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Published in:
European Journal of Pharmacology

DOI:
10.1016/j.ejphar.2010.02.020

2010

Up-regulation of bradykinin receptors in rat bronchia via IκB Kinase-mediated inflammatory signaling pathway

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Abstract

IκB kinase (IKK)-mediated intracellular signaling mechanisms may be involved in airway hyperresponsiveness through up-regulation of bradykinin receptors. This study was designed to examine if organ culture of rat bronchial segments induces airway hyperresponsiveness to bradykinin and if inhibition of IKK can abrogate the airway hyperresponsiveness to bradykinin via suppressing the expression of bradykinin B_1 and B_2 receptors. Rat bronchi were isolated and cut into ring segments. The segments were then organ cultured in the presence or absence of IKK inhibitors, BMS-345541 or TPCA-1. des-Arg^9-bradykinin (B_1 receptor agonist) and bradykinin (B_2 receptor agonist) induced contractions of the segments as monitored by a sensitive organ bath system. The expression of bradykinin B_1 and B_2 receptors, inflammatory mediators and phosphorylated IKK were studied by a real-time PCR and/or by immunohistochemsity using confocal microscopy. Organ culture of the bronchial segments induced a time-dependent up-regulation of bradykinin B_1 and B_2 receptors. The IKK inhibitors abolished the organ culture-induced up-regulation of bradykinin B_1 and B_2 receptor-mediated contractions in a concentration-dependent manner. This was paralleled with inhibition of IKK activity (phosphorylation), reduced mRNA and protein expressions of bradykinin B_1 and B_2 receptors and decreased mRNA expression of inflammatory mediators (interleukin-6, inducible nitric oxide synthase, cyclooxygenase 2 and matrix metalloproteinase 9). Our results show that organ culture induces IKK-mediated inflammatory changes in airways which subsequently results in airway hyperresponsiveness to bradykinin via the up-regulated bradykinin receptors. Thus, IKK inhibition might be a promising approach for treatment of airway inflammation and airway hyperresponsiveness that are often seen in asthmatic patients.

Keywords: IκB kinase, bradykinin, receptors, inflammatory mediators, BMS-345541, TPCA-1.

1. Introduction

Airway hyperresponsiveness and chronic airway inflammation are two major characteristics of asthmatic bronchia. Bradykinin and its related kinins have been implicated in the development of asthma bronchiale and airway inflammation (Barnes, 1986; Proud, 1994). In patients with asthma, inhalation of bradykinin causes bronchial contraction, while it has no such effects in health subjects (Fuller et al., 1987; Polosa et al., 1993). Asthmatic subjects show a greater degree of airway hyperresponsiveness to bradykinin than to methacholine after allergen challenge (Berman et al., 1995), suggesting that bradykinin plays an important role in the asthmatic airways.

Kinins exert their biological activities through two main bradykinin receptor subtypes, named as bradykinin B_1 and B_2 receptors (Hall, 1992; Regoli and Barabe, 1980). The bradykinin B_1 receptor, characterized by binding to des-Arg^9-bradykinin, is absent in the healthy airways, but can be induced during airway inflammation (Regoli et al., 1978), whereas bradykinin B_2 receptors show a high affinity for bradykinin and is constitutively expressed in airways (Hall, 1992; Regoli and Barabe, 1980). Bradykinin receptor expressions are up-regulated in sensitized rat lungs (Huang et al., 1999), in asthmatic airway inflammation (Christiansen et al., 2002) and in murine airways under interleukin-4 (IL-4) stimulation (Bryborn et al., 2004). However, there is limited knowledge about how airway bradykinin receptor expressions are regulated.

Previously, we have demonstrated that interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) induced transcriptional up-regulation of bradykinin B_1 and B_2 receptors in murine airways (Zhang et al., 2004; 2007), which could be abrogated by the anti-inflammatory drug dexamethasone, an inhibitor of NF-κB activity (Zhang et al., 2005). The majority of NF-κB is bound to an IκB inhibitory protein, which holds the complex in an inactive form in the cytoplasm. A critical phosphorylation of the IκB protein, in the classical pathway, is performed by the IκB kinase (IKK) complex, which consists of at least three subunits; two catalytic subunits (IKK-1 and IKK-2, also known as IKK-α and IKK-β) and a regulatory subunit, IKK-γ (Karin, 1999; Scheidereit, 1998; Whiteside and Israel, 1997). Given the importance of the catalytic subunit IKK-α and

IKK-β in regulating activation of the NF-κB dependent inflammatory process, identification of selective IKK inhibitors has received considerable interest. A highly selective inhibitor of IκB kinase, BMS-345541, blocks both joint inflammation and destruction in collagen-induced arthritis in mice (McIntyre et al., 2003). An IκB kinase-2 inhibitor TPCA-1 is identified and blocks inflammation in human airway smooth muscle cells and in a rat model of asthma (Birrell et al., 2005).

The present study was designed to examine if the IκB kinase-mediated inflammatory signaling pathway is involved in up-regulation of airway bradykinin B_1 and B_2 receptors. We demonstrate that bronchial segments after organ culture exhibited increased bradykinin B_1 and B_2 receptor-mediated contractions with enhanced expression of bradykinin B_1 and B_2 receptors in airway smooth muscle cells. The IKK inhibitors, BMS-345541 and TPCA-1, abrogated the up-regulation of airway bradykinin B_1 and B_2 receptors at functional, mRNA and protein levels, and in parallel the elevated mRNA expression of inflammatory mediators in the bronchial cells.

2. Materials and Methods

2.1 Tissue preparation and organ culture procedure

Male Sprague Dawley rats (body weight 250 g, M&B, Denmark) were acclimatized for one week under standardized temperature (21–22°C), humidity (50–60%) and light (12:12 light-dark) conditions in the Animal Department of Wallenberg center, Lund University, Lund (Sweden). The rats were killed by CO_2 and exsanguinated. The lungs were immersed in cold buffer solution (NaCl 119 mM; $NaHCO_3$ 15 mM; KCl 4.6 mM; $MgCl_2$ 1.2 mM; NaH_2PO_4 1.2 mM; $CaCl_2$ 1.5 mM and glucose 5.5 mM) and the bronchi were freed of adhering lung tissue down to the second generation by dissection under a microscope. Circular segments were cut from the bronchi with an outer diameter of 0.3 mm. The experimental protocol was approved by Lund University Animal Ethic's Committee (M161-07).

After the dissection, the segments were placed individually into wells of a 96-well

plate with 200 µl serum free DMEM culture medium containing L-glutamine (584 mg/L) and supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml). Incubation was performed at 37 °C in humidified 5 % CO_2 in air for the required time intervals (24, 48 or 96 h) in the presence and absence of the intracellular signal inhibitors. Segments were transferred into new wells containing fresh medium every 24 h. The signal inhibitors were not present during the experiments.

2.2 In vitro pharmacology

Bronchial segments were immersed into temperature controlled (37 °C) organ baths (Organ Bath Model 610M, J.P. Trading, Aarhus, Denmark) containing 5 ml bicarbonate buffer solution. The solution was continuously aerated with 5 % CO_2 in O_2 resulting in a pH of 7.4. The bronchial segments were mounted on two prongs for continuous recording of isometric tension by the Chart software (Chart 4, AD Instruments, Hastings, UK). A resting tone of 1.0 mN was applied to each segment, and the segments were allowed to stabilize at this tension for at least 1.5 h before being exposed to a potassium-rich (60 mM potassium) buffer solution with the same composition as the standard solution except that sodium chloride was replaced by an equimolar concentration of potassium chloride. The potassium-induced contraction was used as a reference for the contractile capacity, and the individual segments were only used for further studies if two strong (>1 mN) reproducible contractions (variation <10 %) elicited. Concentration–response curves for bradykinin receptor agonists were obtained by cumulative administration of the reagents. At a point 30 min before cumulative concentrations were administered, 3 µmol of indomethacin and 100 µmol of L-NG-monometylarginin (L-NMMA) were added to block the modifying effects of epithelial prostaglandin production and NO synthesis (Alm et al., 2002).

2.3 Real-time polymerase chain reaction (real-time PCR)

2.3.1 Total RNA isolation and reverse transcription into cDNA

Fresh or cultured bronchial segments were collected and immersed into RNA*later*$^{@}$ (Applied Biosystems) overnight at 4 °C, retrieve the segments from RNA*later* solution

then snap frozen in liquid nitrogen for RNA isolation. Following the manufacturer's protocol, total RNA preparations were obtained using the Trizol RNA isolation kit (Invitrogen, Sweden). The RNA was then resuspended in 10 µl of nuclease free water and the 260/280 values were measured by means of a Eppendorf Biophotometer (Hamburg,Germany). Reverse transcription of total RNA to cDNA was carried out using TaqMan Reverse Transcription Reagents (PE Applied Biosystems, CA, USA) in a Perkin-Elmer 2400 PCR (Perkin-Elmer, MA, USA) machine at 42 °C for 30 min.

2.3.2 Real-time PCR investigation

The real-time PCR was performed in a GeneAmp 7300 Sequence Detection system (Perkin-Elmer, Applied Biosystems) using the GeneAmp SYBR® Green kit (Perkin-Elmer, Applied Biosystems) with a 25 µl reaction volume. The PCR reaction started at a temperature of 50 °C for 2 min; 95 °C for 10 min and the following 40 PCR cycles with 95 °C for 15 s and 60 °C for 1 min. Dissociation curves were run after the real-time PCR to identify the specific PCR products. β-actin, elongation factor 1 (EF-1) were used as housekeeping genes. The gene expressions were normalized versus the housekeeping genes to account for differences in the starting material and in the cDNA reaction efficiency. The system automatically monitors the binding of a fluorescent dye to double-strand DNA by real-time detection of the fluorescence during each cycle of PCR amplification.

All primers were designed using the Primer Express 2.0 software (PE Applied Biosystems, CA, USA) and synthesized by TAGCopenhagen A/S (Copenhagen, Denmark). Total gene specificity of the nucleotide sequences chosen for primers was confirmed by results of BLAST searches (GenBank database sequences). The nucleotide sequences of the primers used in the investigation are shown in Table 1.

2.4 Immunohistochemistry

After organ culture, the bronchial segments were immersed in a fixative solution consisting of 4 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 3 h at 4°C. After fixation, the specimens were dehydrated in 20 % sucrose of phosphate buffer (0.1

M, pH 7.4) for 24 h at 4°C, and then frozen in Tissue-Tek (Sakura Finetek Europe B.V., Zoeterwoude, Netherlands) and stored at −80 °C. Sections were cut at 10 μm thickness in a cryostat and mounted on SuperFrost Plus slides. Immunohistology staining with primary antibody against rat bradykinin B_1 receptor (Santa Cruz, Biotechnology, CA, USA), bradykinin B_2 receptor (Santa Cruz, Biotechnology, CA, USA) and phospho-IκB kinase α/β (Ser 176/180, monoclonal antibody, Cell Signaling). Briefly, the sections were incubated with the primary antibody (dilution: B1R 1:50; B2R 1:50; phospho-IKK α/β 1:100) overnight at 4°C, thereafter the secondary antibody donkey anti-rabbit IgG conjugated to Cy^{TM}2 (Jackson ImmunoResearch, 1:200 dilution) was applied for 1 hr at room temperature in dark. To identify the smooth muscle cell layer of the bronchial segments, immunohistology staining with the primary antibody against rat smooth muscle actin (Santa Cruz, 1:200 dilution) and the secondary antibody donkey anti-mouse IgG (H+L) conjugated to Texas Red (Jackson ImmunoResearch, 1:200 dilution) were also performed. In the control experiments, either the primary antibody or the secondary antibody was omitted. The stained bronchial segments were observed under a confocal microscope (Nikon, C1plus, Nikon Instruments Inc., NY, USA) and analysed by Image J software (http://rsb.info.nih.gov/ij). The fluorescence intensity was measured on the smooth muscle cells. For each bronchial segment, six randomly selected sections were studied. In each section, the fluorescence intensity was measured at six preset areas.

2.5 Chemicals

Bradykinin B_1 receptor agonist des-Arg^9-bradykinin, bradykinin B_2 receptor agonist bradykinin, IKK inhibitors TPCA-1([5-(p-Fluorophenyl)-2-ureido]thiophene-3-carboxa--mide) and BMS 345511(N-(1,8-Dimethylimidazo[1,2-a]quinoxalin-4-yl)-1,2-ethane--diaminehydrochloride), acetylcholine, serotonin, sarafotoxin6c, endothelin-1, L-NG-monometylarginin (L-NMMA), indomethacin, actinomycin D and cycloheximide (Sigma).

2.6 Statistics

All data are expressed as mean values ± S.E.M. Contractile responses to bradykinin receptor agonists in each segment were expressed as percent of maximal contraction induced by 60 mM potassium buffer solution. Each agonist concentration-effect curve was fitted to the Hill equation using an iterative, least square method (GraphPad Prism 4, San Diego, U.S.A) to provide estimates of maximal contraction (E_{max}) and pEC_{50} values (negative logarithm of the agonist concentration that produces 50% of the maximum effect). Two-way analysis of variance (ANOVA) with Bonferroni post-test was used to compare the two corresponding data points at each concentration of the two curves. The amount of receptor mRNA is expressed relative to housekeeping gene mRNA. The one-way analysis of variance (ANOVA) with Dunnet post-test was used for comparison of more than two data sets. The data and statistical analysis was performed with Graph-Pad Prism 4. $P < 0.05$ was considered as statistically significant.

3. Results

3.1 In vitro pharmacology

3.1.1 Up-regulation of des-Arg^9-bradykinin and bradykinin induced airway contraction

Basal contractile responses to des-Arg^9-bradykinin (selective B_1 receptor agonist) and bradykinin (selective B_2 receptor agonist) on rat bronchial ring segments were studied in freshly isolated segments. The fresh segments exhibited a negligible contractile effect induced by des-Arg^9-bradykinin (Fig. 1A) and a weak contraction was induced by bradykinin (Fig.1B).

The bronchial ring segments were organ cultured in serum free medium for 24, 48 or 96 h in order to study the time course effects. Compared with control (fresh segments), organ culture resulted in a time-dependent enhanced contractions to both des-Arg^9-bradykinin and bradykinin (Table 2, Fig. 1A-B). The maximal effects were reached at 48 h for des-Arg^9-bradykinin and at 96 h for bradykinin. Leftwards shifts of the concentration effect curves were seen for both des-Arg^9-bradykinin and bradykinin (Fig. 1A-B, Table 2). The maximal contractile responses (E_{max}) to des-Arg^9-bradykinin and bradykinin at 24 h increased from 0.89 ± 0.18 % to 44.73± 5.4 % for

des-Arg^9-bradykinin, and from 41.34 ± 6.65 % to 103.3 ± 17.82 % for bradykinin. This up-regulation of contractile responses were further enhanced (for des-Arg^9-bradykinin, E_{max} from 44.73± 5.4 % to 73.91± 3.31 %; for bradykinin, E_{max} from 103.3 ± 17.82 % to 141.7± 9.80 %), when the culture periods extended to 48 h. Organ culture for 96 h did not cause a further up-regulation of des-Arg^9-bradykinin-induced contraction (Table 2, Fig. 1A), while it further enhanced the up-regulation of bradykinin-induced contraction (Table 2, Fig. 1B). However, we did not see significant variations of the KCl-induced contractions during organ culture up to 96 h (fresh: 2.02 ± 0.19 mN versus 24 h: 2.17 ± 0.36 mN , 48 h: 2.10 ± 0.18 mN and 96 h: 2.07 ± 0.19 mN).

In addition, contractile responses to serotonin, sarafotoxin 6c, endothelin-1 and acetylcholine were also examined in the 48 h organ culture. There were no significant changes in contractile response to serotonin, sarafotoxin 6c and endothelin-1 (Table 3), while the acetylcholine-induced contractile response was enhanced at 48 h of organ culture (Table 3).

3.1.2 Role of transcriptional and translational mechanisms in up-regulation of des-Arg^9-bradykinin- and bradykinin-induced contractions

Actinomycin D (general transcriptional inhibitor, AcD, 5 mg/L) and cycloheximide (general translational inhibitor, CHX, 10^{-5} M) were added to organ culture for 24 h, respectively, in order to block *de novo* transcription or translation of bradykinin receptors induced by organ culture. The results showed that in comparison with control (vehicle), the up-regulation of des-Arg^9-bradykinin-induced contraction was completely abolished by AcD (Fig. 2 A) or CHX (Fig. 2 C). While,both AcD (by 55%, Fig. 2 B) and CHX (by 28%, Fig. 2 D) only partly abrogated the up-regulation of the bradykinin-induced contraction.

In addition, the enhanced contractile response to acetylcholine was not affected by the transcriptional inhibitor AcD (vehicle, E_{max}: 188.6 ± 2.98, pEC_{50}: 6.58 ± 0.04; AcD, E_{max}: 182.1 ± 3.24, pEC_{50}: 6.49 ± 0.04) or the translational inhibitor CHX (vehicle, E_{max}: 188.6 ± 4.51, pEC_{50}: 6.42 ± 0.05; AcD, E_{max}: 183.1 ± 2.56, pEC_{50}: 6.56 ± 0.03).

3.1.3 Effects of epithelial removal

In order to ascertain if the epithelium is involved in the up-regulation of des-Arg^9-bradykinin- or bradykinin-induced contraction, the epithelium was removed before the bronchial segments were organ cultured for 48 h: denudation of epithelium was confirm by immunohistochemistry (data not shown). Removal of epithelium did not modify the up-regulation of contractile responses to des-Arg^9-bradykinin (Fig.1C, epithelium intact E_{max} 59.68 ± 4.17 %, and epithelium denuded E_{max} 63.41 ± 3.19 %) or bradykinin (Fig.1D, epithelium intact E_{max} 156.5 ± 15.61 %, and epithelium denuded E_{max} 135.2 ± 12.77 %).

3.1.4 Effects of IκB kinase (IKK) inhibitors on organ culture-induced up-regulation of des-Arg^9-bradykinin- and bradykinin- induced contractions

In order to examine the role of IKK-mediated intracellular signal pathway in the up-regulation of des-Arg^9-bradykinin- and bradykinin-induced contractions in the airway, bronchial segments were organ cultured in the presence of IKK inhibitors, BMS-345541 (1 μM ~ 10 μM) or TPCA-1 (0.3 μM ~ 10 μM) or vehicle (same volume) for 48 h. des-Arg^9-bradykinin- and bradykinin-induced contractions were concentration-dependently inhibited by either BMS-345541 or TPCA-1. Both BMS-345541 (Table 4, Fig. 3A) and TPCA-1 at 10 μM (Table 4, Fig. 3C) completely abolished the up-regulation of des-Arg^9-bradykinin-induced contractions. TPCA-1 at 3 μM and 10 μM significantly inhibited the enhanced bradykinin-induced contraction (by 27 % at 3 μM, by 67 % at 10 μM, Table 4, Fig. 3D), whereas only 10 μM BMS-345541 significantly inhibited the enhanced bradykinin-induced contraction (by 78%, Table 4, Fig. 3B).

In addition, the increased contractile responses to acetylcholine was not affected by either 10 μM BMS-345541 (vehicle, E_{max}: 230.4 ± 14.91, pEC_{50}: 5.93 ± 0.14; BMS-345541, E_{max}: 216.5 ± 8.80, pEC_{50}: 6.15 ± 0.08) or 10 μM TPCA-1 (vehicle, E_{max}: 236.8 ± 14.53, pEC_{50}: 5.99 ± 0.14; TPCA-1, E_{max}: 231.6 ± 12.9, pEC_{50}: 5.87 ± 0.11) in bronchial segments organ cultured for 48 h.

To examine the direct effect of the IKK inhibitors BMS-345541 (10 μM) or

TPCA-1 (10 μM) on des-Arg^9-bradykinin and bradykinin-induced bronchial contractions, the inhibitors were applied 30 min before obtaining the des-Arg^9-bradykinin or bradykinin concentration effect curves in segments cultured for 48 h. 10 μM BMS-345541 did not modify the bronchial contractile responses to des-Arg^9-bradykinin or bradykinin; while 10 μM TPCA-1 significantly decreased the bronchial contractile responses to des-Arg^9-bradykinin and bradykinin (Table 5). In order to demonstrate that this direct effect of TPCA-1 can be abolished by washing, we performed a series of experiments applying 10 μM TPCA-1 into the organ bath for 30 min and then followed by wash-out. The direct effects of TPCA-1 on bronchial contractile responses to des-Arg^9-bradykinin and bradykinin were completely abrogated by the washing (Table 5).

3.2 Alteration of mRNA expressions of bradykinin receptors and inflammatory mediators

The total RNA was extracted from fresh and 48 h of organ culture in the absence and presence of IKK inhibitors BMS-345541 (10 μM) or TPCA-1 (10 μM). Compared with control (fresh), mRNA expressions of bradykinin B_1 (Fig. 4A) and B_2 receptors (Fig. 4B) were significantly enhanced in segments after 48 h of organ culture. Either BMS-345541 (10 μM) or TPCA-1 (10 μM) administration significantly inhibited the enhanced mRNA expressions for both bradykinin B_1 and B_2 receptors (BMS-345541: by 66% for bradykinin B_1 receptor, by 61% for bradykinin B_2 receptor; TPCA-1: by 77% for bradykinin B_1 receptor, by 58% for bradykinin B_2 receptor; Fig. 4 A-B).

In addition, the mRNA expression of inflammatory mediators, TNF-α, IL-1β, IL-6, ICAM-1, MMP-9, COX-2 and iNOS were studied. The mRNA expression of IL-6 (Fig. 5A), COX-2 (Fig. 5B), iNOS (Fig. 5C) and MMP-9 (Fig. 5D) were significantly up-regulated after 48 h of organ culture, while organ culture did not affect TNF-α and IL-1β (data not shown) mRNA expressions. The IKK inhibitor TPCA-1 (10 μM) significantly inhibited the increased mRNA expression of IL-6 (by 92%), MMP-9 (by 59%), COX-2 (by 43%) and iNOS (by 93%), whereas BMS-345541 (10 μM) only had an inhibitory effects on the increased mRNA expression of IL-6 (by 54%, Fig. 5A, $p<0.05$). The mRNA expression of ICAM-1 was not detected in fresh and 48 h organ

cultured groups (data not shown).

3.3 Alteration of bradykinin B_1 and B_2 receptor and phosphorylation of IKK α/β protein expression

The protein expression of bradykinin B_1 and B_2 receptors and phosphorylated IKK α/β were visualized in bronchial smooth muscle cells and bronchial epithelium cells by immunohistochemistry using confocal microscopy.

The bradykinin B_1 (Fig. 6) and B_2 receptors (Fig. 7), and the phosphorylated IKK α/β protein (Fig. 8) were clearly observed in green color in the smooth muscle cell layer (identified by smooth muscle actin staining, data not shown) and epithelium layer in bronchial segments. There was only a weak expression of phosphorylated IKK α/β protein in bronchial smooth muscle cells in the fresh group (Fig. 8A), while the phosphorylated IKK α/β protein was increased at 48 h of organ culture in the absence (Fig. 8B) and presence of vehicle (DMSO) (Fig. 8C). The IKK inhibitor BMS-345541 (Fig. 8D) and TPCA-1 (Fig. 8E) decreased the enhanced phosphorylated IKK α/β protein expression in the bronchial smooth muscle layer. There were no significant changes of phosphorylated IKK α/β protein in the epithelium layer (Fig. 8A-8E). The fresh bronchial segments showed a weak B_1 receptor immunoreactivity (Fig. 6A) and a positive B_2 receptor immunoreactivity (Fig. 7A) localized to the smooth muscle cell layer. The immunoreacivity of both receptors were more pronounced in bronchial segments after 48 h of organ culture in the absence (Fig. 6B, Fig. 7B) and presence of vehicle (DMSO) (Fig. 6C, Fig. 7C) and appeared fainter in bronchial segments organ cultured for 48 h with IKK inhibitor BMS-345541 (Fig. 6D, Fig. 7D) or TPCA-1 (Fig. 6E, Fig. 7E), respectively. However, there were no significant changes of bradykinin B_1 and B_2 receptor protein expressions in the epithelium layer (Fig. 6A-6E, Fig. 7A-7E). Measurements of bradykinin B_1 and B_2 receptor protein and phosphorylated IKK α/β protein density showed that bradykinin B_1 ($p<0.01$, Fig. 6 F) and B_2 receptor protein ($p<0.01$, Fig. 7 F) and phosphorylated IKK α/β protein ($p<0.01$, Fig. 8 F) expression in bronchial smooth muscle layer were significantly enhanced after 48 h of organ culture in the absence or presence of vehicle (DMSO). Treatment with 10 μM BMS-345541 or

10 μM TPCA-1 significantly decreased the enhanced bradykinin B_1 (BMS-345541 $p<0.01$, TPCA-1 $p<0.01$, Fig. 6 F) and B_2 receptor (BMS-345541 $p<0.05$, TPCA-1 $p<0.05$, Fig. 7 F) protein and phosphorylated IKK α/β protein (BMS-345541 $p<0.01$, TPCA-1 $p<0.01$, Fig. 8 F) expression in bronchial smooth muscle layer in comparison with 48 h of organ culture in the presence of vehicle. Measurements of bradykinin B_1 (Fig. 6F) and B_2 receptor (Fig. 7F) protein and phosphorylated IKK α/β protein (Fig. 8F) density in bronchial epithelium layer did not show any differences.

4. Discussion

Airway hyperresponsiveness is characterized by an increased sensitivity of airway smooth muscle cells to bronchio-constrictor agents, which can be demonstrated in almost all patients with current symptomatic asthma (Cockcroft and Davis, 2006). The increased sensitivity of the airways to constrictor agonists results in a steeper slope of the dose-response relationship and a greater maximal response to the agonist (O'Byrne and Inman, 2003). Both bradykinin B_1 and B_2 receptors are well-recognized to play an important role in allergic airway hyperresponsiveness and airway inflammation (Christiansen et al., 2002; Farmer and Burch, 1991; Kusser et al., 2001). In the present study we demonstrated that organ culture of the bronchial segments induced a time-dependent up-regulation of bradykinin B_1 and B_2 receptor-mediated contractions with enhanced mRNA and protein expressions for bradykinin B_1 and B_2 receptors. The IKK inhibitors, BMS-345541 and TPCA-1, abolished the organ culture-induced up-regulation of bradykinin B_1 and B_2 receptors and abrogated the airway hyperresponsiveness to des-Arg^9-bradykinin and bradykinin. This occurred with a parallel inhibition of the IKK activity (phosphorylation) and decreased mRNA expression of the inflammatory mediators IL-6, COX-2, MMP-9 and iNOS.

There was no contractile response to the bradykinin B_1 receptor agonist des-Arg^9-bradykinin and only a weak contractile effect of the bradykinin B_2 receptor agonist bradykinin in fresh bronchial segments, which is in concert with other reports (Polosa and Holgate, 1990; Reynolds et al., 1999). We have previously characterized

the selectivity of the responses mediated by des-Arg^9-bradykinin and bradykinin in an *in vitro* model of chronic airway inflammation; it was demonstrated that the des-Arg^9-bradykinin induced contraction is mediated by the bradykinin B_1 receptor; bradykinin induced contraction occur via the bradykinin B_2 receptor, while bradykinin at high concentration may in addition activate the bradykinin B_1 receptor (Zhang et al., 2004). It is commonly believed that in contrast to the constitutive expression of bradykinin B_2 receptor, the cell surface expression of bradykinin B_1 receptor is inducible. However, there is evidence which show that it is possible that both B_2 and B_1 receptors are expressed in the murine airways (Li et al., 1998). Here we observed strong immunoreactivity of B_1 and B_2 receptors in the epithelium of fresh rat bronchi; a positive B_2 receptor immunoreactivity and a weak B_1 receptor expression were observed in fresh bronchial smooth muscle layer. This confirms that both bradykinin B_1 and B_2 receptors are expressed in normal rat bronchi. In the present study, we found that after organ culture for up to 48 h, both bradykinin B_1 and B_2 receptors were up-regulated at functional, mRNA and protein levels. The epithelial removal did not affect the up-regulation of des-Arg^9-bradykinin- and bradykinin-induced contractions, which suggest that the enhanced contractile responses to des-Arg^9-bradykinin and bradykinin are mediated via bronchial smooth muscle cells. This is supported by the unchanged immunoreactivity of B_1 and B_2 receptors in bronchial epithelium after 48 h of organ culture. Therefore, we have provided an *in vitro* model of airway smooth muscle hyperresponsiveness to des-Arg^9-bradykinin and bradykinin in this study, which is mediated by up-regulation of bradykinin B_1 and B_2 receptors in airway smooth muscle cells. Interestingly, the contractile responses to serotonin, sarafotoxin 6c and endothelin-1 were not affected by 48 h of organ culture; the contractile response to acetylcholine was enhanced by 48 h of organ culture but it does not seem to be attributed to the transcription or translation mechanisms. This may suggest that the organ culture-induced airway hyperresponsivenss to bradykinin and the up-regulation of bradykinin receptors are rather selective.

Up-regulation of the bradykinin B_1 receptor has been found in airway and other tissues during inflammation (Christiansen et al., 2002; Hara et al., 2008; Vianna et al.,

2003); the hyperresponsiveness to bradykinin or up-regulation of the bradykinin B_2 receptor has been reported in airway inflammation models (Ellis et al., 2004; Kim et al., 2005). Data obtained previously have demonstrated that NF-κB signaling plays an important role in the process up-regulation of the bradykinin B_1 receptor (Moreau et al., 2007; Ni et al., 1998; Sabourin et al., 2002; Schanstra et al., 1998). Others have also shown that the NF-κB pathway is important for inflammatory cytokine enhanced expression of bradyinin B_1 and B_2 receptors in osteoblasts and fibroblasts (Brechter et al., 2008). The catalytic subunit IKK-1 and IKK-2 of IκB kinase (IKK) complex exert the important regulating effects upon activation of the NF-κB. Therefore, we tested the two selective IKK inhibitors BMS-345541 and TPCA-1 in the present study to explore if the IKK-mediated intracellular inflammatory signal pathway is involved in up-regulation of bradykinin receptors. As expected, BMS-345541 and TPCA-1 concentration-dependently inhibited the up-regulation of bradykinin B_1 and B_2 receptors at mRNA, protein and functional levels. BMS-345541 and TPCA-1 have been demonstrated to be highly selective inhibitors of IKK and NF-κB dependent transcription *in vitro* and *in vivo.* BMS-345541 is recognized as a high selective IκB kinase1/2 inhibitor, IC_{50}= 0.3 μM on IKK-2 and IC_{50}= 4 μM on IKK-1.(Burke et al., 2003); TPCA-1 is a highly selective IκB kinase 2 inhibitor, the results from 57 assays gave a mean IC_{50} = 17.9 nM on IKK-2 and has 22-fold selectivity over IKK-1 (Podolin et al., 2005).

In the present study, TPCA-1, at 0.3 μM and 3 μM started to show an inhibitory effect; while BMS-345541, at 1 μM and 10 μM, started to show an inhibitory effect. This is in concert with previous findings that TPCA-1 has higher inhibitory potency on either IKK-1 or IKK-2 than BMS-345541(Burke et al., 2003; Podolin et al., 2005). Both 10 μM BMS-345541 and 10 μM TPCA-1 exerted maximal inhibitory effects on the up-regulation of des-Arg^9-bradykinin- and bradykinin -induced contraction, according to the inhibitory selectivity on IKK subtypes by BMS-345541 and TPCA-1, which suggests that both IKK-1 and IKK-2 subtype are involved in the transcriptional up-regulation of bradykinin B_1 and B_2 receptors. Moreover, the up-regulation of bradykinin-induced contraction was not completely inhibited by 10 μM BMS-345541 or

10 μM TPCA-1.This is most likely due to the fact that the up-regulation of bradykinin B_2 receptor also involves other mechanisms besides IKK dependent signals. In addition, BMS-345541 has no direct effects on des-Arg^9-bradykinin- and bradykinin -induced contraction in the organ bath, while TPCA-1 has a direct effect on decrease in contractile response to des-Arg^9-bradykinin or bradykinin, which could be completely abrogated by the washing, and that not relative to transcription/translation.

The expression of inflammatory genes of IL-6, MMP-9, COX-2 and iNOS in bronchial segments were significantly increased after 48 h of organ culture. IL-6 is a potent pro-inflammatory cytokine that exerts inflammatory effects by activating both leukocytes and structural cells including pulmonary epithelial cells. The levels of IL-6 are increased in the induced sputum, bronchoalveolar lavage, and in peripheral blood of patients with COPD (Bhowmik et al., 2000; Bucchioni et al., 2003; Kim et al., 2008). Increased expressions of COX-2, iNOS and MMP-9 have been observed in airway inflammation (Birrell et al., 2006; Redington et al., 2001). It has been reported that recombinant human IL-6 significant leftward shifts the concentration-response curve of des-Arg^9-bradykinin in human umbilical vein (Sardi et al., 2002); COX-2 may participate in the up-regulation of B_1 receptor-mediated contraction of the rabbit aorta (Medeiros et al., 2001); inhibition of iNOS significantly reduced the up-regulation of B_1 receptor mediated contraction in a murine colitis model (Hara et al., 2008). Thus, the up-regulation of B_1 receptor mediated contraction in the present setup may also be regulated by the overexpression of the inflammatory gene. However, the expression of inflammatory genes including IL-6, MMP-9, COX-2 and iNOS were considerably sensitive to repression by bradykinin B_2 receptor antagonists (Hellal et al., 2003; Hsieh et al., 2008; Lee et al., 2008; Zhang et al., 2008), which may suggest an important role of B_2 receptors in regulating of these genes. Although the present study did not provide enough evidence to reveal the relationship between the up-regulation of bradykinin receptors and the overexpression of inflammatory genes, a practical model has been provided here to further studies on airway inflammation and airway hyperresponsivness. TPCA-1, a highly selective IKK-2 inhibitor, has been identified as an effective inhibitor of airway inflammation *in vitro* as well as *in vivo* (Birrell et al., 2005). BMS-345541, a

highly selective IKK 1/2 inhibitor, has been shown to reduce joint inflammation and destruction in collagen-induced arthritis in mice (McIntyre et al., 2003). Here, we report that the IKK inhibitor TPCA-1 markedly inhibited the organ culture-induced inflammatory gene overexpression of IL-6, MMP-9, COX-2 and iNOS, whereas BMS-345541 only exerted a significant inhibitory effect on IL-6 up-regulation.

In summarize, we have demonstrated that activation of the IKK-mediated inflammatory signal pathway results in airway hyperresponsiveness to des-Arg^9-bradykinin and bradykinin via transcriptional up-regulation of bradykinin B_1 and B_2 receptors. The IKK inhibitors, BMS-345541 and TPCA-1, exert markedly inhibitory effects on airway hyperresponsiveness to bradykinin B_1 and B_2 receptor agonists and on overexpression of inflammatory genes in the rat bronchi. The present findings may address a possible pathway of organ culture-induced airway hyperresponsivess (Fig. 9). Understanding the molecular mechanisms that lead to airway hyperresponsiveness and airway inflammation may provide new options for the treatment.

Acknowledgements

The present work was supported by the Swedish Research Council (grant no. 5958), the Swedish Heart-Lung Foundation (grant no. 20070273) and the Flight Attendant Medical Research Institute, USA.

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Table 1 Accession numbers and primer sequence for the genes that were investigated

Gene name	Abbreviation	Accession No.	Primer sequence
Beta-actin	ACTB	NM_031144.2	Fwd: 5’-GTAGCCATCCAGGCTGTGTTG-3’ Rev: 5’-TGCCAGTGGTACGACCAGAG-3’
Elongatin factor 1	EF-1	NM_175838.1	Fwd: 5’-GCAAGCCCATGTGTGTTGAA-3’ Rev: 5’-TGATGACACCCACAGCAACTG-3
Bradykinin B1 receptor	BKB1R	NM_030851.1	Fwd: 5’-CTGGCCCTTCGGAACTGA-3’ Rev: 5’-CAAACAGGTTGGCCTTGATGAC-3’
Bradykinin B2 receptor	BKB2R	NM_173100.1	Fwd: 5’-ATCACCATCGCCAATAACTTCGA-3’ Rev: 5’-CACCACGCGGCACAG-3’
Tumor necrosis factor-alpha	TNF-α	NM_012675.2	Fwd: 5’-AAATGGGCTCCCTCTCATCAGTTC-3’ Rev: 5’-TCTGCTTGGTGGTTTGCTACGAC-3’
Interleukin-1beta	IL-1β	NM_031512.2	Fwd: 5’- TTGTGCAAGTGTCTGAAGCA-3’ Rev: 5’- TGTCAGCCTCAAAGAACAGG-3’
Interleukin 6	IL-6	NM_012589.1	Fwd: 5’-AAGAGACTTCCAGCCAGTTGCC-3’ Rev: 5’- ACTGGTCTGTTGTGGGTGGTATC-3’
Matrix metalloproteinase 9	MMP-9	NM_031055.1	Fwd: 5’-AAGCCTTGGTGTGGCACGAC-3’ Rev: 5’-TGGAAATACGCAGGGTTTGC-3’
Cyclooxygenase 2	COX-2	AF233596.1	Fwd: 5’-TGTATGCTACCATCTGGCTTCGG-3’ Rev: 5’-GTTTGGAACAGTCGCTCGTCATC-3’
Inducible nitric oxide synthase	iNOS	NM_012611.3	Fwd: 5’-CAATGGCTTGAGGCAGAAGC-3’ Rev: 5’-GCCACCTCGGATATCTATTGC-3’

Table 2 Maximal contractile response (E_{max}) and pEC_{50} values to des-Arg^9-bradykinin and bradykinin in fresh segments and following organ culture

Incubation time	des-Arg^9-bradykinin			bradykinin		
	n	E_{max} (% of 60mM K^+)	pEC_{50}	n	E_{max} (% of 60mM K^+)	pEC_{50}
Fresh	16	0.89 ± 0.18	N.D.	7	41.34 ± 6.65	6.12 ± 0.25
24 h	7	44.73 ± 5.40 [b]	6.20±0.17	12	103.4 ±17.82 [b]	6.27 ± 0.38
48 h	15	73.91 ± 3.31 [b]	6.72±0.07	13	141.7 ± 9.8 [b]	7.56 ±0.17 [b]
96 h	8	59.02 ± 4.39 [b]	6.88±0.13	12	149.6 ± 3.97 [b]	8.45 ±0.08 [b]

Values are expressed as mean ± S.E.M.. Statistical analysis was performed with one-way ANOVA and Dunnet post-test [b]P <0.01 was compared with fresh segments. N.D. = not determined

Table 3 Maximal contractile response (E_{max}) and pEC_{50} values to serotonin (5-HT), sarafotoxin6c (S6c), endothelin-1(ET-1) and acetylcholine (ACh) in fresh segments and 48 h organ culture

Agonists	Fresh			48 h organ culture		
	n	E_{max} (% of 60mM K^+)	pEC_{50}	n	E_{max} (% of 60mM K^+)	pEC_{50}
5-HT	8	98.70 ± 7.18	5.71± 0.07	8	90.24 ± 4.09	5.87± 0.04
S6c	8	163.6 ± 24.0	8.35± 0.30	8	149.4 ± 10.7	8.53± 0.12
ET-1	8	152.7 ± 17.14	8.40 ± 0.21	8	149.5 ± 10.67	8.53 ± 0.13
ACh	8	161.8 ± 8.44	5.16± 0.08	8	226.0 ± 13.15[b]	5.65 ± 0.11 [b]

Values are expressed as mean ± S.E.M.. Statistical analysis was performed with unpaired t-test. [b]P <0.01 was compared with fresh segments.

Table 4 Effects of IKK inhibitors BMS-345541 and TPCA-1 on the maximal contraction (E_{max}) and pEC_{50} values of des-Arg^9-bradykinin and bradykinin in the segments cultured for 48 h in the presence of vehicle (DMSO) or BMS-345541 or TPCA-1.

Inhibitors	des-Arg^9-bradykinin			bradykinin		
	n	E_{max} (% of 60mM K^+)	pEC_{50}	n	E_{max} (% of 60mM K^+)	pEC_{50}
Vehicle	12	72.72 ± 3.21	7.34 ± 0.09	15	146.9 ± 6.31	7.72±0.11
BMS-345541 1μM	6	45.08 ± 3.42 [b]	7.28 ± 0.15	6	134.6 ± 11.6	7.93±0.23
BMS-345541 3μM	6	33.63 ± 2.40 [b]	7.09 ± 0.11	7	133.7 ± 9.28	7.67±0.18
BMS-345541 10μM	9	0.97 ± 0.24 [b]	N.D.	9	32.36 ± 1.55 [b]	8.39±0.15
TPCA-1 0.3μM	9	58.17 ±2.43 [b]	7.19 ± 0.08	6	135.2 ± 12.1	7.83 ± 0.23
TPCA-1 1 μM	7	44.35 ± 2.57 [b]	7.11± 0.10	7	123.1± 4.51 [a]	8.19 ± 0.11
TPCA-1 3 μM	7	14.49 ± 1.91 [b]	6.29 ± 0.18 [b]	7	108.2 ± 3.64 [b]	7.65±0.08
TPCA-1 10μM	8	0.98 ± 0.20 [b]	N.D.	8	64.49 ± 1.97 [b]	7.62±0.07

Values are expressed as mean ± S.E.M.. Statistical analysis was performed with one-way ANOVA and Dunnet post-test [a]P <0.05, [b]P <0.01 was compared with vehicle group. N.D. =not determined

Table 5 The direct effects of IKK inhibitors BMS-345541 and TPCA-1 on the maximal contraction (E_{max}) and pEC_{50} values of des-Arg^9-bradykinin and bradykinin in the bronchial segments

Inhibitors	des-Arg^9-bradykinin			bradykinin		
	n	E_{max} (% of 60mM K^+)	pEC_{50}	n	E_{max} (% of 60mM K^+)	pEC_{50}
Vehicle	10	69.68 ± 3.52	7.39 ± 0.11	10	133.7 ± 6.24	7.70 ± 0.14
BMS-345541 10 μM (30 min)	6	73.64 ± 1.85	7.42 ± 0.05	6	124.5 ± 3.30	7.57 ± 0.21
TPCA-1 10 μM (30 min)	6	9.12 ± 0.28 [b]	7.17 ± 0.05	6	114.1 ± 15.7	6.23 ± 0.33[b]
TPCA-1 10 μM (after washing)	6	74.16 ± 3.10	7.37 ± 0.09	6	136.2 ± 6.27	7.52 ±0.13

The bronchial segments were organ cultured. The IKK inhibitor (BMS-345541 or TPCA-1) was added to the organ bath for 30 min. Thereafter the inhibitor was kept in contact with the segments or wash out. The des-Arg^9-bradykinin or bradykinin concentrations effects were obtained in the segments without washing and with washing, respectively. The Values are expressed as mean ± S.E.M.. Statistical analysis was performed with one-way ANOVA and Dunnet post-test [b]P <0.01 was compared with vehicle group.

Figure legends

Figure 1

Time course of organ culture on the contractile responses to des-Arg^9-bradykinin (d-BK) (A) and bradykinin (BK) (B). The effects of epithelium denuded on the contractile responses to des-Arg^9-bradykinin (d-BK) (C) and bradykinin (BK) (D).Each data point is derived from 7–16 experiments and presented as mean ± S.E.M.. Statistical analysis was performed by two-way ANOVA with Bonferroni post-test. **P* <0.05, ***P* <0.01, ****P* <0.001 was compared with fresh segments. OC: organ culture.

Figure 2

Effects of actinomycin D (A, B) and cycloheximide (C, D) on des-Arg^9-bradykinin (d-BK) (A, C) and bradykinin (BK) (B, D) induced contractions at 24 h of organ culture. Each data point is derived from 8-10 experiments and presented as mean ± S.E.M.. Statistical analysis was performed by two-way ANOVA with Bonferroni post-test. **P* <0.05, ***P* <0.01, ****P* <0.001 was compared with vehicle. ACD: actinomycin D, CHX: cycloheximide.

Figure 3

Effects of BMS-345541 (A, B) and TPCA-1 (C, D) on des-Arg^9-bradykinin (d-BK) (A, C) and bradykinin (BK) (B, D) induced contractions at 48 h of organ culture. Each data point is derived from 6-15 experiments and presented as mean ± S.E.M.. Statistical analysis was performed by two-way ANOVA with Bonferroni post-test. **P* <0.05, ***P* <0.01, ****P* <0.001 was compared with vehicle (DMSO) group. BMS: BMS-345541.

Figure 4

mRNA expression of bradykinin B1 (A) and B2 (B) receptor in the bronchial segments. The bronchial segments were cultured for 48 h in the absence and presence of vehicle (DMSO), 10 μM BMS-345541 and 10 μM TPCA-1. Each data point is derived from six

experiments and presented as mean ± S.E.M.. Statistical analysis was performed by one-way ANOVA with Dunnet post-test. N.S. = not significant; *P <0.05, **P <0.01.OC: organ culture, BMS: BMS-345541.

Figure 5

mRNA expression of inflammatory gene IL-6 (A), COX-2 (B), iNOS (C) and MMP-9 (D) in the bronchial segments. The bronchial segments were cultured for 48 h in the absence and presence of vehicle (DMSO), 10 μM BMS-345541 and 10 μM TPCA-1. Each data point is derived from six experiments and presented as mean ± S.E.M.. Statistical analysis was performed by one-way ANOVA with Dunnet post-test. N.S. = not significant; *P <0.05, **P <0.01. OC: organ culture, BMS: BMS-345541.

Figure 6

Bradykinin B1 receptor protein expressions in bronchial epithelium and smooth muscle are assessed by immunohistochemisty in bronchial segments of fresh (A), 48 h organ culture (B), 48 h organ culture in the presence of vehicle (DMSO) (C) , BMS-345541 10 μM (D) and TPCA-1 10 μM (E). The size bar corresponds to 100 μm. SMC; smooth muscle cell, EP: epithelium. Semi-quantitation of bradykinin B1 receptor protein in bronchial epithelium layer and bronchial smooth muscle layer (F), each data is derived from 6 experiments and presented as mean ± S.E.M., n= 6. Statistical analysis was performed by one-way ANOVA with Dunnet post-test. *P <0.05, **P <0.01.OC: organ culture, BMS: BMS-345541.

Figure 7

Bradykinin B2 receptor protein expressions in bronchial epithelium and smooth muscle are assessed by immunohistochemisty in bronchial segments of fresh (A), 48 h organ culture (B), 48 h organ culture in the presence of vehicle (DMSO) (C), BMS-345541 10 μM (D) and TPCA-1 10 μM (E). The size bar corresponds to 100 μm. SMC; smooth muscle cell, EP: epithelium. Semi-quantitation of bradykinin B2 receptor protein in bronchial epithelium layer and bronchial smooth muscle layer (F), each data is derived

from 6 experiments and presented as mean ± S.E.M., n=6. Statistical analysis was performed by one-way ANOVA with Dunnet post-test. *P <0.05, **P <0.01.OC: organ culture, BMS: BMS-345541.

Figure 8

Phospho-IKKα/β (Ser176/180) protein expressions in bronchial epithelium and bronchial smooth muscle are assessed by immunohistochemisty in bronchial segments of fresh (A), 48 h organ culture (B), 48 h organ culture in the presence of vehicle (DMSO) (C) , BMS-345541 10 μM (D) and TPCA-1 10 μM (E). The size bar corresponds to 100 μm. SMC; smooth muscle cell, EP: epithelium. Semi-quantitation of phospho-IKKα/β (Ser176/180) protein in bronchial epithelium layer and bronchial smooth muscle layer (F), each data is derived from 6 experiments and presented as mean ± S.E.M., n=6. Statistical analysis was performed by one-way ANOVA with Dunnet post-test. *P <0.05, **P <0.01. OC: organ culture, BMS: BMS-345541.

Figure 9.

Schematic diagram of hypothesis and experimental designs of the present study. IL-6: interleukin 6, ACD: actinomycin D, CHX: cycloheximide.

Figure 1

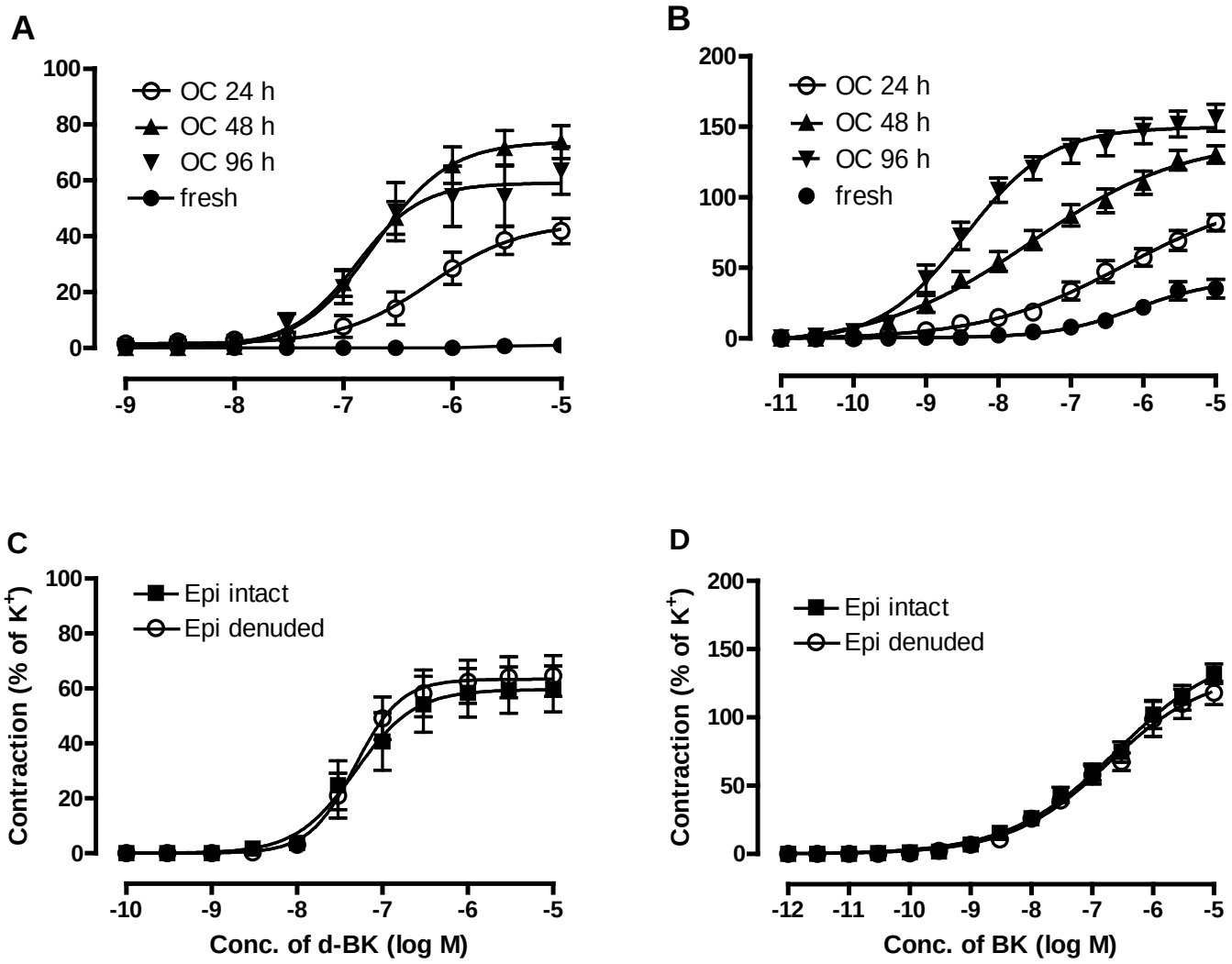
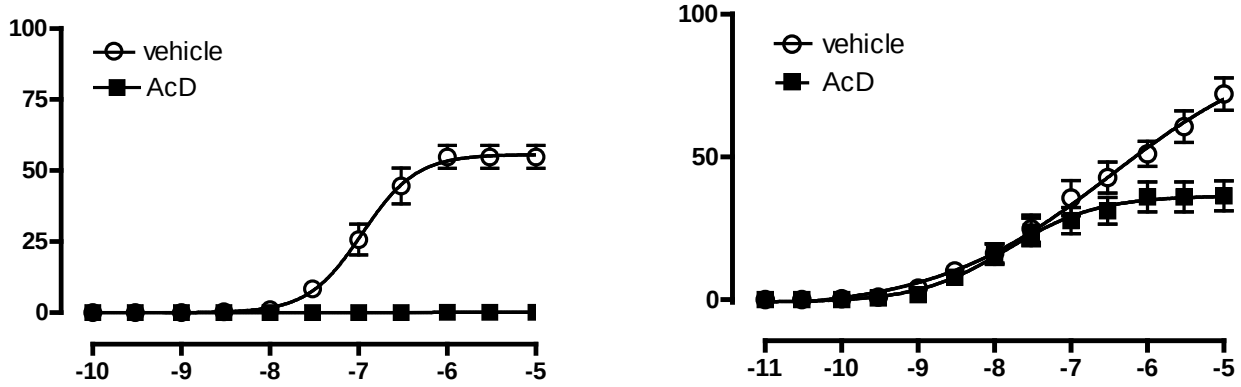


Figure 2



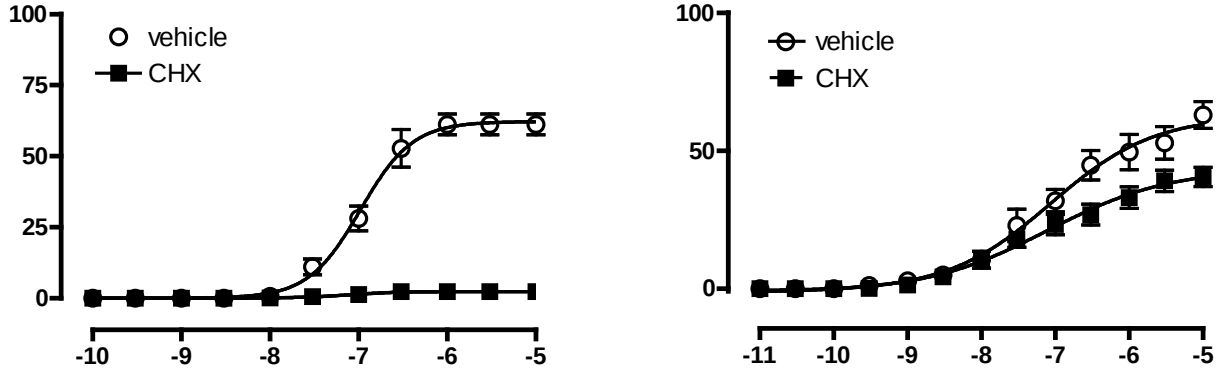

100
75
50
25
0
vehicle
CHX
-10
-9
-8
-7
-6
-5
100
50
0
vehicle
CHX
-11
-10
-9
-8
-7
-6
-5

Figure 3

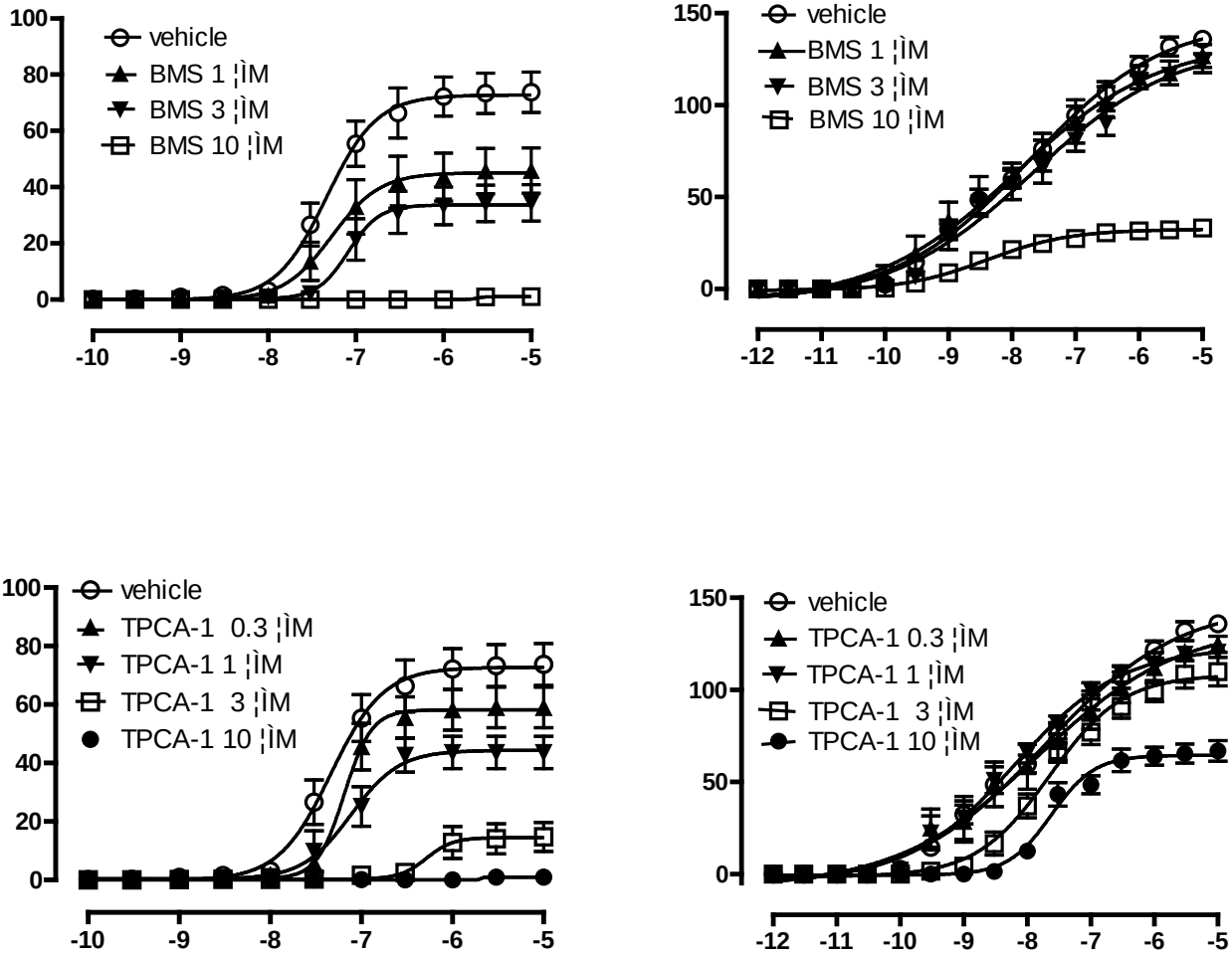

100
80
60
40
20
0
vehicle
BMS 1 ¦ÌM
BMS 3 ¦ÌM
BMS 10 ¦ÌM
-10
-9
-8
-7
-6
-5
150
100
50
0
vehicle
BMS 1 ¦ÌM
BMS 3 ¦ÌM
BMS 10 ¦ÌM
-12
-11
-10
-9
-8
-7
-6
-5
100
80
60
40
20
0
vehicle
TPCA-1 0.3 ¦ÌM
TPCA-1 1 ¦ÌM
TPCA-1 3 ¦ÌM
TPCA-1 10 ¦ÌM
-10
-9
-8
-7
-6
-5
150
100
50
0
vehicle
TPCA-1 0.3 ¦ÌM
TPCA-1 1 ¦ÌM
TPCA-1 3 ¦ÌM
TPCA-1 10 ¦ÌM
-12
-11
-10
-9
-8
-7
-6
-5

Figure 4

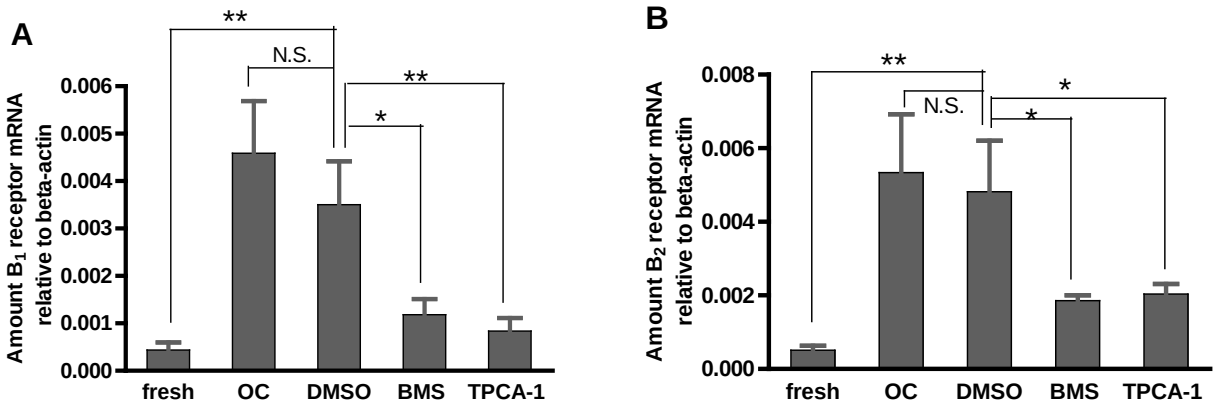


Figure 5

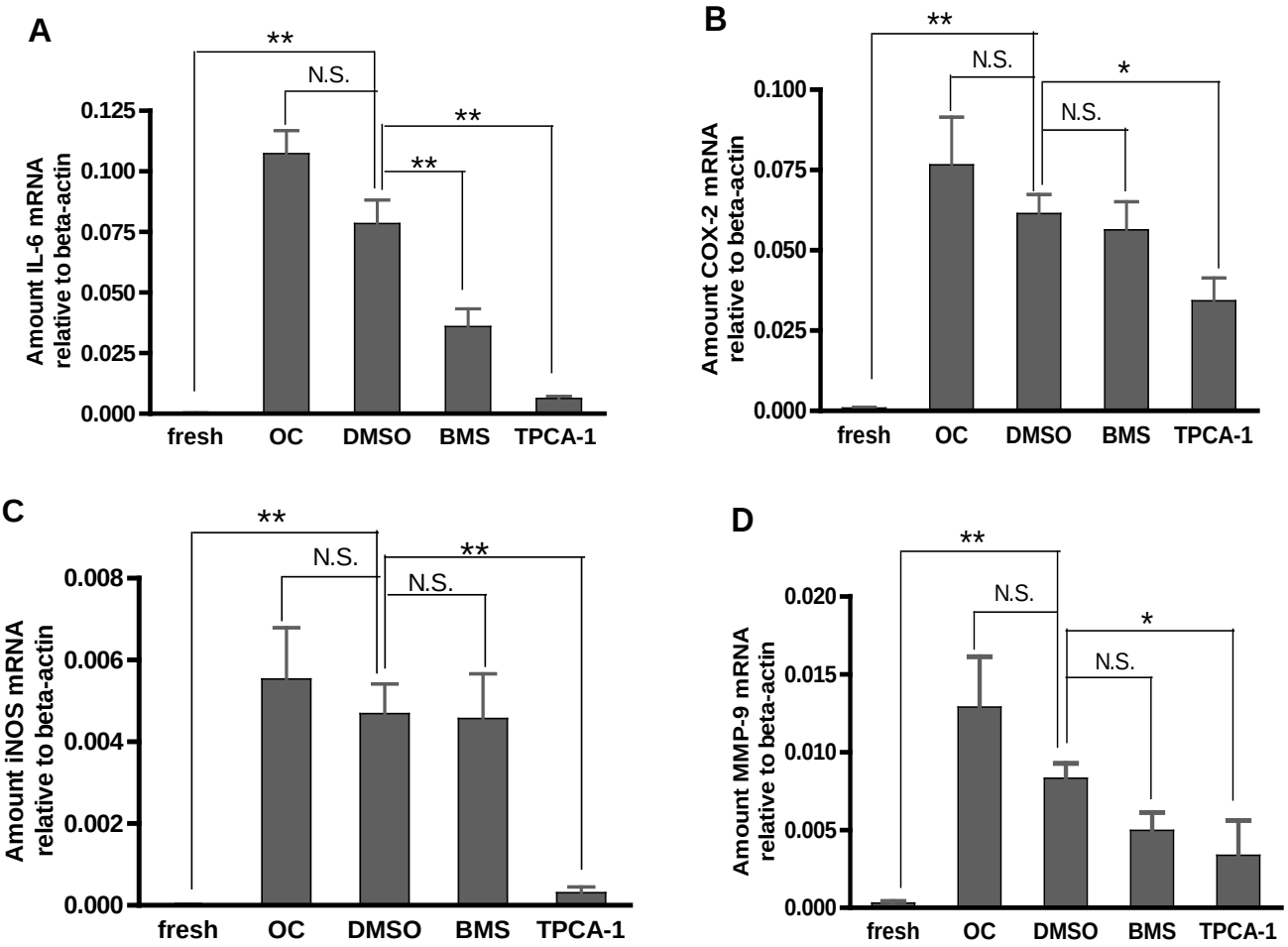


Figure 6

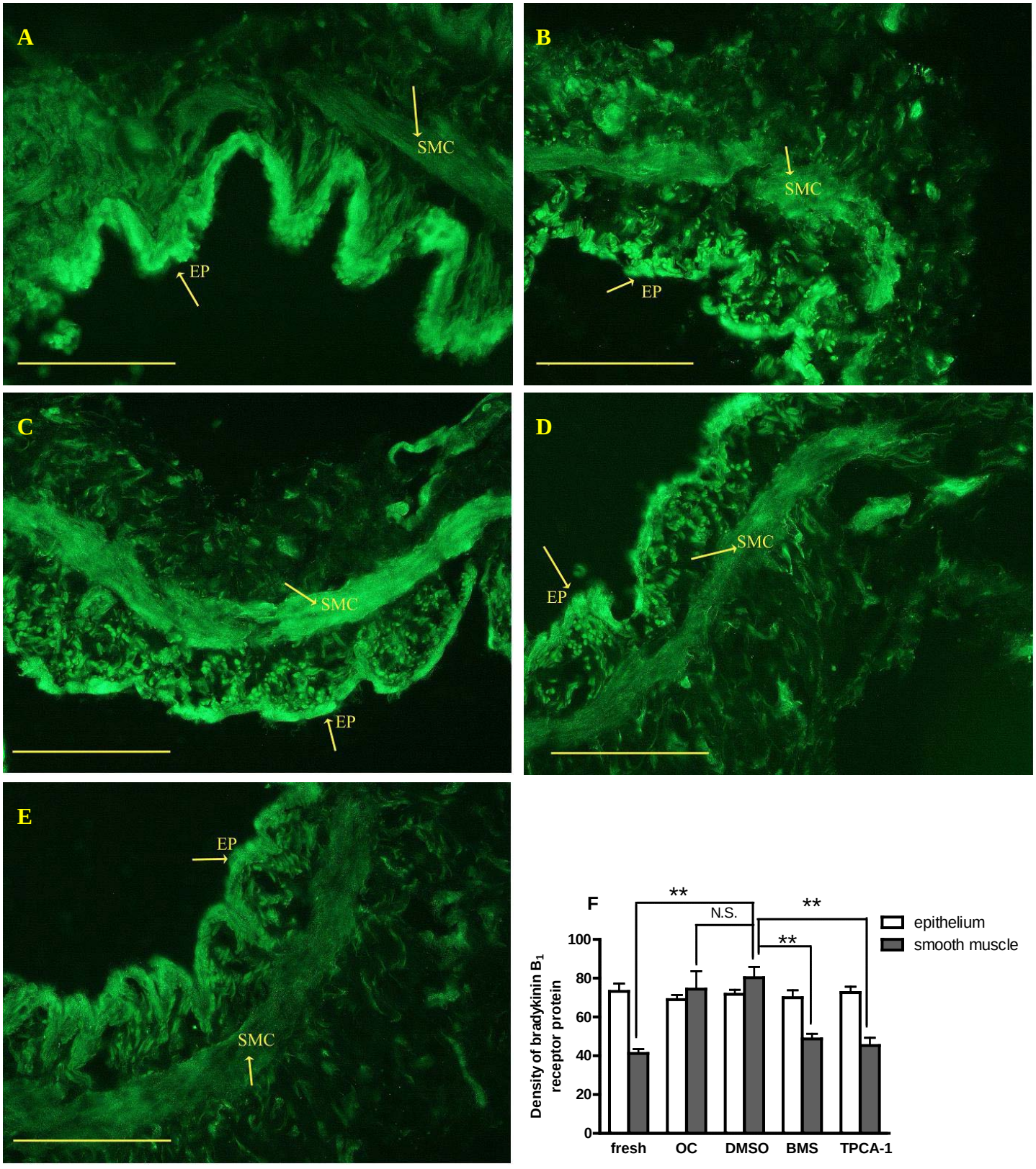

A
SMC
EP
B
SMC
EP
C
SMC
EP
D
SMC
EP
E
EP
SMC
F
**
N.S.
**
**
epithelium
smooth muscle
Density of bradykinin B1 receptor protein
100
80
60
40
20
0
fresh
OC
DMSO
BMS
TPCA-1

Figure 7

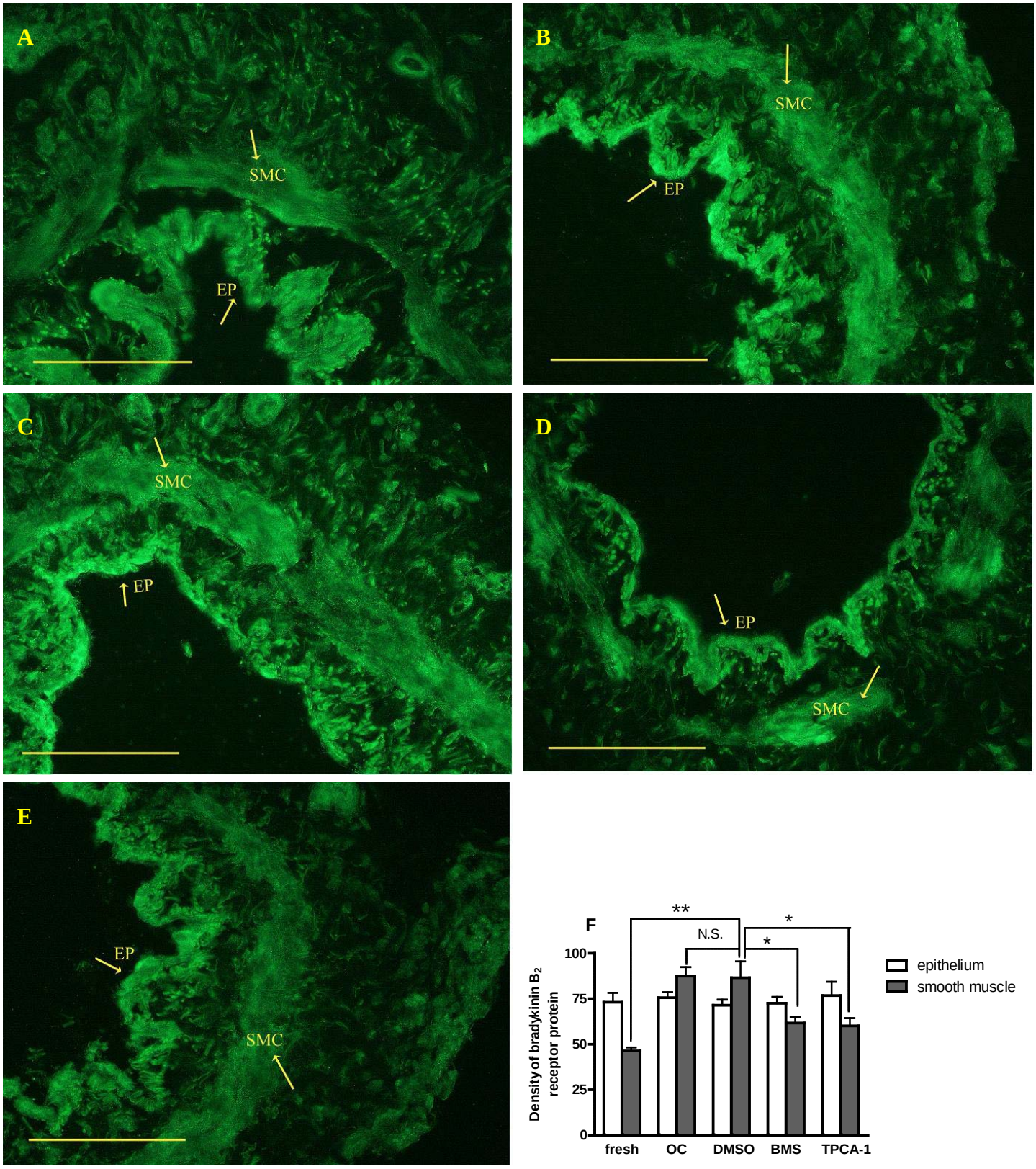

A
SMC
EP
B
SMC
EP
C
SMC
EP
D
EP
SMC
E
EP
SMC
F
**
N.S.
*
*
Density of bradykinin B2 receptor protein
100
75
50
25
0
fresh
OC
DMSO
BMS
TPCA-1
epithelium
smooth muscle

Figure 8

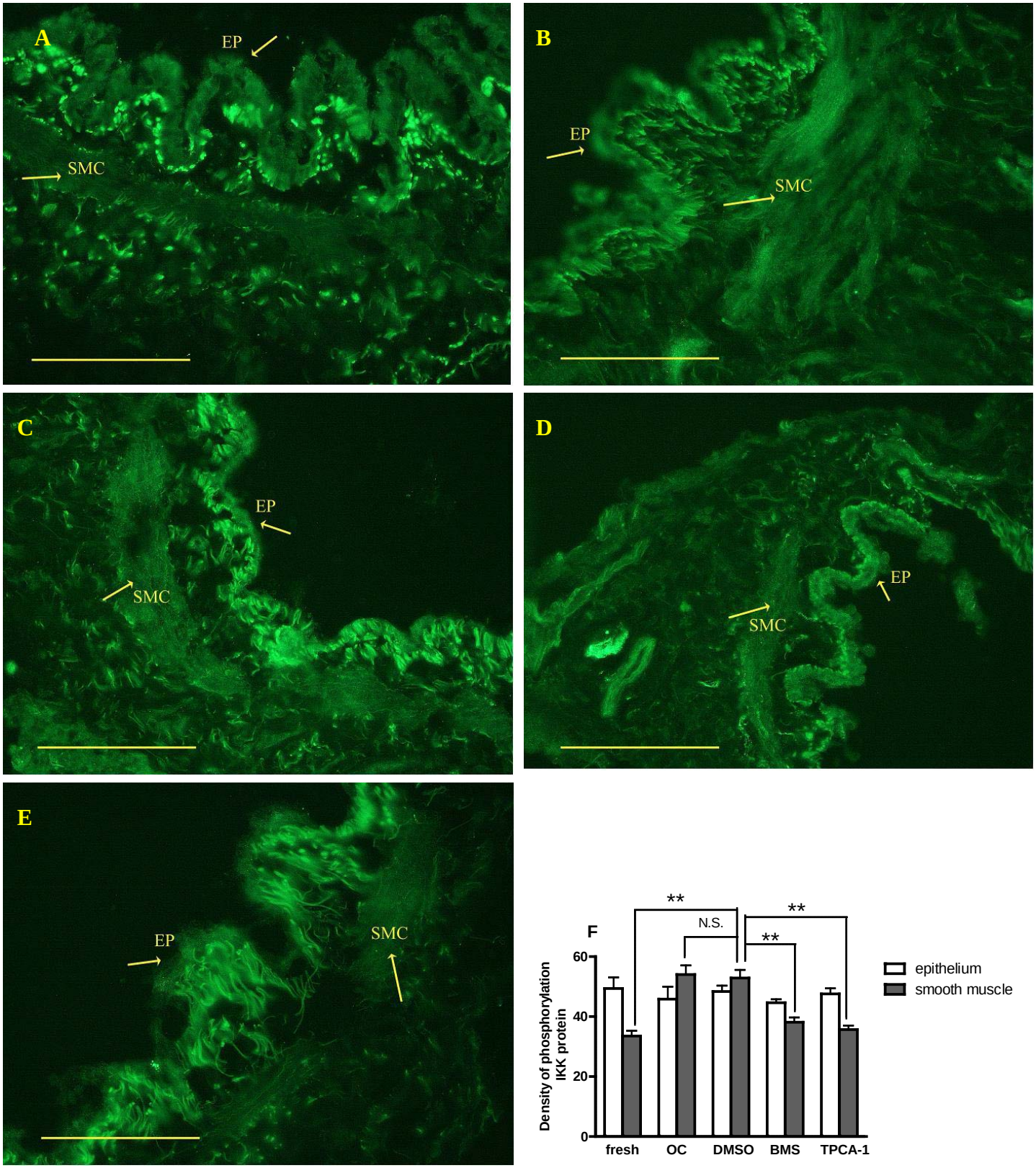
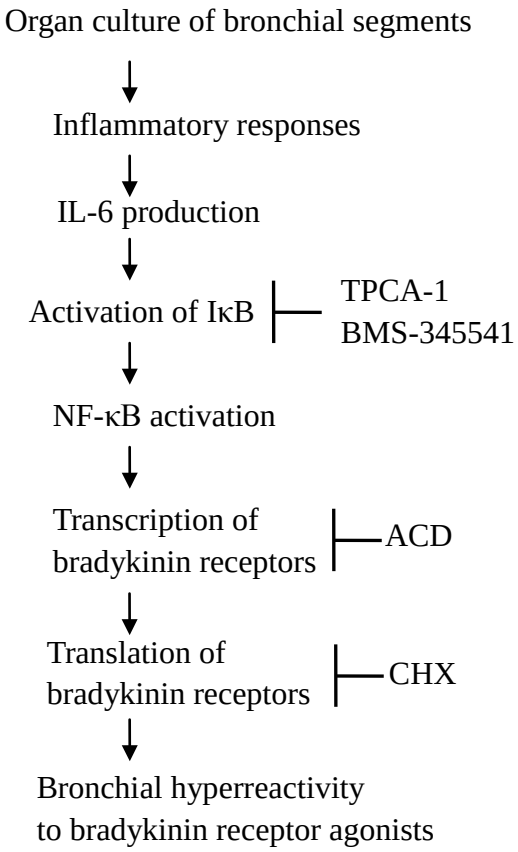

A
EP
SMC
B
EP
SMC
C
EP
SMC
D
EP
SMC
E
EP
SMC
F
**
N.S.
**
**
Density of phosphorylation IKK protein
60
40
20
0
fresh
OC
DMSO
BMS
TPCA-1
epithelium
smooth muscle

Figure 9


Organ culture of bronchial segments
Inflammatory responses
IL-6 production
Activation of IκB
TPCA-1
BMS-345541
NF-κB activation
Transcription of bradykinin receptors
ACD
Translation of bradykinin receptors
CHX
Bronchial hyperreactivity to bradykinin receptor agonists