

## Glucocorticoids suppress transcriptional up-regulation of bradykinin receptors in a murine *in vitro* model of chronic airway inflammation

Y. Zhang, M. Adner and L.-O. Cardell

Department of Otorhinolaryngology, Laboratory of Clinical and Experimental Allergy Research, Malmö University Hospital, Lund University, SE-205 02 Malmö, Sweden

### Summary

**Background** Glucocorticoids are effective drugs for controlling symptoms and airway inflammation in respiratory diseases such as asthma and chronic obstructive pulmonary disease. However, the mechanisms behind their effects are not fully understood. We have recently demonstrated that prolonged exposure to the pro-inflammatory mediator tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) markedly enhanced contractile responses to des-Arg<sup>9</sup>-bradykinin (selective bradykinin B<sub>1</sub> receptor agonist) and bradykinin (selective bradykinin B<sub>2</sub> receptor agonist) in murine airways. This increase was paralleled with elevated mRNA levels for bradykinin B<sub>1</sub> and B<sub>2</sub> receptors, a process involving intracellular mitogen-activated protein kinase pathways.

**Objective** To investigate the effects of glucocorticoids on the TNF- $\alpha$  up-regulated bradykinin B<sub>1</sub> and B<sub>2</sub> receptor response.

**Methods** Tracheal segments from BALB/c J mice were cultured with and without TNF- $\alpha$ , in the absence and presence of the transcriptional inhibitor actinomycin D or the glucocorticoid, dexamethasone. The contractile response induced by des-Arg<sup>9</sup>-bradykinin and bradykinin was subsequently assessed in a myograph system and mRNA for bradykinin B<sub>1</sub> and B<sub>2</sub> receptors was quantified using real-time polymerase chain reaction.

**Results** Actinomycin D abolished and dexamethasone concentration-dependently suppressed the TNF- $\alpha$ -induced enhancement of the des-Arg<sup>9</sup>-bradykinin and bradykinin responses. This was paralleled by a reduction of the mRNA expression for the bradykinin B<sub>1</sub> and B<sub>2</sub> receptors.

**Conclusion** The presented data suggests the involvement of transcriptional mechanisms in the up-regulation of bradykinin B<sub>1</sub> and B<sub>2</sub> receptors during asthmatic airway inflammation, as well as in their down-regulation following glucocorticoid treatment.

**Keywords** airway, asthma, bradykinin, glucocorticoid, hyper-responsiveness, inflammation, TNF- $\alpha$ , transcription

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### Introduction

Hyper-responsiveness to bronchoconstrictors is a major pathophysiological feature in asthma. It can be defined as an increase in the ease and degree of airway narrowing in response to various bronchoconstrictor stimuli [1, 2]. TNF- $\alpha$  is recognized as an important pro-inflammatory cytokine with a large spectrum of activities, including the ability to induce airway hyper-responsiveness [3–5]. Kinins are mediators, produced in blood and tissues during inflammation and act by stimulating distinct receptors, such as bradykinin B<sub>1</sub> and B<sub>2</sub> receptors [6]. Bradykinin, a prominent member of the kinin family, is a potent bronchoconstrictor in asthmatic patients but has no such effects in healthy subjects [7].

Increased levels of TNF- $\alpha$  and bradykinin in bronchoalveolar lavage fluids from symptomatic asthmatic patients have suggested a relationship between TNF- $\alpha$  and bradykinin in the pathogenesis of asthma [8, 9]. We have recently, using a murine *in vitro* model of chronic airway inflammation, demonstrated that prolonged exposure to TNF- $\alpha$  markedly enhanced contractile responses to des-Arg<sup>9</sup>-bradykinin and bradykinin in tracheal smooth muscle. This increase was paralleled with elevated mRNA levels for the bradykinin B<sub>1</sub> and B<sub>2</sub> receptors. In addition, mitogen-activated protein kinase (MAPK) pathways c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase 1 and 2 (ERK 1/2) pathways were shown to be involved in this process [5]. Activation of MAPK pathway is known to induce gene transcription via stimulation of 'down-stream' transcriptional factors [10–12]. TNF- $\alpha$  induced up-regulation of bradykinin B<sub>1</sub> and B<sub>2</sub> receptors might also partly depend on *de novo* transcription and synthesis of bradykinin B<sub>1</sub> and B<sub>2</sub> receptors.

Glucocorticoids have a wide range of inhibitory effects on the inflammatory process and the related immune response

Correspondence: Yaping Zhang, Department of Otorhinolaryngology, Laboratory of Clinical and Experimental Allergy Research, Malmö University Hospital, Lund University, SE-205 02 Malmö, Sweden.  
E-mail: Yaping.Zhang@oron.mas.lu.se

[13]. They bind to a single class of glucocorticoid receptors and cause either increase or decrease of gene expressions [14]. Glucocorticoids have been demonstrated to inhibit TNF- $\alpha$ - and IL-1 $\beta$ -induced up-regulation of bradykinin B<sub>1</sub> and B<sub>2</sub> receptors in human airway fibroblast and smooth muscle cells [15, 16]. Glucocorticoids have also been shown to enhance the gene transcription for bradykinin B<sub>2</sub> receptors in cultured airway smooth muscle cells [17]. Thus, the mechanisms behind the glucocorticoid effect on bradykinin receptors are not clear. The present study was designed to ascertain the effects of dexamethasone, a glucocorticoid, on the TNF- $\alpha$ -induced up-regulation of bradykinin B<sub>1</sub> and B<sub>2</sub> receptors in murine tracheal smooth muscle and to define the transcriptional mechanisms involved.

## Material and methods

### Tissue preparation

Ten-week-old male BALB/c J mice (MB A/S, Ry, Denmark) were sacrificed by cervical dislocation. The whole trachea was rapidly removed and placed into Dulbecco's Modified Eagle's Medium (DMEM; 4500 mg/L D-glucose, 110 mg/L sodium pyruvate, 584 mg/L L-glutamine) supplemented with penicillin (100 U/mL) and streptomycin (100  $\mu$ g/mL). The trachea was then dissected free of adhering tissue under a microscope and cut into three to four segments, each with three cartilages per ring, for subsequent organ culture.

### Organ culture

After the dissection, the segments were placed individually into wells of a 96-well plate (Ultra-low attachment; Sigma, St Louis, MO, USA) with 300  $\mu$ L serum free DMEM incubated at 37 °C in humidified 5% CO<sub>2</sub> in air with and without TNF- $\alpha$  (100 ng/mL), in the absence and presence of actinomycin D (5  $\mu$ g/mL), a general transcriptional inhibitor, for 1 day or dexamethasone (0.01, 0.1 or 1  $\mu$ M), a glucocorticoid, for 4 days. Segments were transferred into new wells containing fresh media including TNF- $\alpha$ , actinomycin D or dexamethasone every day.

### In vitro pharmacology

The cultured segments were immersed in temperature-controlled (37 °C) myograph bath (Organ Bath Model 700MO, J.P. Trading, Aarhus, Denmark) containing 5 mL Krebs-Henseleit buffer solution (143 mM Na<sup>+</sup>, 5.9 mM K<sup>+</sup>, 1.5 mM Ca<sup>2+</sup>, 2.5 mM Mg<sup>2+</sup>, 128 mM Cl<sup>-</sup>, 1.2 mM H<sub>2</sub>PO<sub>4</sub><sup>-</sup>, 1.2 mM SO<sub>4</sub><sup>-</sup>, 25 mM HCO<sub>3</sub><sup>-</sup> and 10 mM D-glucose). The solution was continuously equilibrated with 5% CO<sub>2</sub> in O<sub>2</sub> to result in a stable pH of 7.4. Each tracheal segment was mounted on two L-shaped metal prongs. One prong was connected to a force-displacement transducer for continuous recording of isometric tension by the Chart software (AD Instruments Ltd., Hastings, UK). The other prong was connected to a displacement device, allowing adjustment of the distance between the two parallel prongs. Following equilibration, a pre-tension about 0.8 mN was applied to each segment and adjusted to this level of tension for at least 1 h. Each segment was contracted with 60 mM KCl to test the

contractile function. To inhibit epithelial prostaglandin release, the segments were incubated with 3  $\mu$ M indomethacin 30 min before administration of des-Arg<sup>9</sup>-bradykinin or bradykinin [18, 19]. At the end of the experiment a concentration-effect curve for carbachol was assessed.

### Data analysis

All data were expressed as mean values  $\pm$  SEM. In the dexamethasone experiments, the contractile responses were expressed as percent of carbachol-induced maximal contraction (% of Cch). In experiments with actinomycin D absolute values (mN) were used. The latter since actinomycin D attenuated the carbachol-induced maximal contraction following organ culture in the presence as well as absence of TNF- $\alpha$  (Fig. 4c). Each agonist concentration-effect curve was fitted to the Hill equation using an iterative, least square method (GraphPad Prism, San Diego, CA, USA), to provide estimates of maximal contraction ( $\alpha$ ) and pEC<sub>50</sub> values (negative logarithm of the agonist concentration that produces 50% of the maximal effect). Unpaired student's *t*-test was used when two sets of data were compared and one-way analysis of variance (ANOVA) with Bonferroni correction was used for comparisons of more than two data sets. *P* < 0.05 were accepted as statistically significant. *n* equals the number of experiments performed.

### Chemicals

Recombinant murine TNF- $\alpha$  was obtained from R&D Systems (Abingdon, UK). Bradykinin and des-Arg<sup>9</sup>-bradykinin was purchased from Neosystem SA (Strasbourg, France). Dexamethasone, actinomycin D, indomethacin, carbachol, DMEM and Krebs-Henseleit Buffer were from Sigma (St. Louis, MO, USA). TNF- $\alpha$ , bradykinin, des-Arg<sup>9</sup>-bradykinin and actinomycin D were dissolved in distilled water with chicken serum albumin (0.1% w/v), indomethacin in 95% ethanol, carbachol and dexamethasone in distilled water.

### mRNA study

The tracheal smooth muscle was isolated mechanically on an ice tray under a microscope and the total RNA was extracted. Briefly, after removal of tracheal epithelium and cartilages, the smooth muscle strip was rinsed with cold phosphate-buffered saline and stored in RNeasy lysis buffer (QIAGEN GmbH, Hilden, Germany) at -80 °C until use for extraction of total RNA. The tracheal smooth muscle strips were homogenized and the total RNA was extracted by using the RNeasy Mini kit following the supplier's instructions (QIAGEN GmbH). The purity of total RNA was checked by a spectrophotometer and the wavelength absorption ratio (260/280 nm) was between 1.6 and 2.0 in all preparations.

Reverse transcription (RT) of total RNA to cDNA was carried out using Omniscript<sup>TM</sup> reverse transcriptase kit (QIAGEN) in 20  $\mu$ L volume reaction at 37 °C for 1 h by using Mastercycler personal PCR machine (Eppendorf AG, Hamburg, Germany).

To quantify mRNA for bradykinin B<sub>1</sub> and B<sub>2</sub> receptors, real-time PCR was performed with the QuantiTect<sup>TM</sup> SYBR<sup>®</sup> Green PCR kit (QIAGEN GmbH) in The Smart Cycler<sup>®</sup> II

system (Cepheid, Sunnyvale, CA, USA). The system automatically monitors the binding of a fluorescent dye SYBR<sup>®</sup> 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 25  $\mu$ L reaction volumes and carried out with heating 95 °C for 15 min followed by touch down PCR i.e. denature at 94 °C for 30 s and annealing at 66 °C for 1 min 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. The data were analysed with the threshold cycle ( $C_T$ ) method and the specificity of the PCR products were checked by the dissociation curves and visualized by agarose electrophoresis. Expected PCR products of bradykinin B<sub>1</sub> receptor 102 bp, bradykinin B<sub>2</sub> receptor 104 bp and  $\beta$ -actin 102 bp with a single band for each product were seen.

All PCR primers used in the present study were designed by using Prime Express<sup>®</sup> 2.0 software (Applied Biosystem, Foster city, CA, USA) and synthesized by DNA Technology A/S. Sequences as follows:

#### Bradykinin B<sub>1</sub> receptor:

Forward: 5'-CCA TAG CAG AAA TCT ACC TGG CTA AC-3'  
Reverse: 5'-GCC AGT TGA AAC GGT TCC-3'

#### Bradykinin B<sub>2</sub> receptor:

Forward: 5'-ATG TTC AAC GTC ACC ACA CAA GTC-3'  
Reverse: 5'-TGG ATG GCA TTG AGC CAA C-3'

#### $\beta$ -actin:

Forward: 5'-TGG GTC AGA AGG ACT CCT ATG TG-3'  
Reverse: 5'-CGT CCC AGT TGG TAA CAA TGC-3'

The  $\Delta\Delta C_T$  method was employed to calculate the relative amount of mRNA for bradykinin B<sub>1</sub> and B<sub>2</sub> receptors. The relative amount of mRNA was obtained by the  $C_T$  values of mRNA for bradykinin B<sub>1</sub> or B<sub>2</sub> receptor in relation to the  $C_T$  values of mRNA for house keep gene  $\beta$ -actin in the same sample. A blank (no template) was included in all the experiments for negative controls.

## Results

### Effects of dexamethasone

Organ culture of the tracheal segments for 4 days in the absence and presence of dexamethasone (0.01, 0.1 or 1  $\mu$ M) revealed that contractions induced by des-Arg<sup>9</sup>-bradykinin (selective bradykinin B<sub>1</sub> receptor agonist) and bradykinin (selective bradykinin B<sub>2</sub> receptor agonist) were concentration-dependently inhibited by dexamethasone (Fig. 1a and b, Table 1).

When tracheal segments were cultured for 4 days in the presence of TNF- $\alpha$  (100 ng/mL) with increasing concentrations of dexamethasone (0.003, 0.01, 0.03, 0.1 or 1  $\mu$ M), dexamethasone produced a concentration-dependent reduction of the maximal contractile responses to des-Arg<sup>9</sup>-bradykinin and bradykinin. The maximal reduction was, for both kinins, reached at 1  $\mu$ M of dexamethasone. For des-Arg<sup>9</sup>-

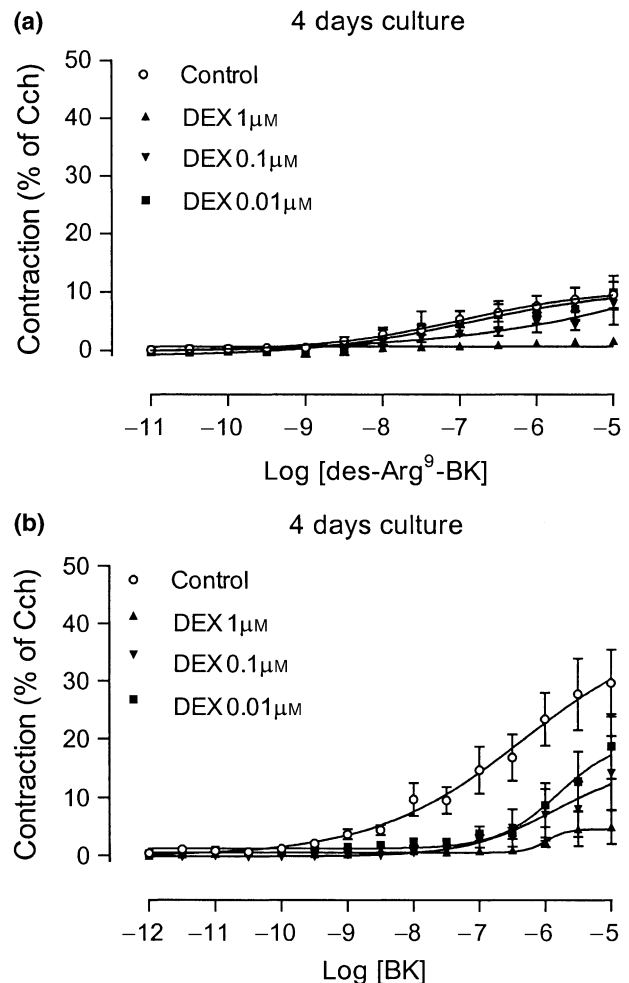


**Fig. 1.** Contractile effects of (a) des-Arg<sup>9</sup>-bradykinin and (b) bradykinin in segments cultured for 4 days in the absence and presence of dexamethasone (DEX) 0.01, 0.1 or 1  $\mu$ M. Each data point is represented as the mean of all segments  $\pm$  SEM ( $n = 6-7$ ).

bradykinin this reduction became significant at 0.03, 0.1 and 1  $\mu$ M of dexamethasone (Fig. 2a, Table 1,  $P < 0.05$ ) and for bradykinin at 0.01, 0.03, 0.1 and 1  $\mu$ M of dexamethasone (Fig. 2b, Table 1,  $P < 0.05$ ). In addition, there was a rightward shift of des-Arg<sup>9</sup>-bradykinin and bradykinin concentration-response curves with significant changes of pEC<sub>50</sub> values at 0.1 and 1  $\mu$ M of dexamethasone for des-Arg<sup>9</sup>-bradykinin (Fig. 2a, Table 1,  $P < 0.05$ ) and 0.01, 0.03, 0.1 and 1  $\mu$ M of dexamethasone for bradykinin (Fig. 2b, Table 1,  $P < 0.001$ ).

To exclude possible toxic effects of dexamethasone, carbachol concentration-response curves were performed in the segments cultured for 4 days with and without TNF- $\alpha$  (100 ng/mL) in the presence of dexamethasone. The maximal contractions and the pEC<sub>50</sub> values were not affected by dexamethasone (Fig. 3a and b). Thus, data derived from the dexamethasone experiments are presented as percent of the carbachol-induced maximal contraction.

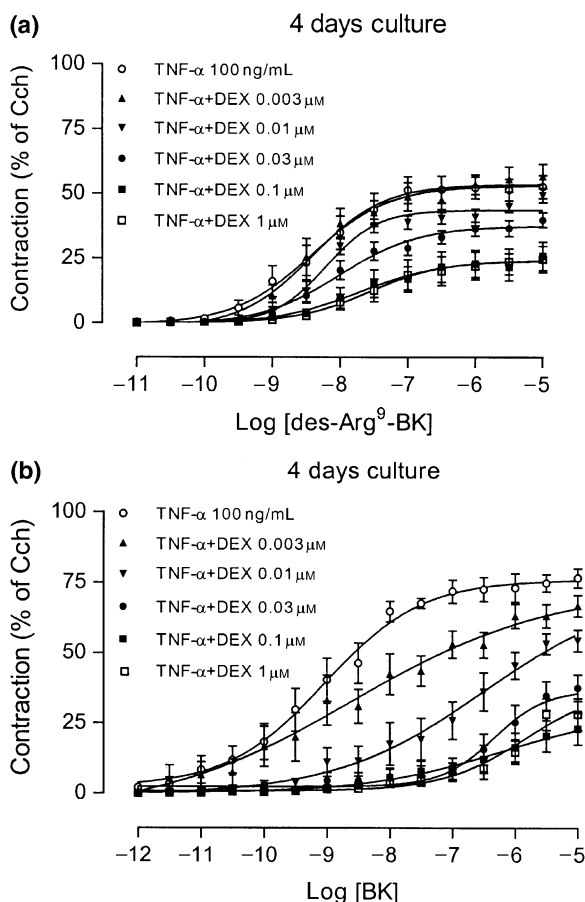
### Effects of actinomycin D

To investigate if the up-regulation of bradykinin B<sub>1</sub> and B<sub>2</sub> receptors were mediated via transcriptional mechanisms,

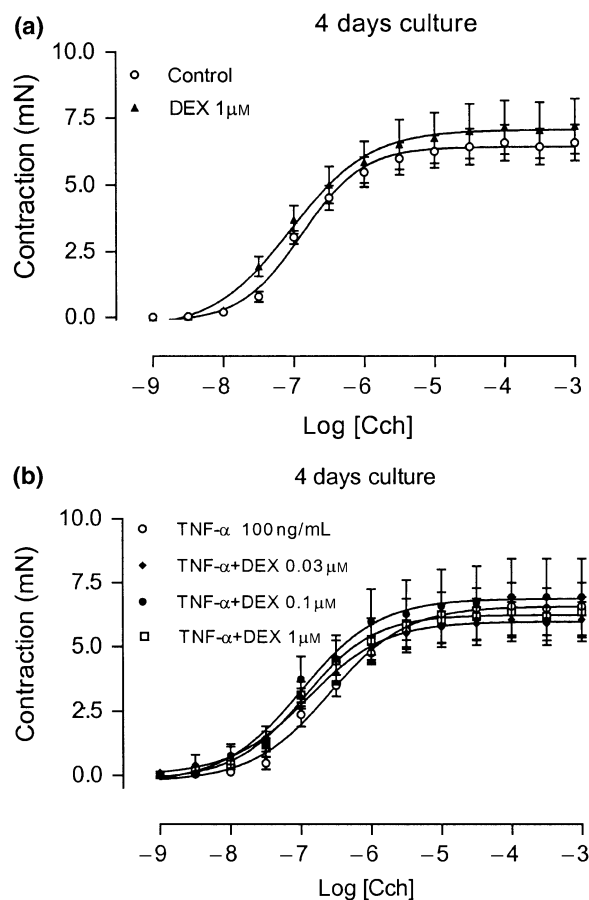
**Table 1.** Effects of dexamethasone on the maximal contraction ( $\alpha$ ) and pEC<sub>50</sub> values of des-Arg<sup>9</sup>-bradykinin and bradykinin in segments cultured for 4 days in the absence and presence of TNF- $\alpha$ 

|                                  | des-Arg <sup>9</sup> -bradykinin |                             |                              | Bradykinin |                             |                              |
|----------------------------------|----------------------------------|-----------------------------|------------------------------|------------|-----------------------------|------------------------------|
|                                  | <i>n</i>                         | $\alpha$ (% of Cch)         | pEC <sub>50</sub>            | <i>n</i>   | $\alpha$ (% of Cch)         | pEC <sub>50</sub>            |
| Control (organ culture)          | 7                                | 9.5 $\pm$ 2.2               | 6.85 $\pm$ 0.16              | 7          | 29.7 $\pm$ 5.8              | 6.08 $\pm$ 0.39              |
| DEX 0.01 $\mu$ M                 | 7                                | 9.9 $\pm$ 2.9               | 6.82 $\pm$ 0.29              | 6          | 18.8 $\pm$ 5.5              | 5.67 $\pm$ 0.26              |
| DEX 0.1 $\mu$ M                  | 6                                | 8.0 $\pm$ 3.6               | 6.03 $\pm$ 0.68              | 6          | 14.2 $\pm$ 6.3              | 5.64 $\pm$ 0.18              |
| DEX 1 $\mu$ M                    | 7                                | 1.6 $\pm$ 0.3               | ND                           | 6          | 4.9 $\pm$ 2.9 <sup>b</sup>  | 5.70 $\pm$ 0.91              |
| TNF- $\alpha$ 100 ng/mL          | 10                               | 53.1 $\pm$ 3.9 <sup>a</sup> | 8.28 $\pm$ 0.23 <sup>a</sup> | 7          | 76.7 $\pm$ 3.4 <sup>a</sup> | 9.19 $\pm$ 0.31 <sup>a</sup> |
| TNF- $\alpha$ +DEX 0.003 $\mu$ M | 6                                | 53.4 $\pm$ 3.8              | 8.32 $\pm$ 0.19              | 6          | 66.9 $\pm$ 3.7              | 8.13 $\pm$ 0.52              |
| TNF- $\alpha$ +DEX 0.01 $\mu$ M  | 6                                | 45.3 $\pm$ 3.1              | 8.14 $\pm$ 0.24              | 6          | 56.1 $\pm$ 3.2 <sup>c</sup> | 7.13 $\pm$ 0.42 <sup>c</sup> |
| TNF- $\alpha$ +DEX 0.03 $\mu$ M  | 6                                | 37.8 $\pm$ 2.3 <sup>c</sup> | 7.92 $\pm$ 0.20              | 6          | 37.6 $\pm$ 4.7 <sup>c</sup> | 6.31 $\pm$ 0.27 <sup>c</sup> |
| TNF- $\alpha$ +DEX 0.1 $\mu$ M   | 6                                | 25.3 $\pm$ 5.1 <sup>c</sup> | 7.31 $\pm$ 0.27 <sup>c</sup> | 9          | 23.1 $\pm$ 5.6 <sup>c</sup> | 6.24 $\pm$ 0.38 <sup>c</sup> |
| TNF- $\alpha$ +DEX 1 $\mu$ M     | 7                                | 25.2 $\pm$ 4.9 <sup>c</sup> | 7.21 $\pm$ 0.19 <sup>c</sup> | 6          | 28.3 $\pm$ 5.6 <sup>c</sup> | 5.82 $\pm$ 0.19 <sup>c</sup> |

Data are represented as percent of carbachol (Cch) induced maximal contraction with the mean  $\pm$  SEM. Statistical analysis was performed with unpaired student's *t*-test (<sup>a</sup>control vs. TNF- $\alpha$ ) or one-way ANOVA and Dunnett's post test (<sup>b</sup>control vs. DEX and <sup>c</sup>TNF- $\alpha$  vs. TNF- $\alpha$ +DEX). *P* < 0.05 were considered to be significant. ND, not determined; DEX, dexamethasone.

**Fig. 2.** Contractile effects of (a) des-Arg<sup>9</sup>-bradykinin and (b) bradykinin in segments cultured for 4 days with TNF- $\alpha$  100 ng/mL in the absence and presence of different concentrations of dexamethasone (DEX). Each data point is represented as the mean of all segments  $\pm$  SEM (*n* = 6–10).

segments were cultured for 1 day with and without TNF- $\alpha$  (100 ng/mL), in the presence of actinomycin D (5  $\mu$ g/mL), a general transcriptional inhibitor. Actinomycin D completely abolished the TNF- $\alpha$  induced enhancement of the contractile response to des-Arg<sup>9</sup>-bradykinin and bradykinin. In addition,

**Fig. 3.** Contractile effects of carbachol in segments cultured for 4 day in (a) absence and (b) presence of TNF- $\alpha$  100 ng/mL with and without dexamethasone (DEX). Each data point is represented as the mean of all segments  $\pm$  SEM (*n* = 4–6).

the contractions seen during control conditions (cultured for 1 day without TNF- $\alpha$ ) were also omitted as the result of the presence of actinomycin D (Fig. 4a and b, Table 2, *P* < 0.05).

To determine whether actinomycin D (5  $\mu$ g/mL) affected the carbachol-induced contraction *per se*, experiments were

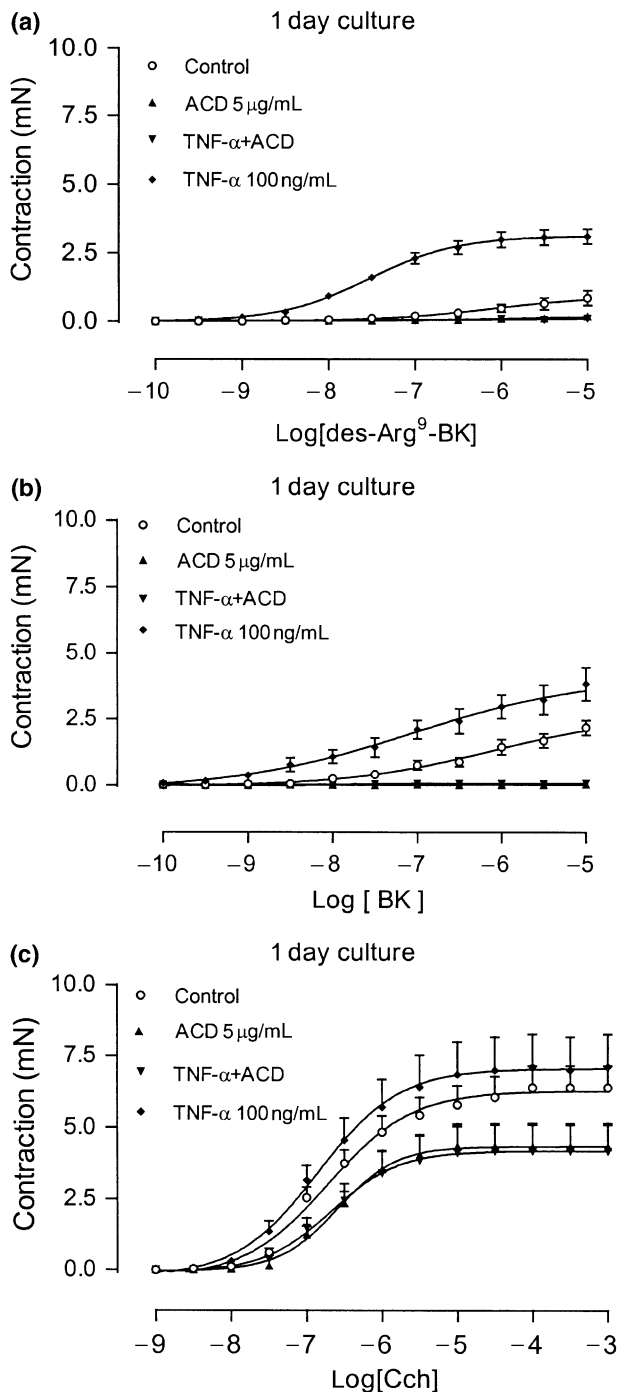


**Fig. 4.** Contractile effects of (a) des-Arg<sup>9</sup>-bradykinin, (b) bradykinin and (c) carbachol in segments cultured for 1 day in the absence and presence of TNF- $\alpha$  100 ng/mL with and without actinomycin D (ACD) 5  $\mu$ g/mL. Each data point is represented as the mean of all segments  $\pm$  SEM ( $n = 6-8$ ).

performed with segments cultured for 1 day, with and without TNF- $\alpha$  100 ng/mL, in the presence of actinomycin D. Actinomycin D reduced the maximal contraction and produced a rightward shift of carbachol concentration-response curves (Fig. 4c,  $P > 0.05$ ). Since actinomycin D affected carbachol-induced contractions, the actinomycin D experiments are presented as absolute values (mN).

**Table 2.** Effects of actinomycin D on the maximal contraction ( $\alpha$ ) and pEC<sub>50</sub> values of des-Arg<sup>9</sup>-bradykinin and bradykinin in segments cultured for 1 day in the absence and presence of TNF- $\alpha$

|                         |   | des-Arg <sup>9</sup> -bradykinin |                          | Bradykinin        |                          |                          |
|-------------------------|---|----------------------------------|--------------------------|-------------------|--------------------------|--------------------------|
|                         |   | <i>n</i>                         | $\alpha$ (mN)            | pEC <sub>50</sub> | <i>n</i>                 | $\alpha$ (mN)            |
| Control (organ culture) | 6 | 0.86 ± 0.28                      | 6.19 ± 0.06              | 7                 | 2.69 ± 0.19              | 5.95 ± 0.23              |
| ACD 5 μg/mL             | 6 | 0.19 ± 0.09 <sup>b</sup>         | ND                       | 7                 | 0.06 ± 0.02 <sup>b</sup> | ND                       |
| TNF-α 100 ng/mL         | 6 | 3.12 ± 0.29 <sup>a</sup>         | 7.56 ± 0.09 <sup>a</sup> | 8                 | 3.96 ± 0.61              | 6.98 ± 0.19 <sup>a</sup> |
| TNF-α+ACD 5 μg/mL       | 6 | 0.11 ± 0.04 <sup>c</sup>         | ND                       | 8                 | 0.09 ± 0.03 <sup>c</sup> | ND                       |

Data are presented as absolute values of contraction (mN) with the mean  $\pm$  SEM. Statistical analysis was performed with unpaired student's *t*-test.  $P < 0.05$  were considered to be significant; <sup>a</sup>control (organ culture) vs. TNF- $\alpha$ , <sup>b</sup>control (organ culture) vs. organ culture with ACD (actinomycin D) and <sup>c</sup>TNF- $\alpha$  vs. TNF- $\alpha$ +ACD. ND, not determined; ACD, actinomycin D.

### Receptor mRNA Study

To investigate if the TNF- $\alpha$  induced up-regulation of the bradykinin receptors were related to an increased *de novo* transcription of the receptor mRNA, the total RNA was extracted from the tracheal smooth muscle strips cultured for 1 or 4 days, in the absence and presence of TNF- $\alpha$  (100 ng/mL). The relative amount of mRNA for the bradykinin B<sub>1</sub> and B<sub>2</sub> receptors was quantified with real-time PCR. Enhanced mRNA levels of the bradykinin B<sub>1</sub> and B<sub>2</sub> receptors were found at 1 day (Fig. 5a,  $P < 0.001$ ) and 4 days (Fig. 5b,  $P < 0.001$ ) of organ culture. These levels were further increased by TNF- $\alpha$  (100 ng/mL) ( $P < 0.05$ ).

To further confirm the involvement of transcriptional mechanisms, the tracheae were cultured with and without TNF- $\alpha$  (100 ng/mL), for 1 day in the presence of actinomycin D (5  $\mu$ g/mL) or for 4 days in presence of dexamethasone (1  $\mu$ M). In both cases, a significant inhibition of mRNA expression for the bradykinin B<sub>1</sub> and B<sub>2</sub> receptors was obtained (Fig. 6a and b,  $P < 0.001$ ).

### Discussion

Airway inflammation induces airway smooth muscle hyper-responsiveness to various contractile mediators [1, 2]. We have previously demonstrated that TNF- $\alpha$  up-regulates bradykinin B<sub>1</sub> and B<sub>2</sub> receptor-mediated contractions in murine airways, a phenomenon that is paralleled with an increased mRNA expression for these receptors. This effect was at least partly mediated via the JNK and ERK 1/2 MAPK pathways [5]. The present study reveals that dexamethasone suppresses TNF- $\alpha$ -induced up-regulation of the bradykinin receptors via a transcriptional mechanism (Fig. 7).

In the airways only small amounts of bradykinin B<sub>2</sub> receptors and no bradykinin B<sub>1</sub> receptors are expressed during physiological conditions [20, 21]. In the present set up no response to des-Arg<sup>9</sup>-bradykinin and only a weak contractile response to bradykinin could be seen in fresh tracheal segments, even though some mRNA for the bradykinin B<sub>1</sub> receptor could be detected [5]. This suggests that the tracheal segments used were in a physiological

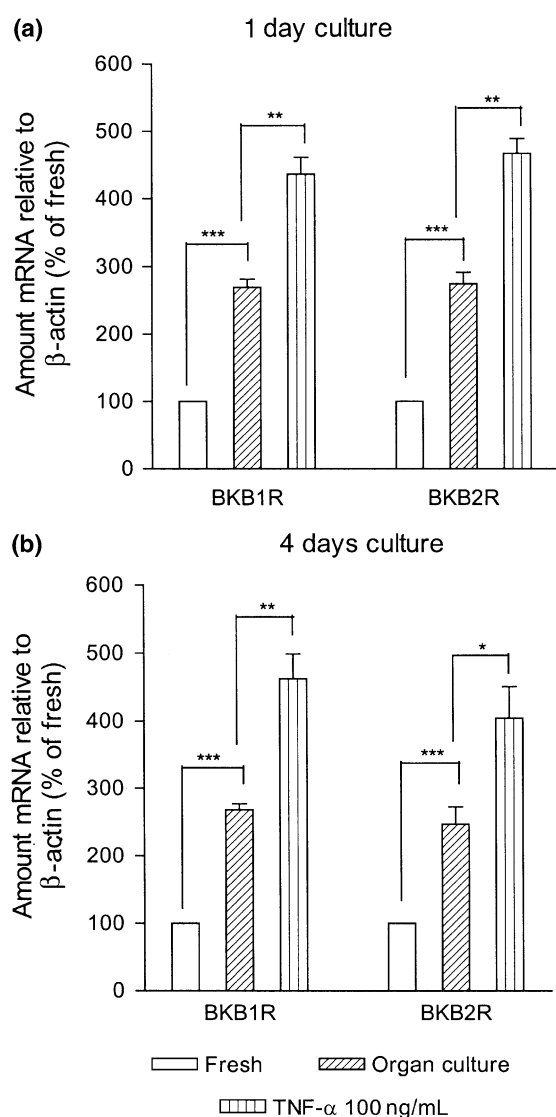


**Fig. 5.** Effects of TNF- $\alpha$  on bradykinin B<sub>1</sub> and B<sub>2</sub> receptor mRNA expression in tracheal smooth muscle strips before and after organ culture, analysed with real-time quantitative PCR. Segments cultured for (a) 1 and (b) 4 days in the absence and presence of TNF- $\alpha$  (100 ng/mL). Each data point is derived from three identical experiments and represented as percent of the fresh segments, mean  $\pm$  SEM. \* $P$  < 0.05, \*\* $P$  < 0.01 and \*\*\* $P$  < 0.001. (Unpaired student's  $t$ -test) BK B<sub>1</sub>R, bradykinin B<sub>1</sub> receptor; BK B<sub>2</sub>R, bradykinin B<sub>2</sub> receptor.

condition. After organ culture of the tracheal segments for 1 or 4 days, bradykinin B<sub>1</sub> and B<sub>2</sub> receptors were up-regulated at the mRNA level as well as at the functional level. Thus, contractile responses to des-Arg<sup>9</sup>-bradykinin and bradykinin appeared. The mechanisms behind this up-regulation are not clear, but since the anti-inflammatory agent dexamethasone inhibited this up-regulation, it is most likely that the tracheal segments underwent changes induced by some kind of inflammatory process. Changes induced by the segment transferred from their natural *in vivo* milieu to culture in serum free medium along with the simultaneous loss of the continuous air pressure might also contribute to this process. The up-regulation of the bradykinin B<sub>1</sub> and B<sub>2</sub> receptors was further enhanced during organ culture in presence of the inflammatory mediator TNF- $\alpha$ . These findings are in line

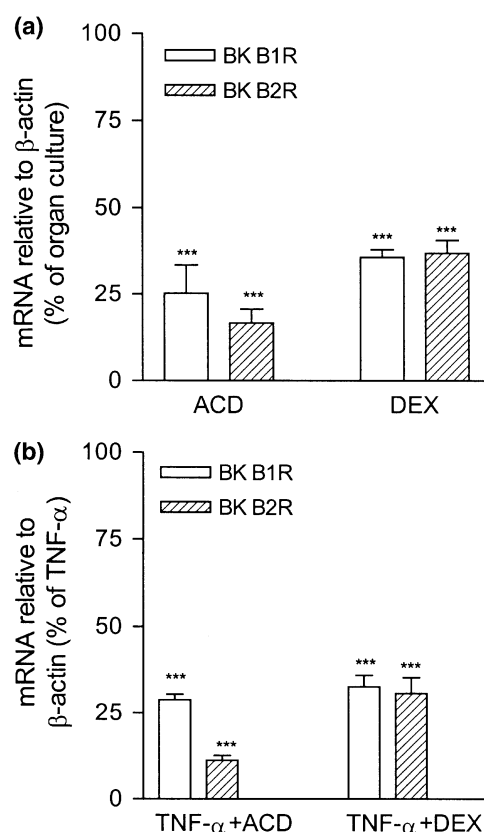


**Fig. 6.** Effects of actinomycin D (ACD) and dexamethasone (DEX) on bradykinin B<sub>1</sub> and B<sub>2</sub> receptor mRNA expression in tracheal smooth muscle strips after organ culture, analysed with real-time quantitative PCR. Segments cultured for 1 day with ACD or 4 days with dexamethasone in (a) absence or (b) presence of TNF- $\alpha$  (100 ng/mL). Each data point is derived from three to four identical experiments and represented as percent of control ((a) organ culture without ACD or dexamethasone and (b) organ culture in presence of TNF- $\alpha$ , but without ACD or dexamethasone), mean  $\pm$  SEM. \*\*\* $P$  < 0.001 (Unpaired student's  $t$ -test, each column compared with control). BK B<sub>1</sub>R, bradykinin B<sub>1</sub> receptor; BK B<sub>2</sub>R, bradykinin B<sub>2</sub> receptor.

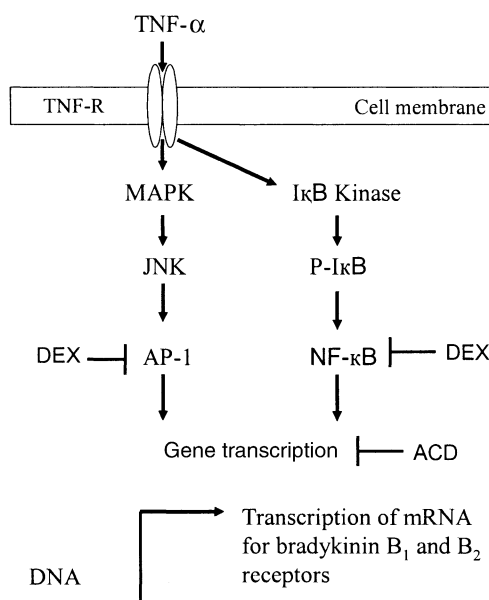


**Fig. 7.** Demonstration of the hypothesis and experimental design for the present study. P-I $\kappa$ B = I $\kappa$ B phosphorylation; TNF-R, TNF-receptors; DEX, dexamethasone; ACD, actinomycin D.

with previous *in vivo* experiments demonstrating an up-regulation of bradykinin-induced contractions during asthmatic conditions [7, 22]. Thus, an inflammatory process seems to be a key element for the increase of responses to bradykinin in airways. TNF- $\alpha$ -induced a 5-fold increase of the bradykinin B<sub>1</sub> receptor-mediated contractions and a threefold increase of the bradykinin B<sub>2</sub> receptor-mediated contractions in the segments cultured for 4 days. The corresponding receptor mRNA was enhanced two to three times. Discrepancies between mRNA expression and protein function are not uncommon and might be because of the fact that one copy of the mRNA can translate into several copies of the protein. The 'time window' between mRNA expression and the completion of the protein synthesis, and differences in the kinetics of mRNA and protein synthesis might also play a role along with differences in degradation.

TNF- $\alpha$  activates the MAPK and I $\kappa$ B kinase pathways resulting in gene transcription via the transcriptional factors NF- $\kappa$ B and AP-1 [12, 23, 24]. Actinomycin D is a transcriptional inhibitor, which binds to double-helical DNA and inhibits DNA-directed RNA synthesis [25]. Dexamethasone is an inhibitor for NF- $\kappa$ B and AP-1 activity [26–30]. In the present study, actinomycin D and dexamethasone both inhibited the TNF- $\alpha$  up-regulated expression of mRNA for bradykinin B<sub>1</sub> and B<sub>2</sub> receptors, confirming a role for transcriptional mechanisms in the up- and down-regulation of bradykinin B<sub>1</sub> and B<sub>2</sub> receptors. The inhibitory effects of dexamethasone were the same on both bradykinin receptor subtypes at the mRNA level, but differed in magnitude at the functional level. At the latter level dexamethasone attenuated des-Arg<sup>9</sup>-bradykinin-induced contractions by 50%, whereas bradykinin induced contractions returned to the control level. This suggests that more than one mechanism might be involved in the up-regulation of the bradykinin B<sub>1</sub> and B<sub>2</sub> receptors. Such an assumption is in line with our previous finding that the two bradykinin receptors only partly use the same intracellular signal transduction pathways for mediation of the TNF- $\alpha$  induced contractile up-regulation. JNK and ERK1/2 pathways are involved in bradykinin B<sub>2</sub> receptor activation, whereas the JNK pathway, but not the ERK 1/2 pathways are associated with bradykinin B<sub>1</sub> receptors activation [5]. Another possibility is that bradykinin B<sub>1</sub> and B<sub>2</sub> receptor coupling might be affected by the TNF- $\alpha$  and/or dexamethasone treatment [31, 32].

Glucocorticoids reduce airway hyper-reactivity in the asthmatic airways [33–36] and diminish airway inflammation via inhibition of the transcription factors AP-1 and NF- $\kappa$ B activities [28, 29, 37–39]. Dexamethasone inhibits the inducible bradykinin receptor expression in cultured human airway fibroblasts and smooth muscle cells [15, 16]. In contrast, methylprednisolone, another glucocorticoid, enhances the mRNA expression for bradykinin B<sub>2</sub> receptors in cultured guinea-pig tracheal smooth muscle cells. This up-regulation is apparent at 3 h, but not 7 h after the methylprednisolone application [17]. Thus, it might be that constitutive and induced bradykinin receptors react differently in response to glucocorticoids. The use of different glucocorticoids as well as the time points chosen for analysis might also affect the outcome.

In summary: As previously demonstrated, long-term exposure to TNF- $\alpha$  enhances the murine airway smooth

muscle response to des-Arg<sup>9</sup>-bradykinin and bradykinin, a phenomenon known to be dependent on the activation of different intracellular MAPK pathways [5]. In the present study this up-regulation is inhibited by dexamethasone and actinomycin D. These data suggest the involvement of transcriptional mechanisms in the up-regulation of bradykinin B<sub>1</sub> and B<sub>2</sub> receptors during airway inflammation, as well as in the down-regulation following glucocorticoids treatment. Further understanding of this intracellular signalling regulation may reveal the key for the 'transcription switch' thereby providing us with new strategies for treatment of asthma and chronic airway inflammation.

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## References

- 1 Amrani Y, Panettieri RA Jr. Cytokines induce airway smooth muscle cell hyperresponsiveness to contractile agonists. *Thorax* 1998; 53:713–6.
- 2 Barnes PJ, Adcock IM. How do corticosteroids work in asthma? *Ann Intern Med* 2003; 139:359–70.
- 3 Thomas PS, Yates DH, Barnes PJ. Tumor necrosis factor- $\alpha$  increases airway responsiveness and sputum neutrophilia in normal human subjects. *Am J Respir Crit Care Med* 1995; 152:76–80.
- 4 Thomas PS. Tumour necrosis factor- $\alpha$ : the role of this multifunctional cytokine in asthma. *Immunol Cell Biol* 2001; 79:132–40.
- 5 Zhang Y, Adner M, Cardell LO. Up-regulation of bradykinin receptors in a murine in-vitro model of chronic airway inflammation. *Eur J Pharmacol* 2004; 489:117–26.
- 6 Regoli D, Barabe J. Pharmacology of bradykinin and related kinins. *Pharmacol Rev* 1980; 32:1–46.
- 7 Polosa R, Holgate ST. Comparative airway response to inhaled bradykinin, kallidin, and [des-Arg<sup>9</sup>]bradykinin in normal and asthmatic subjects. *Am Rev Respir Dis* 1990; 142:1367–71.
- 8 Cembrzynska-Nowak M, Szklarz E, Inglot AD, Teodorczyk-Injeyan JA. Elevated release of tumor necrosis factor- $\alpha$  and interferon- $\gamma$  by bronchoalveolar leukocytes from patients with bronchial asthma. *Am Rev Respir Dis* 1993; 147:291–5.
- 9 Christiansen SC, Proud D, Sarnoff RB, Juergens U, Cochrane CG, Zuraw BL. Elevation of tissue kallikrein and kinin in the airways of asthmatic subjects after endobronchial allergen challenge. *Am Rev Respir Dis* 1992; 145:900–5.
- 10 Zhou L, Hershenson MB. Mitogenic signaling pathways in airway smooth muscle. *Respir Physiol Neurobiol* 2003; 137:295–308.
- 11 Gerthoffer WT, Singer CA. MAPK regulation of gene expression in airway smooth muscle. *Respir Physiol Neurobiol* 2003; 137: 237–50.
- 12 Aggarwal BB. Tumour necrosis factors receptor associated signalling molecules and their role in activation of apoptosis, JNK and NF- $\kappa$ B. *Ann Rheum Dis* 2000; 59 Suppl. 1:i6–16.
- 13 Wilckens T. Glucocorticoids and immune function: physiological relevance and pathogenic potential of hormonal dysfunction. *Trends Pharmacol Sci* 1995; 16:193–7.

- 14 Beato M, Herrlich P, Schutz G. Steroid hormone receptors: many actors in search of a plot. *Cell* 1995; 83:851–7.
- 15 Haddad EB, Fox AJ, Rousell J et al. Post-transcriptional regulation of bradykinin B<sub>1</sub> and B<sub>2</sub> receptor gene expression in human lung fibroblasts by tumor necrosis factor- $\alpha$ : modulation by dexamethasone. *Mol Pharmacol* 2000; 57:1123–31.
- 16 Schmidlin F, Scherrer D, Landry Y, Gies JP. Glucocorticoids inhibit the bradykinin B<sub>2</sub> receptor increase induced by interleukin-1 $\beta$  in human bronchial smooth muscle cells. *Eur J Pharmacol* 1998; 354:R7–8.
- 17 Scherrer D, Schmidlin F, Haddad EB, Kassel O, Landry Y, Gies JP. Glucocorticoids increase bradykinin B<sub>2</sub> receptor gene transcription in cultured guinea-pig tracheal smooth muscle cells. *Naunyn Schmiedebergs Arch Pharmacol* 1999; 359:153–9.
- 18 Li L, Vaali K, Paakkari I, Vapaatalo H. Involvement of bradykinin B<sub>1</sub> and B<sub>2</sub> receptors in relaxation of mouse isolated trachea. *Br J Pharmacol* 1998; 123:1337–42.
- 19 van Heuven-Nolsen D, Westra-De Vlieger JF, Muis T, Denee JH, Rivas TO, Nijkamp FP. Pharmacology and mode of action of bradykinin on mouse-isolated trachea. *Naunyn Schmiedebergs Arch Pharmacol* 1997; 356:134–8.
- 20 Marceau F, Hess JF, Bachvarov DR. The B<sub>1</sub> receptors for kinins. *Pharmacol Rev* 1998; 50:357–86.
- 21 Regoli D, Rizzi A, Calo G, Nsa Allogho S, Gobeil F. B<sub>1</sub> and B<sub>2</sub> kinin receptors in various species. *Immunopharmacology* 1997; 36:143–7.
- 22 Fuller RW, Dixon CM, Cuss FM, Barnes PJ. Bradykinin-induced bronchoconstriction in humans. Mode of action. *Am Rev Respir Dis* 1987; 135:176–80.
- 23 Wajant H, Pfizenmaier K, Scheurich P. Tumor necrosis factor signaling. *Cell Death Differ* 2003; 10:45–65.
- 24 Westwick JK, Weitzel C, Minden A, Karin M, Brenner DA. Tumor necrosis factor  $\alpha$  stimulates AP-1 activity through prolonged activation of the c-Jun kinase. *J Biol Chem* 1994; 269:26396–401.
- 25 Goldberg IH, Friedman PA. Antibiotics and nucleic acids. *Annu Rev Biochem* 1971; 40:775–810.
- 26 Cato AC, Wade E. Molecular mechanisms of anti-inflammatory action of glucocorticoids. *Bioessays* 1996; 18:371–8.
- 27 Gougat C, Jaffuel D, Gagliardo R et al. Overexpression of the human glucocorticoid receptor  $\alpha$  and  $\beta$  isoforms inhibits AP-1 and NF- $\kappa$ B activities hormone independently. *J Mol Med* 2002; 80:309–18.
- 28 Jaffuel D, Demoly P, Gougat C et al. Transcriptional potencies of inhaled glucocorticoids. *Am J Respir Crit Care Med* 2000; 162:57–63.
- 29 Adcock IM. Glucocorticoid-regulated transcription factors. *Pulm Pharmacol Ther* 2001; 14:211–9.
- 30 Gottlicher M, Heck S, Herrlich P. Transcriptional cross-talk, the second mode of steroid hormone receptor action. *J Mol Med* 1998; 76:480–9.
- 31 AbdAlla S, Lothar H, Quitterer U. AT<sub>1</sub>-receptor heterodimers show enhanced G-protein activation and altered receptor sequestration. *Nature* 2000; 407:94–8.
- 32 Barki-Harrington L, Bookout AL, Wang G, Lamb ME, Leeb-Lundberg LM, Daaka Y. Requirement for direct cross-talk between B<sub>1</sub> and B<sub>2</sub> kinin receptors for the proliferation of androgen-insensitive prostate cancer PC3 cells. *Biochem J* 2003; 371:581–7.
- 33 Meijer RJ, Kerstjens HA, Arends LR, Kauffman HF, Koeter GH, Postma DS. Effects of inhaled fluticasone and oral prednisolone on clinical and inflammatory parameters in patients with asthma. *Thorax* 1999; 54:894–9.
- 34 Overbeek SE, Rijnbeek PR, Vons C, Mulder PG, Hoogsteden HC, Bogaard JM. Effects of fluticasone propionate on methacholine dose–response curves in nonsmoking atopic asthmatics. *Eur Respir J* 1996; 9:2256–62.
- 35 Trifileff A, El-Hashim A, Bertrand C. Time course of inflammatory and remodeling events in a murine model of asthma: effect of steroid treatment. *Am J Physiol Lung Cell Mol Physiol* 2000; 279:L1120–8.
- 36 De Bie JJ, Hessel EM, Van Ark I et al. Effect of dexamethasone and endogenous corticosterone on airway hyperresponsiveness and eosinophilia in the mouse. *Br J Pharmacol* 1996; 119:1484–90.
- 37 Hakonarson H, Halapi E, Whelan R, Gulcher J, Stefansson K, Grunstein MM. Association between IL-1 $\beta$ /TNF- $\alpha$ -induced glucocorticoid-sensitive changes in multiple gene expression and altered responsiveness in airway smooth muscle. *Am J Respir Cell Mol Biol* 2001; 25:761–71.
- 38 Jeon YJ, Han SH, Lee YW, Lee M, Yang KH, Kim HM. Dexamethasone inhibits IL-1  $\beta$  gene expression in LPS-stimulated RAW 264.7 cells by blocking NF- $\kappa$ B/Rel and AP-1 activation. *Immunopharmacology* 2000; 48:173–83.
- 39 De Bosscher K, Vanden Berghe W, Haegeman G. The interplay between the glucocorticoid receptor and nuclear factor- $\kappa$ B or activator protein-1: molecular mechanisms for gene repression. *Endocr Rev* 2003; 24:488–522.