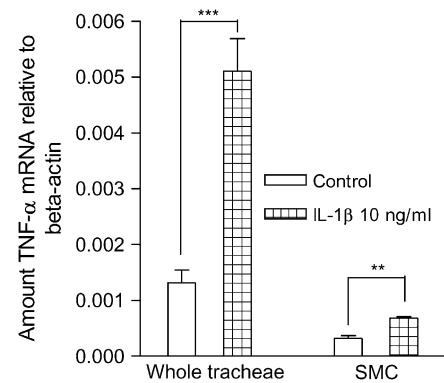


Figure 7. TNF-α mRNA expression in the tracheal smooth muscle (4 d of culture) and the whole trachea with epithelium (2 d of culture). The tracheae were cultured in the absence (control) and presence of 10 ng/ml IL-1β for 2 or 4 d. Each data point is derived from three to five experiments and presented as mean ± SEM. Unpaired Student's *t* test was used. ***P* < 0.01, ****P* < 0.001.

Remicade is a mouse Fv-human IgG₁-k antibody with high affinity for human TNF-α (23). It binds with specificity to both soluble and membrane-bound forms of TNF-α, but does not interact with TNF-β (24). Remicade is successfully used in the treatment of rheumatoid arthritis and Crohn's disease (25, 26). In the present study, we demonstrated that both mTNF-α and hTNF-α enhanced des-Arg⁹-bradykinin- and bradykinin-induced airway contraction and that Remicade could abolish this enhancement with different degrees. Remicade at 1 mg/ml almost completely abolished hTNF-α–increased bradykinin B₁ and B₂ receptor-mediated contraction, while it inhibited only 50–60% of the corresponding response in the mTNF-α-treated group. This difference in effects of Remicade is probably due to a higher affinity to hTNF-α than to mTNF-α.

Proinflammatory cytokines are mainly produced *de novo* in response to inflammatory stimuli. They generally act over a short distance (intercellular space) and exists therefore at low circulating levels (27). In the present study, the concentrations of the cytokines used in culture medium and the agonists in tissue bath seem relatively high, but presumably, the concentrations gained in the intercellular space should reflect paracrine or autocrine secretion. Since the levels of enhanced bradykinin responses by IL-1β and TNF-α were very similar and given the ability of Remicade to inhibit the effect of IL-1β, the TNF-α concentration given in the well might reflect the IL-1β-induced secretion of TNF-α in the intercellular space. Our finding of IL-1β-enhanced bradykinin B₁ and B₂ receptor expression is well in line with *in vivo* data demonstrating that bradykinin B₁ and B₂ receptors were involved in allergen-induced airway hyperresponsiveness in rat (28), and bradykinin-induced airway contraction was enhanced in human subjects with asthma (8). Thus, our *in vitro* finding is relevant to whole animal and human pathophysiology.

The present study showed that IL-1β increased TNF-α mRNA expression in the whole tracheae with epithelium. Similarly, IL-1β increased TNF-α mRNA expression in the tracheal smooth muscle; however, the level of TNF-α mRNA expression in the tracheal smooth muscle was much lower than in the whole tracheae with epithelium. The up-regulation of TNF-α mRNA expression by IL-1β in the tracheae suggests a local increase in TNF-α production under IL-1β stimulation. It has been reported that IL-1β induces an increase of TNF-α secretion in an alveolar macrophage cell line (MH-S murine) (29) and an increase in the

production of TNF- α in antigen-challenged guinea pig airways (30). However, we were not able to measure any TNF- α in the cultured medium by enzyme-linked immunosorbant assay (ELISA) after organ culture of the tracheal segments in the presence and absence of IL-1 β . This might be due to the fact that the ELISA is rather insensitive, or the release of TNF- α is similar to the release of endothelin-1, which is mainly directed toward the smooth muscle cells (31).

Both IL-1 β and TNF- α can activate MAPK. The intracellular MAPK signal pathways consist of JNK, ERK1/2, and p38 and their downstream transcription factors (12, 32). The effectors of these pathways include proliferation, contraction, migration, apoptosis, receptor expressions, and peptides synthesis in airway (33–35). We have recently demonstrated that the intracellular JNK and ERK 1/2 pathways were involved TNF- α -induced up-regulation of bradykinin B₂ receptors (14). In the present study, SP600125 has powerfully inhibitory effects on IL-1 β -enhanced up-regulation of bradykinin B₁ and B₂ receptors, while the intracellular ERK 1/2 inhibitor PD98059 had no effects. Interestingly, the lack of inhibition by PD98059 on the IL-1 β -induced up-regulation of B₂ receptors can partly be due to a direct effect of IL-1 β on the smooth muscle and partly due to a secretion of TNF- α , since it was not blocked by more than 60% by Remicade. Conversely, Yang and coworkers has reported that the intracellular ERK 1/2 pathway was involved in IL-1 β -induced up-regulation of bradykinin B₂ receptors in canine cultured tracheal smooth muscle cells (36). The long-term culture procedure along with the use of different species and different models might be one explanation for this dissimilarity.

SP600125 was developed as an inhibitor of JNK, but it may inhibit other kinases as well (16). Thus, a more selective and specific JNK inhibitor, TAT-TI-JIP_{153–163} (a small peptide, cell membrane permeable and structurally unrelated to SP600125), was used to confirm the involvement of JNK pathway. TAT-TI-JIP_{153–163} had a significant inhibitory effect on the IL-1 β -induced up-regulation of bradykinin B₁ and B₂ receptor expression, suggesting that the JNK pathway was involved. Since the signaling transduction inhibitors had been washed away before the tracheal segments were mounted in tissue bath, and then the tracheal segments have incubated more than 2 h before the administration of agonists, it abolishes a direct effect of the inhibitors on contractility of the segments (data not shown). The contractility of the tracheal segments was tested on carbachol-induced contraction, with no differences in the presence or absence of the inhibitors (SP600125 and PD98059) (14). In addition, we have previously revealed that PD98059 and SP600125 could inhibit organ culture (the control)-induced up-regulation of bradykinin receptor expression (14). The present study showed that IL-1 β further enhanced this up-regulation. SP600125, but not PD98059, could inhibit IL-1 β -induced up-regulation of bradykinin B₁ and B₂ receptor expression in the tracheal smooth muscle.

The intracellular JNK inhibitor SP600125 exhibited a much stronger inhibition on bradykinin B₁ receptor-mediated contraction than on bradykinin B₁ receptor mRNA expression. This difference is most likely due to a “time-window” between transcription of the receptor mRNA and translation of the receptor protein for the function of the receptors. However, there was no evidence that angiotensin-converting enzyme (ACE), endothelial function and cholinergic activity were involved in our model (14). In the present study, higher levels of bradykinin B₁ receptor mRNA than bradykinin B₂ receptor mRNA were in all groups, while the level of bradykinin B₁ and B₂ receptors-mediated contractions were more or less identical. Particularly, in the fresh tracheal segments, there was a considerable amount of bradykinin B₁ receptor mRNA (data not shown), but bradykinin B₁ receptor only elicited a negligible contraction (14). This

indicates that the levels of receptor mRNA is not necessarily in concert with the level of the achieved receptor-mediated contraction, although we in most cases found that changes of the receptor mRNA paralleled with the altered levels of the receptor-mediated contraction (37).

In conclusion, IL-1 β induced a transcriptional up-regulation of bradykinin B₁ and B₂ receptor expression in mouse tracheal smooth muscle. The intracellular JNK signal transduction pathway was involved in this up-regulation. In addition, TNF- α was at least partly participated in this process. Finding a link between airway hyperresponsiveness and inflammatory mediators during airway inflammation may provide a new way for the treatment of asthmatic inflammation.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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