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Up-regulation of bradykinin receptors in a murine in-vitro model of chronic airway inflammation

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Abstract

Tumour necrosis factor-alpha (TNF-α) is a mediator with a likely role in chronic airway inflammation and airway hyperresponsiveness. In the present study, mouse tracheal segments were cultured for 1, 4 or 8 days in the absence and presence of TNF-α. Contractile response of cultured segments to des-Arg^9-bradykinin and bradykinin was assessed in myographs and mRNA for bradykinin B_1 and B_2 receptors was quantified by real-time polymerase chain reaction. Both contraction to des-Arg^9-bradykinin and bradykinin, mediated via bradykinin B_1 and B_2 receptors, respectively, and mRNA levels for these receptors were up-regulated following culture. These responses were markedly increased in segments treated with TNF-α. Experiments with SP600125 (anthrax(1,9-*cd*)pyrazol-6(2*H*)-one) and PD98059 (2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one) demonstrated that both intracellular c-Jun N-terminal kinase and extracellular signal-regulated kinase 1/2 pathways were implicated in this process. Thus, TNF-α causes an increase of bradykinin contractility in mouse trachea, which at least partly is due to a transcriptional increase of bradykinin receptors.

Keywords: TNF-α; Bradykinin B_1 and B_2 receptors; Chronic airway inflammation; Airway hyperresponsiveness; MAPK pathway

1. Introduction

Chronic airway inflammation is associated with the development of airway hyperresponsiveness (Foresi et al., 1990; Laprise et al., 1999). The release of different mediators, such as tumour necrosis factor-alpha (TNF-α), during the inflammatory process, is known to cause both functional and structural alterations of the airways (Bousquet et al., 2000). The airway smooth muscle cells are of importance in this process (Boulet et al., 1998; Halayko and Amrani, 2003). We have recently demonstrated how the long-term influence of inflammatory mediators on the isolated airway smooth muscle could induce an increased sensitivity to serotonin (5-hydroxytryptamine, 5-HT) (Adner et al., 2002).

Bradykinin and related kinins play important roles in the pathogenesis of inflammation, tissue damage and repair (Dray and Perkins, 1993; Proud and Kaplan, 1988). Bradykinin is formed from the kininogen precursor after proteolytic cleavage by kallikrein, and can further be converted by carboxypeptidase N to des-Arg^9-bradykinin. Both kinins can be degraded by kininase II that is identical to angiotensin-converting enzyme (ACE) (Barnes, 1992; Kaplan et al., 2002). Bradykinin is a weak constrictor in isolated human bronchi, while, in vivo, bradykinin is a potent bronchoconstrictor in asthmatic patients. This may be due to bradykinin-induced activation of cholinergic nerves (Fuller et al., 1987; Reynolds et al., 1999). Intravenous administration of bradykinin causes intense bronchoconstriction in guinea pigs (Ichinose et al., 1990). The effects of kinins are mediated by two G-protein-coupled receptors (bradykinin B_1 and B_2 receptors) (Regoli and Barabe, 1980), present in both airway smooth muscle and epithelial cells (Li et al., 1998). Both bradykinin receptor subtypes seem to contribute to allergen-induced bronchial hyperresponsiveness of rat (Huang et al., 1999). However, patients with asthma exhibit a hyperresponsiveness to aerosolized bradykinin (a bradykinin B_2 receptor agonist), but not to des-Arg^9-bradyknin (a bradykinin B_1 receptor agonist) (Polosa and Holgate, 1990; Reynolds et al., 1999).

Both TNF-α and bradykinin are synthesized and released during chronic airway inflammation and augmented

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TNF-α and bradykinin levels are found in airway biopsies as well as bronchoalveolar lavage fluids from asthmatic patients (Cembrzynska-Nowak et al., 1993; Christiansen et al., 1992). TNF-α can be derived from mast cells and macrophages via immunoglobulin E (IgE)-dependent mechanisms (Thomas, 2001) and has been demonstrated to cause an increase of airway hyperresponsiveness (Thomas et al., 1995). In addition, recent studies have shown that cytokines such as TNF-α and interleukin-1β increase bradykinin B_1 receptor expression in human lung fibroblasts (Phagoo et al., 2000) and that bradykinin stimulates the release of TNF-α and interleukin-1β from macrophages (Tiffany and Burch, 1989). Altogether, the above-mentioned results prompted us to further investigate the possibility of a role for TNF-α and bradykinin interaction in the development of airway hyperresponsiveness in asthma and chronic airway inflammation. For this purpose, we used a newly developed organ culture assay (Adner et al., 2002), validated as a suitable in-vitro assay for evaluation of long-term effects induced by inflammatory mediators (Johnson, 2002). Special attention was given the role of the mitogen-activated protein kinase (MAPK) pathways as a possible link between TNF-α and the bradykinin B_1 and B_2 receptors.

2. Materials and methods

2.1. Chemicals

Recombinant murine TNF-α was obtained from R&D Systems (Abingdon), UK. des-Arg^9-bradykinin and bradykinin were purchased from Neosystem (Strasbourg, France). HOE140 (D-Arg-[Hyp^3, Thi^5, D-Tic^7, Oic^8]bradykinin), [Des-Arg^{10}]HOE140 (DesArg^9-D-Arg[Hyp^3,Thi^5,D-Tic^7,Oic^8]bradykinin), indomethacin, carbachol, PD98059 (2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one), atropine, *N*-monomethyl-L-arginine (L-NMMA), captopril, dimethyl sulfoxide (DMSO), Dulbecco's modified Eagle's medium (DMEM; 4500 mg/l D-glucose, 110 mg/l sodium pyruvate, 584 mg/l L-glutamine) and Krebs-Henseleit buffer were from Sigma (St. Louis, USA). SP600125 (anthrax (1,9-*cd*)pyrazol-6(2*H*)-one), penicillin and streptomycin were obtained from Calbiochem (Bad Sodem, Germany) and Life Technologies (Gaithersburg, MD, USA), respectively.

2.2. Tissue preparation and organ culture

Male BALB/c J strain mice (10 weeks old; MB, Ry, Denmark) were killed by cervical dislocation. Whole tracheae were placed into Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). Under sterile conditions, the tracheae were cut into segments with three cartilage rings each for organ culture or directly used. Segments were placed individually into wells of a 96-well plate (Ultra-low attachment; Sigma) with 300 μl serum-free DMEM and incubated at 37 °C in humidified 5% CO_2 in air for 1, 4 or 8 days in the absence and presence of TNF-α (1, 10 or 100 ng/ml). Segments were moved into a new well containing fresh media everyday. To study the intracellular mechanisms, the segments were incubated with SP600125 (anthrax (1,9-*cd*)pyrazol-6(2*H*)-one) or PD98059 (2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one) in the absence and presence of TNF-α for 4 days. Control segments received the same amount of TNF-α and, in addition, dimethyl sulfoxide (DMSO) as vehicle since SP600125 and PD98059 was dissolved in DMSO. After incubation, des-Arg^9-bradykinin- and bradykinin-induced concentration curves on the segments were obtained and the smooth muscle isolated for bradykinin B_1 and B_2 receptors mRNAs.

2.3. In-vitro pharmacology

Tracheal smooth muscle reactivity was recorded in temperature-controlled (37 °C) myographs (Organ Bath Model 700MO, J.P. Trading, Denmark) containing 5 ml Krebs–Henseleit buffer with 10 mM D-glucose. The solution was continuously equilibrated with 5% CO_2 in O_2 resulting in a stable pH of 7.4. The tracheal segments were mounted for continuous recording of isometric tension by the Chart software (AD Instruments, UK). One-hour equilibration was performed by washing every 15 min and adjusting the basal tension to 0.8 mN (Adner et al., 2002). Each segment was contracted with 60 mM KCl to test the contractile function. To inhibit epithelial prostaglandin release, the segments were incubated with 3 μM indomethacin 30 min before administration of des-Arg^9-bradykinin or bradykinin (Li et al., 1998; van Heuven-Nolsen et al., 1997). At the end of the experiment, the contractile response of 1 mM carbachol was assessed. Pharmacological characterisation of bradykinin receptors was obtained using the receptor antagonists [Des-Arg^{10}]HOE140 (DesArg^9-D-Arg[Hyp^3,Thi^5,D-Tic^7,Oic^8]bradykinin) or HOE140 (D-Arg-[Hyp^3, Thi^5, D-Tic^7, Oic^8]bradykinin) administration before 30 min of des-Arg^9-bradykinin or bradykinin. In other experiments, to exclude that release of nitric oxide (NO) or cholinergic nerve activation affected the responses to des-Arg^9-bradykinin and bradykinin, a nitric oxide synthase (NOS) inhibitor *N*-monomethyl-L-arginine (L-NMMA; 100 μM) and a muscarinic receptor antagonist (atropine; 1 μM) were added 30 min before bradykinin or des-Arg^9-bradykinin. Furthermore, to evaluate the possibility of a breakdown of the kinins, the ACE inhibitor (captopril; 1 μM) was added 1 h before the experiments.

2.4. Analysis

All data are expressed as mean values ± S.E.M. The responses of des-Arg^9-bradykinin and bradykinin are presented as percentage of contraction to 1 mM carbachol and in mN. This concentration of carbachol has been shown to

Table 1
Maximal contractile response (α) to des-Arg^9-bradykinin and bradykinin and the p[A]$_{50}$ values in fresh segments and following organ culture in the absence and presence of different concentrations of TNF-α

Culture time	TNF-α (ng/ml)	des-Arg^9-bradykinin				Bradykinin			
		n	α (% of Cch)	α (mN)	p[A]$_{50}$	*n*	α (% of Cch)	α (mN)	p[A]$_{50}$
Day 0	0	7	0.08 ± 0.03	0.05 ± 0.03	ND	7	4.2 ± 1.5	0.11 ± 0.03	ND
Day 1	0	5	17.6 ± 2.6[a]	1.15 ± 0.34[a]	6.79 ± 0.29	5	25.4 ± 9.6	1.69 ± 0.52	5.78 ± 0.25
	1	5	26.7 ± 6.9	1.96 ± 0.46	6.60 ± 0.27	5	27.6 ± 4.7	2.23 ± 0.61	5.83 ± 0.31
	10	6	35.9 ± 3.9[b]	2.95 ± 0.56[b]	7.41 ± 0.07	6	33.8 ± 9.4	2.60 ± 1.14	6.18 ± 0.41
	100	6	45.6 ± 5.4[b]	3.08 ± 0.65[b]	7.64 ± 0.16[b]	6	47.6 ± 6.2	3.16 ± 0.56	6.83 ± 0.25
Day 4	0	6	6.8 ± 2.2	0.54 ± 0.17	6.92 ± 0.27	6	30.5 ± 7.1[a]	2.15 ± 0.57[a]	6.56 ± 0.22
	1	7	10.4 ± 2.5	0.67 ± 0.12	6.81 ± 0.27	7	41.4 ± 6.1	2.22 ± 0.39	6.35 ± 0.29
	10	7	35.7 ± 7.3[b]	3.64 ± 0.90[b]	7.80 ± 0.15	7	55.2 ± 7.0[b]	3.54 ± 1.09[b]	7.62 ± 0.30[b,c]
	100	6	50.3 ± 5.5[b]	3.90 ± 0.78[b]	8.14 ± 0.35[b]	6	67.8 ± 2.9[b,c]	3.94 ± 0.29[b]	8.98 ± 0.14[b,c]
Day 8	0	6	11.0 ± 4.9	0.63 ± 0.10	6.87 ± 0.21	6	27.7 ± 6.5[a]	1.14 ± 0.20	6.45 ± 0.32
	1	5	12.0 ± 5.1	0.69 ± 0.10	6.81 ± 0.25	5	52.7 ± 13.3	1.90 ± 0.39	7.31 ± 0.24[c]
	10	5	57.8 ± 5.1[b,c]	3.27 ± 0.34[b]	7.22 ± 0.20	5	70.3 ± 5.6[b,c]	2.99 ± 0.42[b]	7.20 ± 0.27
	100	6	65.4 ± 7.5[b]	2.81 ± 0.43[b]	8.42 ± 0.17[b]	6	87.6 ± 3.1[b,c]	3.42 ± 0.49[b]	9.63 ± 0.24[b,c]

Data are presented as mean ± S.E.M. Cch denotes carbachol. Statistical analysis was performed with one-way ANOVA and Dunnet's post test. $P < 0.05$ were considered to be significant and marked by [a,b,c]. [a]Fresh (day 0) vs. organ culture 1, 4 and 8 days without TNF-α; [b]organ culture without TNF-α vs. organ culture with TNF-α among day 1, day 4 or day 8; [c]culture day 1 with TNF-α (1, 10 and 100 ng/ml) vs. culture day 4 with TNF-α (1, 10 and 100 ng/ml) or culture day 8 with TNF-α (1, 10 and 100 ng/ml). ND = not determined.

induce a contraction close to the maximal capacity of both fresh and cultured segments and is not altered by treatment of TNF-α (Adner et al., 2002). Agonist concentration–effect curves were fitted to the Hill equation using an iterative, least square method (GraphPad Prism), to provide estimates of maximal contraction (α) and midpoint location (p[A]$_{50}$). Statistical analysis was performed using one-way analysis of variance (ANOVA) with Dunnett's post-test. $P < 0.05$ was considered to be significant.

pK_B values for the HOE140 and [Des-Arg10]HOE140 were estimated by fitting the individual log[A]$_{50}$ values obtained in the absence (log[A]$_{50}$) and presence (log[A]$_{50B}$) of antagonist concentrations [B] to the following derivation of the Schild equation (Black et al., 1985).

$$\log[A]_{50B} = \log[A]_{50} + \log\left(1 + \frac{[B]^b}{10^{-pK_B}}\right)$$

In the first fit, the Schild slope parameter (b) was allowed to vary. If the value of b was found not to be significantly different from unity, a second fit was performed with b constrained to unity.

2.5. Real-time polymerase chain reaction (real-time PCR)

In order to evaluate whether TNF-α affects transcription of bradykinin B_1 and B_2 receptors, real-time PCR analysis was performed on cDNA reverse transcripted from total RNA extracted from tracheal smooth muscle. The smooth muscle was isolated mechanically on an ice tray under a microscope. After removal of tracheal epithelium and cartilages, the smooth muscle was rinsed with cold PBS and stored in the RNAlater™ (QIAGEN, Germany) at − 80 °C until extraction of total RNA. Thereafter, the tracheal smooth muscle was homogenized and the total RNA was extracted by using the RNeasy Mini following the kit instructions (QIAGEN). The purity of total RNA was checked by a spectrophotometer and the wavelength absorption ratio (260/280 nm) was between 1.8 and 2.0 in all preparations. Reverse transcription of total RNA to cDNA

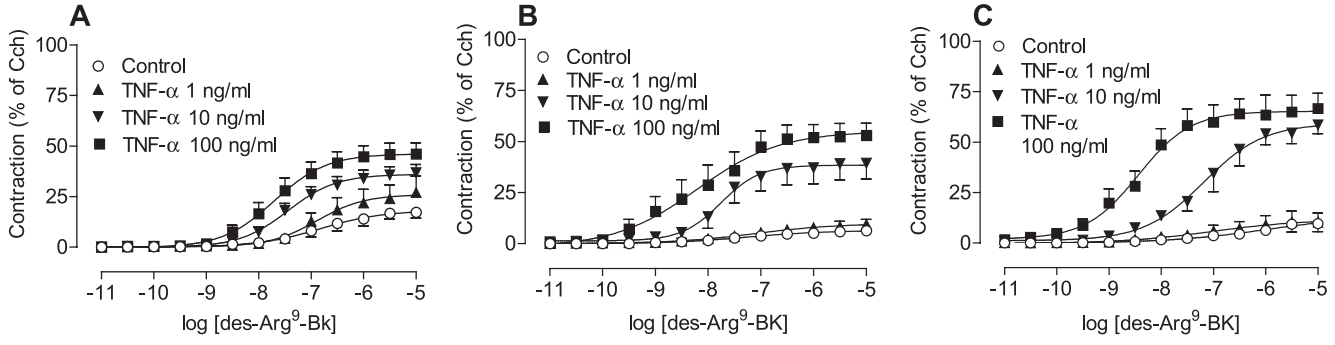

Fig. 1. Concentration–effect curves of des-Arg^9-bradykinin obtained on mouse tracheal segments incubated for 1 day (A), 4 days (B) and 8 days (C) in the absence and presence of TNF-α (1, 10 and 100 ng/ml). Each point represents the mean of all segments ± S.E.M ($n = 5–6$).

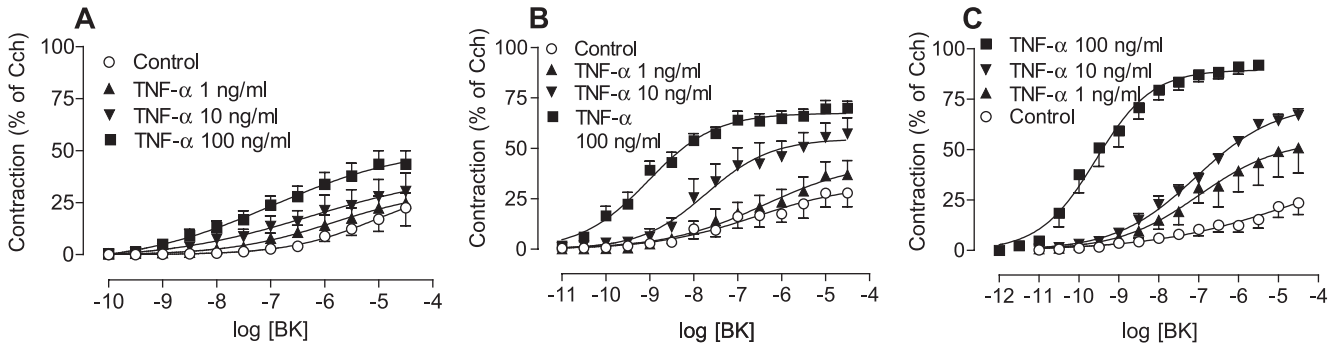

Fig. 2. Concentration–effect curves of bradykinin obtained on mouse tracheal segments incubated for 1 day (A), 4 days (B) and 8 days (C) in the absence and presence of TNF-α (1, 10 and 100 ng/ml). Each point represents the mean of all segments ± S.E.M ($n = 5–7$).

was carried out using Omniscript™ reverse transcriptase kit (QIAGEN) in 20 μl volume reaction by using Mastercycler personal PCR machine (Eppendorf, Germany). The RNA was incubated at 65 °C for 5 min to denature. After the denaturation, the RNA was immediately cooled and the reverse transcription master mix was added into the tube. The reaction of reverse transcription was carried out at 37 °C for 1 h.

The real-time quantitative PCR was performed with the QuantiTect™ SYBR® Green PCR kit (QIAGEN) in the ABI PRISM 7700 sequence detection system (PE Applied Biosystem, USA). The system automatically monitors the binding of a fluorescent dye SYBR® Green to double-stranded DNA by real-time detection of the fluorescence during each cycle of PCR amplification. The real-time PCR was performed in 50 μl reaction volume and carried out with heating 95 °C for 15 min followed by 40 PCR cycles with 94 °C for 30 s and 55 °C for 1 min. The data were analyzed with cycle threshold (CT) method and the specific of PCR products were analysed by the dissociation curves and visualized by agarose electrophoresis. Expected PCR products of bradykinin B_1 receptor 102 basepairs, bradykinin B_2 receptor 104 basepairs and β-actin 102 basepairs with a single band for each product were seen.

All the PCR primers used in the present study were designed by using Prime Express® 2.0 software (Applied

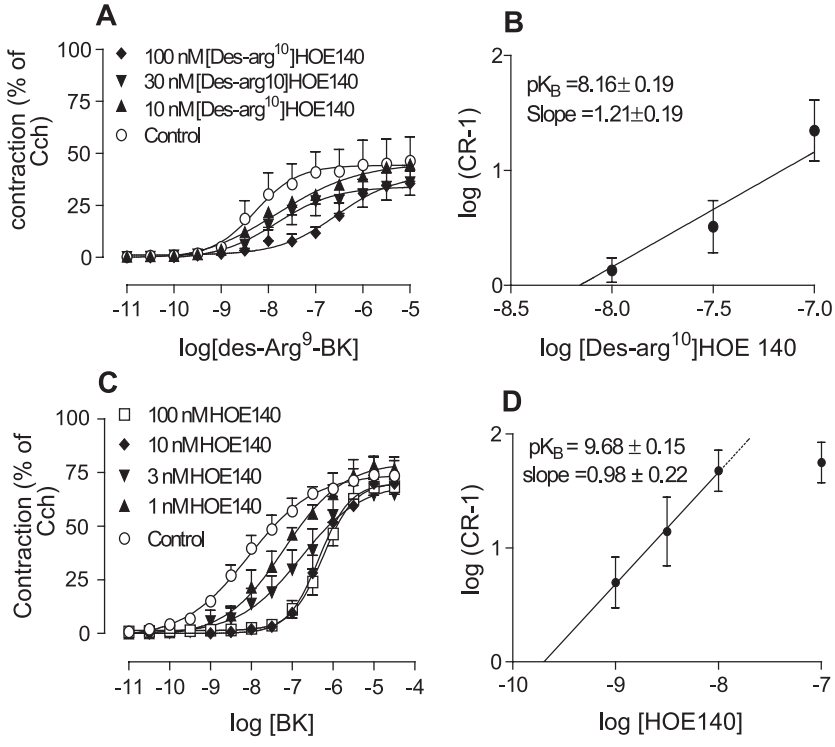

Fig. 3. Contractile responses to des-Arg^9-bradykinin (A) or bradykinin (C) in the absence and presence of bradykinin B_1 receptor antagonist [Des-Arg^{10}]HOE140 or bradykinin B_2 receptor antagonist HOE140 30 min before the agonist curves in mouse tracheal segments. The segments were incubated for 4 days with TNF-α (100 ng/ml). Schild analysis was performed for [Des-Arg^{10}]HOE140 (B) and for HOE140 (D). Each point represents the mean of all segments ± S.E.M ($n = 5–6$).

Biosystem, USA) and synthesized by DNA Technology (Aarhus, Denmark). Below are the sequences:

Bradykinin B_1 receptor
Forward: 5′-CCATAGCAGAAATCTACCTGGC-TAAC-3′
Reverse: 5′-GCCAGTTGAAACGGTTCC-3′
Bradykinin B_2 receptor
Forward: 5′-ATGTTCAACGTCACCACACAAGTC-3′
Reverse: 5′-TGGATGGCATTGAGCCAAC-3′
β-actin
Forward: 5′-TGGGTCAGAAGGACTCCTATGTG-3′
Reverse: 5′-CGTCCCAGTTGGTAACAATGC-3′.

The ΔΔCT method was employed to calculate a relative amount of mRNA for bradykinin B_1 and B_2 receptors. This is the CT value of mRNA for housekeeping gene β-actin as a reference to normalize a relative amount of mRNA for bradykinin B_1 and B_2 receptors. The relative amount of mRNA was obtained by the CT values of mRNA for bradykinin B_1 or B_2 receptor in relation to the CT values of mRNA for β-actin mRNA in the same sample. A blank control (no template) was included in all the experiments for negative controls.

3. Results

3.1. Contractile effects of des-Arg^9-bradykinin and bradykinin on freshly isolated mouse tracheal segments

Basal contractile responses of des-Arg^9-bradykinin and bradykinin on isolated mouse trachea were studied in freshly isolated segments. The segments elicited a negligible contractile effect by des-Arg^9-bradykinin, whereas bradykinin induced a weak contraction (Table 1).

3.2. Organ culture, with and without TNF-α: effects on des-Arg^9-bradykinin-induced contractions

Tracheal segments were cultured for 1, 4 or 8 days. Subsequent exposure to des-Arg^9-bradykinin elicited a minor contractile response in the tested segments. The responses were small both in mN and expressed as a in percentage of carbachol and did, for 4 and 8 days, not differ significantly from the small response seen in fresh segments ($P>0.05$). Segments cultured for 1 day exhibited a significant increase of the des-Arg^9-bradykinin contractions in comparison with fresh segments (Fig. 1A–C, Table 1, $P<0.05$).

When segments were cultured for 1, 4 or 8 days with TNF-α (1, 10 and 100 ng/ml), the subsequent response to des-Arg^9-bradykinin were changed. Treatment with 10 and 100 ng/ml TNF-α caused a significant increase of the maximal contractile responses to des-Arg^9-bradykinin after 1, 4 and 8 days, but not the treatment with 1 ng/ml of TNF-α (Fig. 1A–C, Table 1). The $p[A]_{50}$ values for des-Arg^9-bradykinin were significantly increased after treatment with 100 ng/ml TNF-α for 1, 4 and 8 days in comparison with organ culture (Table 1, $P<0.05$).

3.3. Organ culture, with and without TNF-α: effects on bradykinin-induced contractions

Tracheal segments were cultured for 1, 4 or 8 days. The subsequent maximal contraction induced by bradykinin was not significantly altered after 1 day of culture in comparison with fresh segments ($P>0.05$), whereas a significant increase was seen after 4 and 8 days (Table 1, $P<0.05$). This increase was relatively small both in percentage of carbachol and in mN (Fig. 2A–C, Table 1). No difference in potency was seen as the result of culture.

Treatment with TNF-α (1, 10 and 100 ng/ml) for 1 day did not significantly alter the concentration–effect curves for bradykinin in comparison with untreated (control segments) (Fig. 2A, Table 1, $P>0.05$), whereas a significant increase of the maximal response was observed in segments treated with 10 and 100 ng/ml TNF-α during 4 and 8 days

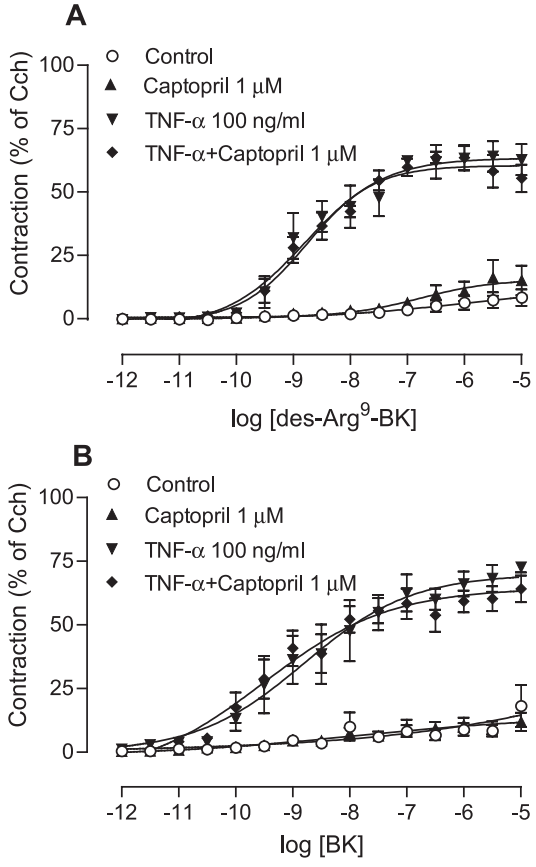

Fig. 4. ACE inhibitor captopril (1 μM) was added 1 h before des-Arg^9-bradykinin (A) or bradykinin (B) concentration–response curves. The segments cultured for 4 days in the absence and presence of TNF-α 100 ng/ml. Each data point represents the mean of all segments ± S.E.M ($n=3–5$).

(Fig. 2B and C, Table 1). A substantial increase of $p[A]_{50}$ values was observed after 4 days treatment with 10 and 100 ng/ml TNF-α (Fig. 2B, Table 1, $P<0.05$). An even more marked increase in $p[A]_{50}$ values was obtained with 100 ng/ml TNF-α in segments cultured for 8 days (Fig. 2C, Table 1, $P<0.05$). This increased potence for bradykinin induced by 100 ng/ml TNF-α was significantly enhanced over time of the culture (Table 1).

3.4. Pharmacological characterisation

In order to investigate the receptors responsible for the contractile effect of des-Arg^9-bradykinin, a pharmacological characterisation was performed in tracheal segments incubated for 4 days with TNF-α 100 ng/ml. For this purpose, a selective bradykinin B_1 receptor antagonist, [Des-Arg^{10}] HOE140, was used. The results showed that [Des-Arg^{10}] HOE140 (10–100 nM) caused a rightward shift of the des-Arg^9-bradykinin concentration–effect curves without significantly altering the maximal level of the contraction (Fig. 3A). Schild analysis gave a slope coefficient not different from unity (1.21 ± 0.19) which therefore could be constrained to unity and a pK_B value for [Des-arg^{10}]HOE140 of 8.16 ± 0.19 was obtained (Fig. 3B). The des-Arg^9-bradykinin-induced contraction was not affected by 100 nM HOE140 (not shown).

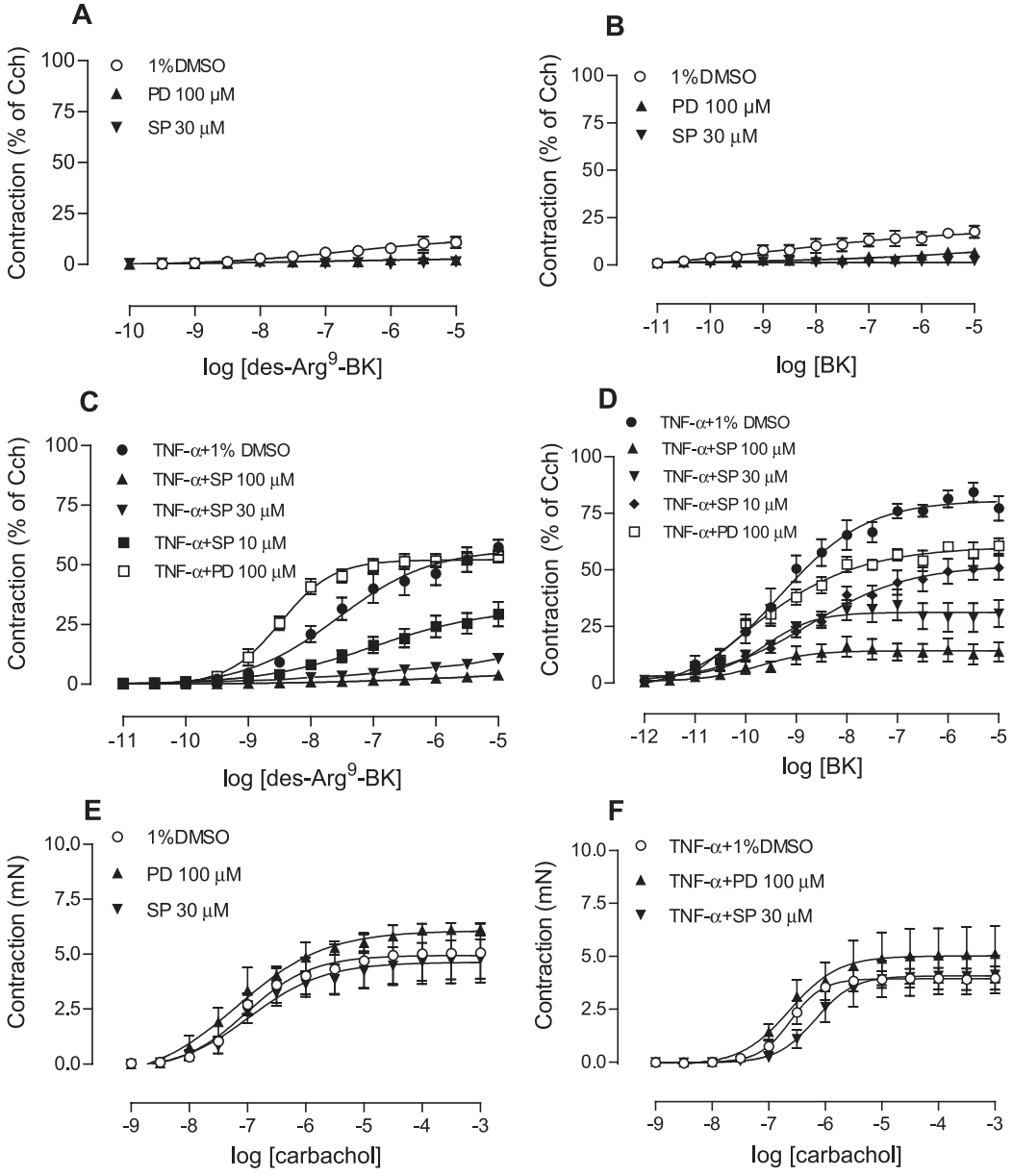

Fig. 5. Concentration–effect curves of des-Arg^9-bradykinin (A, C), bradykinin (B, D) and carbachol (E, F; $n=3-4$) were obtained on the segments cultured for 4 days in the absence and presence of TNF-α (100 ng/ml) with SP600125 (SP) or PD98059 (PD) or vehicle (DMSO). Each data point represents the mean of all segments ± S.E.M ($n=5-6$).

To confirm that bradykinin-induced contraction of the tracheal segments was mediated by bradykinin B_2 receptor, a selective antagonist, HOE140, was used in the segments cultured for 4 days with TNF-α 100 ng/ml. In the concentration range of 1–10 nM, HOE140 caused a concentration-dependent parallel rightward shift of the bradykinin concentration–effect curves without altering the maximal level of the contraction (Fig. 3C). However, 100 nM HOE140 did not cause a further rightward shift, indicating that another receptor is responsible for this contraction. Using both bradykinin B_1 and B_2 receptors antagonists, [Des-Arg^{10}]HOE140 (100 nM) and HOE140 (100 nM), simultaneously caused a further rightward shift and depressed maximal contraction of bradykinin concentration–effect curves ($p[A]_{50} = 5.47 \pm 0.09$, $\alpha = 35.5 \pm 1.8\%$, $n = 2$), suggesting that bradykinin at high concentrations also activates bradykinin B_1 receptors. Schild analysis, excluding 100 nM HOE140, gave a slope coefficient not different from unity (0.98 ± 0.22) and a pK_B value for HOE140 of 9.68 ± 0.15 was obtained when the slope was constrained to unity (Fig. 3D).

3.5. Four days of organ culture, with and without TNF-α: effects of atropine, L-NMMA and captopril on des-Arg^9-bradykinin- and bradykinin-induced contractions

To exclude that release of NO, cholinergic nerve activation or ACE activity were involved in the up-regulation of the des-Arg^9-bradykinin and bradykinin contractions seen following TNF-α treatment, experiments were performed in the presence of the NOS inhibitor, L-NMMA (100 μM), the muscarinic receptor antagonist, atropine (1 μM) and the ACE inhibitor captopril (1 μM). None of these three antagonists affected the contractile response induced by des-Arg^9-bradykinin and bradykinin in TNF-α treated segments (captopril: Fig. 4A and B; L-NMMA and atropine, not shown). Neither did these antagonists affect contractions induced by des-Arg^9-bradykinin and bradykinin in untreated, cultured segments.

3.6. Four days of organ culture, with and without TNF-α: effects of des-Arg^9-bradykinin and bradykinin in relation to the MAPK pathways

Specific inhibitors for c-Jun N-terminal kinase (JNK) SP600125 and extracellular signal-regulated kinase 1/2 (ERK 1/2) PD98059 were used to investigate if JNK and ERK 1/2 signal transduction pathways were required for the up-regulation of bradykinin B_1 and B_2 receptor-mediated contraction. Both the inhibitors were incubated with the segments for 4 days in the absence and presence of TNF-α. Clearly, they significantly attenuated the contraction induced by des-Arg^9-bradykinin and bradykinin in the segments without TNF-α (Fig. 5A and B). In the segments treated with TNF-α, SP600125 had a concentration-dependent inhibitory effect on both des-Arg^9-bradykinin and bradykinin concentration–response curves (Fig. 5C and D), while PD98059 only inhibited the contraction induced by bradykinin ($\alpha = 79.5 \pm 3.8\%$ vs. $60.5 \pm 3.3\%$, $P < 0.05$, Fig. 5D), but not des-Arg^9-bradykinin ($\alpha = 54.9 \pm 5.9\%$ vs. $52.1 \pm 2.9\%$, $P > 0.05$, Fig. 5C).

In addition, both PD98059 and SP600125 did not affect the concentration–response curves of carbachol in the maximal contraction and $p[A]_{50}$ values in the segments cultured for 4 days in the absence and presence of TNF-α (Fig. 5E and F).

3.7. Four days of organ culture, with and without TNF-α: effects on the expression of bradykinin B_1 and B_2 receptors

The relative amount of mRNA for bradykinin B_1 and B_2 receptors was quantified with real-time PCR with and without TNF-α treatment (100 ng/ml). Both receptors were up-regulated following culture (twofold) and this up-regulation was further enhanced following TNF-α treatment (threefold) (Fig. 6, $P < 0.05$).

3.8. Four days of organ culture, with and without TNF-α: effects on the expression of bradykinin B_1 and B_2 receptors in relation to the MAPK pathways

To find an intracellular link between inflammatory mediators TNF-α and the enhanced mRNA expression for bradykinin B_1 and B_2 receptors, the segments were cultured for 4 days with PD98059 or SP600125 or vehicle (DMSO) in the absence and presence of TNF-α. The up-regulated mRNA expression for bradykinin B_1 and B_2 receptors in the smooth muscle in both organ culture and the organ culture with TNF-α was significantly attenuated by both inhibitors SP600125 and PD98059 (Fig. 7A and B, $P < 0.05$), except

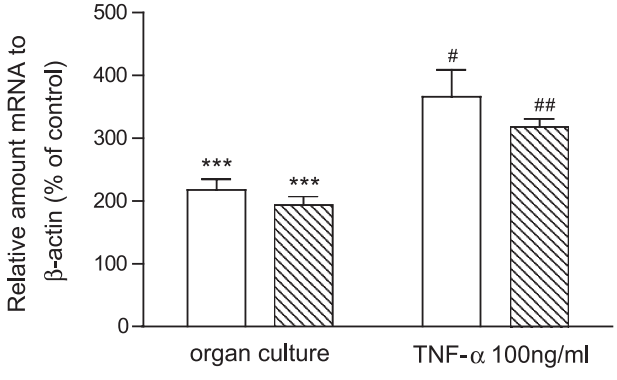

Fig. 6. Effects of organ culture in the absence and presence of TNF-α (100 ng/ml) on mRNA expressions for bradykinin B_1 and B_2 receptors in smooth muscle isolated from the tracheal cultured for 4 days. Bradykinin B_1 and B_2 receptor mRNAs were quantified by real-time PCR. Each data point was derived from three to four identical experiments and represent as percentage of control (fresh segments) with the mean ± S.E.M. Empty bars are bradykinin B_1 receptor; dashed bars are bradykinin B_2 receptor. Two-way unpaired Student's *t*-test was used for statistic. $P < 0.05$ were considered to be significant. Fresh vs. organ culture (***$P < 0.001$) and organ culture vs. TNF-α treatment ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$).

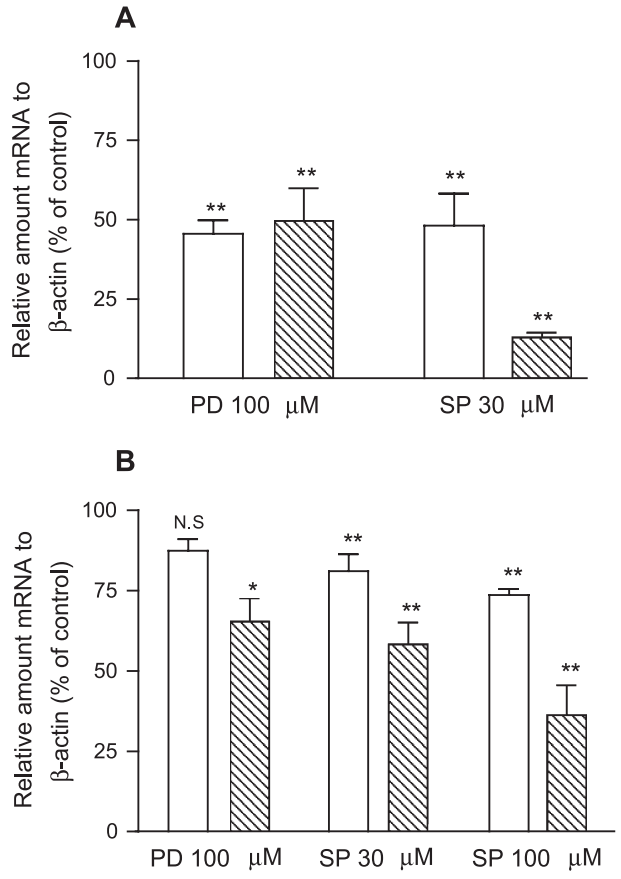

Fig. 7. (A) Effects of PD98059 and SP600125 on the enhanced mRNA expressions for bradykinin B_1 and B_2 receptors in the smooth muscle during the organ culture without TNF-α. Each data point was derived from four to five identical experiments and represent as percentage of control (organ culture plus DMSO) with the mean ± S.E.M. (B) Effects of PD98059 and SP600125 on the enhanced mRNA expression for bradykinin B_1 and B_2 receptors in the smooth muscle during the organ culture with TNF-α 100 ng/ml. Each data point was derived from four to five identical experiments and represent as percentage of control (TNF-α plus DMSO) with the mean ± S.E.M. The experiments were performed on the tracheal cultured for 4 days in the absence and presence of TNF-α 100 ng/ml with SP600125 or PD98059 or vehicle (DMSO). Empty bars are bradykinin B_1 receptor; dashed bars are bradykinin B_2 receptor. Statistical analysis was performed with one-way ANOVA and Dunnet's post test. $P<0.05$ was considered to be significant. Compared with their own controls, $*P<0.05$, $**P<0.01$. PD = PD98059; SP = SP600125.

PD98059 did not significantly inhibit the up-regulation of mRNA for bradykinin B_1 receptors in the presence of TNF-α (Fig. 7B, $P>0.05$).

4. Discussion

We have recently developed a novel in-vitro assay for the evaluation of inflammatory-mediators effects on mouse airways (Adner et al., 2002; Johnson, 2002). Using this culture model, we demonstrated that long-term exposure to TNF-α enhanced 5-HT_{2A} receptor-mediated airway contractions (Adner et al., 2002). In the present study, the effect of long-term exposure to TNF-α on des-Arg^9-bradykinin- and bradykinin-induced airway contractions were investigated using the same model. Both the functional response to des-Arg^9-bradykinin and bradykinin, mediated via the bradykinin B_1 and B_2 receptors, and the mRNA levels for these receptors were up-regulated following the TNF-α treatment. Experiment with a specific JNK inhibitor SP600125 and the ERK 1/2 inhibitor PD98059 revealed the involvement of MAPK pathways in this up-regulation. An up-regulation following the culture procedure per se was also noted and found to activate, similar intracellular pathways.

Bradykinin B_1 and B_2 receptors display low sequence homology and they exhibit different expression patterns. The expression levels of the bradykinin B_1 receptor are generally low during normal circumstances, but are up-regulated or synthesized de novo during inflammatory conditions (Marceau et al., 1980; Marceau et al., 1998; Regoli and Barabe, 1980). Our findings of TNF-α-induced bradykinin receptor up-regulations are in line with in vivo data, demonstrating that both bradykinin B_1 and B_2 receptors are involved in allergen-induced airway hyperresponsiveness in rat (Huang et al., 1999). However, in humans, airway hyperresponsiveness only occurs to aerosolized bradykinin (a bradykinin B_2 receptor agonist) but not to des-Arg^9-bradykinin (a bradykinin B_1 receptor agonist) (Polosa and Holgate, 1990; Reynolds et al., 1999). However, it seems to be a marked difference between the strong effect seen following bradykinin inhalation and the relatively small effects obtained in isolated human bronchus (Fuller et al., 1987). In analogy, the effects of both des-Arg^9-bradykinin and bradykinin in the present study were small in freshly isolated murine airways. The response to des-Arg^9-bradykinin was almost negligible, whereas the response to bradykinin was slightly more marked.

Following long-term exposure to TNF-α, the contractile response induced by both des-Arg^9-bradykinin and bradykinin was substantially increased. The increases were related to the dosage of TNF-α in a concentration-dependent way. A parallel increase in mRNA levels for the bradykinin B_1 and B_2 receptors indicated that the TNF-α-induced enhancement of the contractile response might be related to increased transcription of these receptors. However, the on-set time for up-regulation was much shorter for the bradykinin B_1 receptor than for the bradykinin B_2 receptor. For bradykinin B_1 receptor maximum response was reached already after 1 day of culture, whereas bradykinin B_2 receptor-mediated response still increased at day 8. In addition, the leftward shift induced by bradykinin was more accentuated for the bradykinin B_2 receptor than for the bradykinin B_1 receptor. These differences indicate that the described up-regulation of the two bradykinin receptor subtypes, at least partly, might be the related to different mechanisms.

According to the literature several factors like release of NO and prostaglandins (Sipahi et al., 1998), cholinergic nerve activation (Fuller et al., 1987; Reynolds et al., 1999) and ACE activity (Ichinose and Barnes, 1990; Schilero et

al., 1994) can interfere with bradykinin-induced airway contractions. However, in the present setup no change of the effects induced by des-Arg^9-bradykinin and bradykinin were seen when the NO inhibitor, L-NMMA, the muscarinic receptor antagonist, atropine and the ACE inhibitor captopril were added to the tissue baths prior to the kinins. Neither did these antagonists affect the results obtained following TNF-α treatment. This indicates that NO, cholinergic nerve reflexes and ACE are of no or only minor importance for the described phenomenon. Eventual interfering effects induced by the release of prostaglandins (including PGI_2) could be ruled out since all experiments were performed after pretreatment (30 min) with indomethacin, a cyclooxygenase inhibitor (Li et al., 1998; van Heuven-Nolsen et al., 1997).

TNF-α and interleukin-1β are well known agonists for MAPK pathways. Signalling along these pathways is known to trigger transcription factors like nuclear factor kappa B (NF-κB) and activating protein-1 (AP-1) (Liacini et al., 2003; Liacini et al., 2002). In cultured rabbit aorta, PD98059, a highly specific inhibitor for mitogen-activated protein kinase kinase (MAPKK) (Alessi et al., 1995; Marcus et al., 2003) attenuated the spontaneous increased response to des-Arg^9-bradykinin and reduced the increased response induced by interleukin-1β (Larrivee et al., 1998). In contrast, the present study did not find any inhibitory effects of PD98059 on the TNF-α-enhanced des-Arg^9-bradykinin response. The long-term culture procedure along with the use of different species might be one explanation for this dissimilarity. The selectivity of PD98059 (100 μM) and SP600125 (30 μM) in the present setup was tested on carbachol-induced contractions in segments cultured for 4 days in the absence and presence of TNF-α. The results indicate that none of the inhibitors displayed nonspecific effects.

A minor increase of the contractile response induced by des-Arg^9-bradykinin and bradykinin following the culture procedure per se was also noted. The mechanism behind this remains unknown, but the phenomenon might the related to the altered environment for epithelial cells or to the loss of breathing pressure. Another factor could be the induction of a nonspecific inflammation induced by tissue injury. However, regardless of its cause, this up-regulation appeared to at least partly be mediated via the same pathways as the much more powerful TNF-α-induced up-regulation.

The present experiments demonstrate that prolonged exposure to TNF-α markedly enhances the contractile response to des-Arg^9-bradykinin and bradykinin in the murine airways, a process involving the transcriptional increase of both bradykinin B_1 and B_2 receptors. Furthermore, the effects induced by the specific JNK and ERK 1/2 inhibitors demonstrated the involvement of the MAPK pathway in the process. It is therefore tempting to speculate in a role for MAPK as mediators of airway hyperresponsiveness in chronic airway inflammation.

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