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

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Abstract

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-α) markedly enhanced contractile responses to des-Arg^9-bradykinin (selective bradykinin B_1 receptor agonist) and bradykinin (selective bradykinin B_2 receptor agonist) in murine airways. This increase was paralleled with elevated mRNA levels for bradykinin B_1 and B_2 receptors, a process involving intracellular mitogen-activated protein kinase pathways.

Objective To investigate the effects of glucocorticoids on the TNF-α up-regulated bradykinin B_1 and B_2 receptor response

Methods Tracheal segments from BALB/c J mice were cultured with and without TNF-α, in the absence and presence of the transcriptional inhibitor actinomycin D or the glucocorticoid, dexamethasone. The contractile response induced by des-Arg^9-bradykinin and bradykinin was subsequently assessed in a myograph system and mRNA for bradykinin B_1 and B_2 receptors was quantified using real-time polymerase chain reaction.

Results Actinomycin D abolished, and dexamethasone concentration-dependently suppressed the TNF-α-induced enhancement of the des-Arg^9-bradykinin and bradykinin responses. This was paralleled by a reduction of the mRNA expression for the bradykinin B_1 and B_2 receptors.

Conclusion The presented data suggests the involvement of transcriptional mechanisms in the up-regulation of bradykinin B_1 and B_2 receptors during asthmatic airway inflammation, as well as in their down-regulation following glucocorticoid treatment.

Key words: TNF-α, bradykinin, glucocorticoid, transcription, airway, inflammation, hyperresponsiveness, asthma

Introduction

Hyperresponsiveness 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]. Tumor necrosis factor-alpha (TNF-α) is recognized as an important pro-inflammatory cytokine with a large spectrum of activities, including the ability to induce airway hyperresponsiveness [3-5]. Kinins are mediators, produced in blood and tissues during inflammation and act by stimulating distinct receptors, such as bradykinin B_1 and B_2 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-α and bradykinin in bronchoalveolar lavage fluids from symptomatic asthmatic patients have suggested a relationship between TNF-α 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-α markedly enhanced contractile responses to des-Arg^9-bradykinin and bradykinin in tracheal smooth muscle. This increase was paralleled with elevated mRNA levels for the bradykinin B_1 and B_2 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-α induced up-regulation of bradykinin B_1 and B_2 receptors might also partly depend on *de novo* transcription and synthesis of bradykinin B_1 and B_2 receptors.

Glucocorticoids have a wide range of inhibitory effects on the inflammatory process and the related immune response [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-α- and interleukin-1β- induced up-regulation of bradykinin B_1 and B_2 receptors in human airway fibroblast and smooth muscle cells [15, 16]. Glucocorticoids have also been shown to enhance the gene transcription for bradykinin B_2 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-α-induced up-regulation of bradykinin B_1 and B_2 receptors in murine tracheal smooth muscle and to define the transcriptional mechanisms involved.

Material and Methods

Tissue preparation

10 weeks 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 μg/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 rings, 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, U.S.A.) with 300 µl serum free DMEM incubated at 37 °C in humidified 5% CO_2 in air with and without TNF-α (100 ng/ml), in the absence and presence of actinomycin D (5 µg/ml), a general transcriptional inhibitor, for 1 day or dexamethasone (0.01, 0.1 or 1 µM), a glucocorticoid, for 4 days. Segments were transferred into new wells containing fresh media including TNF-α, actinomycin D or dexamethasone every day.

In-vitro pharmacology

The cultured segments were immersed in temperature-controlled (37 ^{o}C) myograph bath (Organ Bath Model 700MO, J.P. Trading, Aarhus, Denmark) containing 5 ml Krebs-Henseleit buffer solution (143 mM Na^+, 5.9 mM K^+, 1.5 mM Ca^{2+}, 2.5 mM Mg^{2+}, 128 mM Cl^-, 1.2 mM $H_2PO_4^{2-}$, 1.2 mM SO_4^{2-}, 25 mM HCO_3^- and 10 mM D-glucose). The solution was continuously equilibrated with 5% CO_2 in O_2 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, U.K.). 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 one hour. 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 [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 ± S.E.M. 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-α (Fig. 4C). Each agonist concentration-effect curve was fitted to the Hill equation using an iterative, least square method (GraphPad Prism, San Diego, U.S.A), to provide estimates of maximal contraction (α) and pEC_{50} values (negative logarithm of the agonist concentration that produces 50% of the maximal effect). Unpaired student *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-α was obtained from R&D Systems (Abingdon, U.K). Bradykinin and des-Arg^9-bradykinin was purchased from Neosystem S.A. (Strasbourg, France). Dexamethasone, actinomycin D, indomethacin, carbachol, DMEM and Krebs-Henseleit Buffer were from Sigma (St. Louis, MO, U.S.A). TNF-α, bradykinin, des-Arg^9-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 PBS and stored in the RNAlater™ (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, Hilden, Germany). 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™ reverse transcriptase kit (QIAGEN GmbH, Hilden, Germany) in 20 µl volume reaction at 37 °C for 1 h by using Mastercycler personal PCR machine (Eppendorf AG, Hamburg, Germany).

To quantify mRNA for bradykinin B_1 and B_2 receptors, real-time polymerase chain reaction (real-time PCR) was performed with the QuantiTect™ SYBR® Green PCR kit (QIAGEN GmbH, Hilden, Germany) in The Smart Cycler® II system (Cepheid, Sunnyvale, CA, 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 25 µl 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 sec 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 sec and 55 °C for 1 min were performed. The data were analyzed 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_1 receptor 102 bp, bradykinin B_2 receptor 104 bp and β-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® 2.0 software (Applied Biosystem, Forster city, CA, USA) and synthesized by DNA Technology A/S (Aarhus, Denmark). Sequences as follows:

Bradykinin B_1 receptor: Forward: 5'-CCA TAG CAG AAA TCT ACC TGG CTA AC-3'
Reverse: 5'-GCC AGT TGA AAC GGT TCC-3'
Bradykinin B_2 receptor: Forward: 5'-ATG TTC AAC GTC ACC ACA CAA GTC-3'
Reverse: 5'-TGG ATG GCA TTG AGC CAA C-3'
β-actin: Forward: 5'-TGG GTC AGA AGG ACT CCT ATG TG-3'
Reverse: 5'-CGT CCC AGT TGG TAA CAA TGC-3'

The $^{\Delta\Delta}C_T$ method was employed to calculate the relative amount of mRNA for bradykinin B_1 and B_2 receptors. The relative amount of mRNA was obtained by the C_T values of mRNA for bradykinin B_1 or B_2 receptor in relation to the C_T values of mRNA for house keep gene β-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 μM) revealed that contractions induced by des-Arg^9-bradykinin (selective bradykinin B_1 receptor agonist) and bradykinin (selective bradykinin B_2 receptor agonist) were concentration-dependently inhibited by dexamethasone (Fig. 1A-B, table 1).

When tracheal segments were cultured for 4 days in the presence of TNF-α (100 ng/ml) with increasing concentrations of dexamethasone (0.003, 0.01, 0.03, 0.1 or 1 μM), dexametasone produced a concentration-dependent reduction of the maximal contractile responses to des-Arg^9-bradykinin and bradykinin. The maximal reduction was, for both kinins, reached at 1 μM of dexamethasone. For des-Arg^9-bradykinin this reduction became significant at 0.03, 0.1 and 1 μM of dexamethasone (Fig. 2A, table 1, $P<0.05$) and for bradykinin at 0.01, 0.03, 0.1 and 1 μM of dexamethasone (Fig. 2B, table 1, $P<0.05$). In addition, there was a rightward shift of des-Arg^9-bradykinin and bradykinin concentration-response curves with significant changes of pEC_{50} values at 0.1 and 1 μM of dexamethasone for des-Arg^9-bradykinin (Fig. 2A, table 1, $P<0.05$) and 0.01, 0.03, 0.1 and 1 μM 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-α (100 ng/ml) in the presence of dexamethasone. The maximal contractions and the pEC_{50}-values were not affected by dexamethasone (Fig. 3A-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_1 and B_2 receptors were mediated via transcriptional mechanisms, segments were cultured for 1 day with and without TNF-α (100 ng/ml), in the presence of actinomycin D (5 μg/ml), a general transcriptional inhibitor. Actinomycin D completely abolished the TNF-α induced enhancement of the contractile response to des-Arg^9-bradykinin and bradykinin. In addition, the contractions seen during control conditions (cultured for 1 day without TNF-α) were also omitted as the result of the presence of actinomycin D (Fig. 4A-B, table 2, $P<0.05$).

To determine whether actinomycin D (5 μg/ml) affected the carbachol-induced contraction *per se*, experiments were performed with segments cultured for 1 day, with and without TNF-α 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).

Receptor mRNA Study

To investigate if the TNF-α 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-α (100 ng/ml). The relative amount of mRNA for the bradykinin B_1 and B_2 receptors was quantified with real-time PCR. Enhanced mRNA levels of the bradykinin B_1 and B_2 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-α (100 ng/ml) (P<0.05).

To further confirm the involvement of transcriptional mechanisms, the tracheae were cultured with and without TNF-α (100 ng/ml), for 1 day in the presence of actinomycin D (5 μg/ml) or for 4 days in presence of dexamethasone (1 μM). In both cases, a significant inhibition of mRNA expression for the bradykinin B_1 and B_2 receptors was obtained (Fig. 6A-B, P<0.001).

Discussion

Airway inflammation induces airway smooth muscle hyperresponsiveness to various contractile mediators [1, 2]. We have previously demonstrated that TNF-α up-regulates bradykinin B_1 and B_2 receptor-mediated contractions in murine airways, a phenomenon that are 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-α-induced up-regulation of the bradykinin receptors via a transcriptional mechanism (Fig. 7).

In the airways only small amounts of bradykinin B_2 receptors and no bradykinin B_1 receptors are expressed during physiological conditions [20, 21]. In the present set up no response to des-Arg^9-bradykinin and only a weak contractile response to bradykinin could be seen in fresh tracheal segments, even though some mRNA for the bradykinin B_1 receptor could be detected [5]. This suggests that the tracheal segments used were in a physiological condition. After organ culture of the tracheal segments for 1 or 4 days, bradykinin B_1 and B_2 receptors were up-regulated at the mRNA level as well as at the functional level. Thus, contractile responses to des-Arg^9-bradykinin and bradykinin appeared. The mechanism 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_1 and B_2 receptors was further enhanced during organ culture in presence of the inflammatory mediator TNF-α. These findings are in line 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-α-induced a 5-fold increase of the bradykinin B_1 receptor-mediated contractions and a 3-fold increase of the bradykinin B_2 receptor-mediated contractions in the segments cultured for 4 days. The corresponding receptor mRNA were enhanced 2-3 times. Discrepancies between mRNA expression and protein function are not uncommon and might be due to the fact that one copy of the mRNA that 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-α activates the MAPK and I*k*B kinase pathways resulting in gene transcription via the transcriptional factors NF-*k*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-*k*B and AP-1 activity [26-30]. In the present study, actinomycin D and dexamethasone both inhibited the TNF-α up-regulated expression of mRNA for bradykinin B_1 and B_2 receptors, confirming a role for transcriptional mechanisms in the up- and down-regulation of bradykinin B_1 and B_2 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^9-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_1 and B_2 receptors. Such an assumption is in line with our previous finding that the two bradykinin receptors only partly uses the same intracellular signal transduction pathways for mediation of the TNF-α induced contractile up-regulation. JNK and ERK1/2 pathways are involved in bradykinin B_2 receptor activation, whereas the JNK pathway, but not the ERK ½ pathways are associated with bradykinin B_1

receptors activation [5]. Another possibility is that bradykinin B_1 and B_2 receptor coupling might be affected by the TNF-α and/or dexamethasone treatment [31, 32].

Glucocorticoids reduce airway hyperreactivity in the asthmatic airways [36-39] and diminish airway inflammation via inhibition of the transcription factors AP-1 and NF-*k*B activities [28, 29, 33-35]. 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_2 receptors in cultured guinea-pig tracheal smooth muscle cells. This up-regulation is apparent at 3 hours, but not 7 hours 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 analyse might also have effect the outcome.

In summary: As previously demonstrated, long-term exposure to TNF-α enhances the murine airway smooth muscle response to des-Arg^9-bradykinin and bradykinin, a phenomenon known to 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_1 and B_2 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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Figure Legends

Figure 1

Contractile effects of (A) des-Arg^9-bradykinin and (B) bradykinin in segments cultured for 4 days in the absence and presence of dexamethasone (DEX) 0.01, 0.1 or 1 μM. Each data point is represented as the mean of all segments ± S.E.M (n=6-7).

Figure 2

Contractile effects of (A) des-Arg^9-bradykinin and (B) bradykinin in segments cultured for 4 days with TNF-α 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 ± S.E.M (n=6-10).

Figure 3

Contractile effects of carbachol in segments cultured for 4 day in (A) absence and (B) presence of TNF-α 100 ng/ml with and without dexamethasone (DEX). Each data point is represented as the mean of all segments ± S.E.M (n=4-6).

Figure 4

Contractile effects of (A) des-Arg^9-bradykinin, (B) bradykinin and (C) carbachol in segments cultured for 1 day in the absence and presence of TNF-α 100 ng/ml with and without actinomycin D 5 μg/ml (ACD). Each data point is represented as the mean of all segments ± S.E.M (n=6-8).

Figure 5

Effects of TNF-α on bradykinin B_1 and B_2 receptor mRNA expression in tracheal smooth muscle strips before and after organ culture, analyzed with real-time quantitative PCR. Segments cultured for (A) 1 and (B) 4 days in the absence and presence of TNF-α (100 ng/ml). Each data point is derived from 3 identical experiments and represented as percent of the fresh segments, mean ± S.E.M. *P<0.05, **P<0.01 and ***P<0.001. (Unpaired student *t* test) BK B1R=bradykinin B_1 receptor, BK B2R=bradykinin B_2 receptor.

Figure 6

Effects of actinomycin D and dexamethasone on bradykinin B_1 and B_2 receptor mRNA expression in tracheal smooth muscle strips after organ culture, analyzed with real-time quantitative PCR. Segments cultured for 1 day with actinomycin D or 4 days with dexamethasone in (A) absence or (B) presence of TNF-α (100 ng/ml). Each data point is derived from 3-4 identical experiments and represented as percent of control (A: organ culture without actinomycin D or dexamethasone and B: organ culture in presence of TNF-α, but without actinomycin D or dexamethasone), mean ± S.E.M. ***P<0.001 (Unpaired student *t* test, each column compared with control). BK B1R=bradykinin B_1 receptor, BK B2R=bradykinin B_2 receptor.

Figure 7

Demonstration of the hypothesis and experimental design for the present study.

P-I*k*B = I*k*B phosphorylation; TNF-R=TNF-receptors; DEX=dexamethasone;

ACD=actinomycin D.

Table 1

Effects of dexamethasone on the maximal contraction (α) and pEC_{50} values of des-Arg^9-bradykinin and bradykinin in segments cultured for 4 days in the absence and presence of TNF-α

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

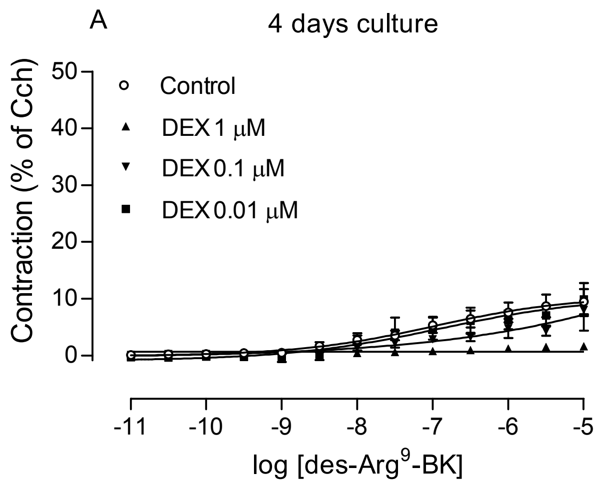
Data are represented as percent of carbachol (Cch) induced maximal contraction with the mean ± S.E.M. Statistical analysis was performed with unpaired student *t* test ([a]control vs. TNF-α) or one-way ANOVA and Dunnet's post test ([b]control vs. DEX and [c]TNF-α vs. TNF-α + DEX). $P < 0.05$ were considered to be significant. ND = not determined, DEX=dexamethasone.

Table 2

Effects of actinomycin D on the maximal contraction (α) and pEC_{50} values of des-Arg^9-bradykinin and bradykinin in segments cultured for 1 day in the absence and presence of TNF-α

	des-Arg^9-bradykinin			Bradykinin		
	n	α (mN)	pEC_{50}	n	α (mN)	pEC_{50}
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[b]	ND	7	0.06±0.02[b]	ND
TNF-α 100 ng/ml	6	3.12±0.29[a]	7.56±0.09[a]	8	3.96±0.61	6.98±0.19[a]
TNF-α + ACD 5 μg/ml	6	0.11±0.04[c]	ND	8	0.09±0.03[c]	ND

Data are presented as absolute values of contraction (mN) with the mean ± S.E.M. Statistical analysis was performed with unpaired student *t* test. $P < 0.05$ were considered to be significant; [a]control (organ culture) vs. TNF-α, [b]control (organ culture) vs. organ culture with ACD (actinomycin D) and [c]TNF-α vs. TNF-α + ACD. ND = not determined, ACD=actinomycin D.


A
4 days culture
Contraction (% of Cch)
50
40
30
20
10
0
Control
DEX 1 μM
DEX 0.1 μM
DEX 0.01 μM
-11
-10
-9
-8
-7
-6
-5
log [des-Arg9-BK]

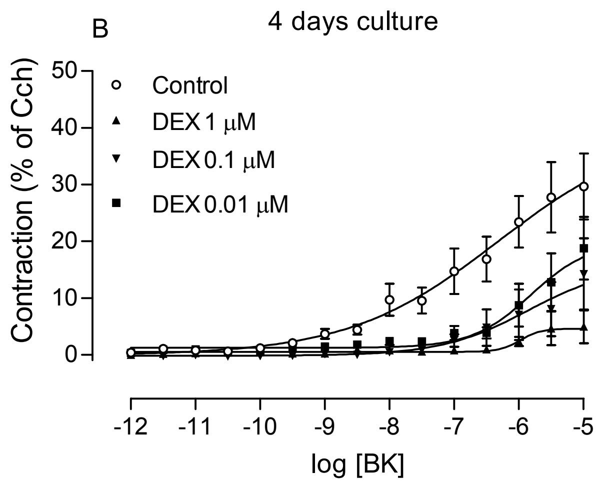

B
4 days culture
Contraction (% of Cch)
50
40
30
20
10
0
Control
DEX 1 μM
DEX 0.1 μM
DEX 0.01 μM
-12
-11
-10
-9
-8
-7
-6
-5
log [BK]

Figure 1 A-B

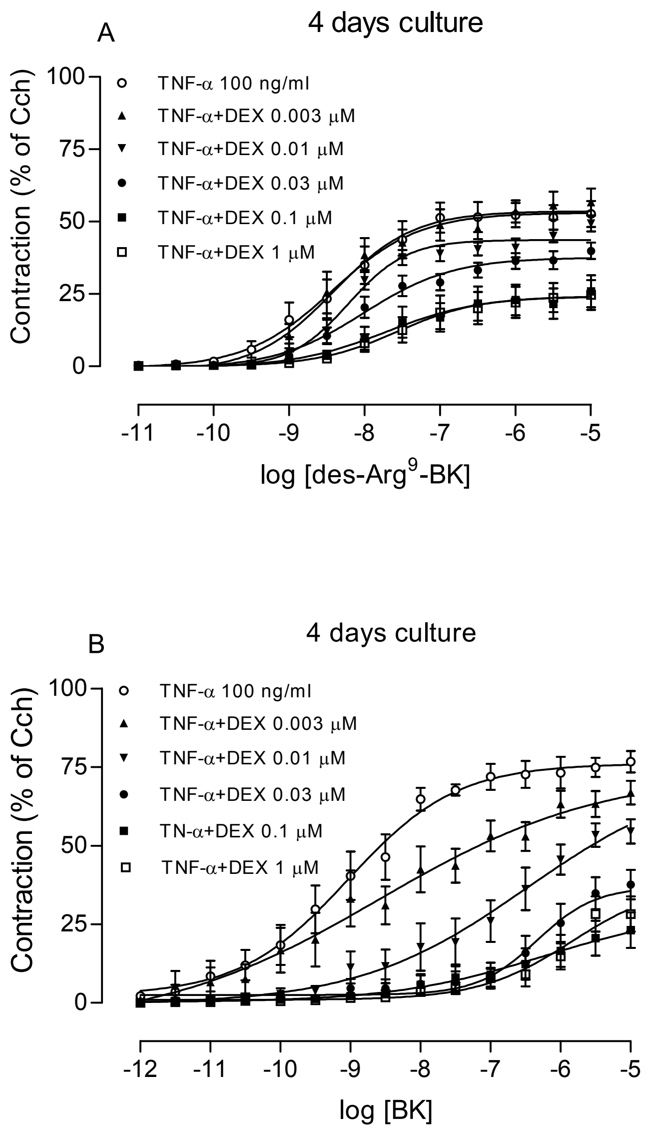

A
4 days culture
Contraction (% of Cch)
100
75
50
25
0
TNF-α 100 ng/ml
TNF-α+DEX 0.003 μM
TNF-α+DEX 0.01 μM
TNF-α+DEX 0.03 μM
TNF-α+DEX 0.1 μM
TNF-α+DEX 1 μM
-11
-10
-9
-8
-7
-6
-5
log [des-Arg9-BK]
B
4 days culture
Contraction (% of Cch)
100
75
50
25
0
TNF-α 100 ng/ml
TNF-α+DEX 0.003 μM
TNF-α+DEX 0.01 μM
TNF-α+DEX 0.03 μM
TN-α+DEX 0.1 μM
TNF-α+DEX 1 μM
-12
-11
-10
-9
-8
-7
-6
-5
log [BK]

Figure 2 A-B

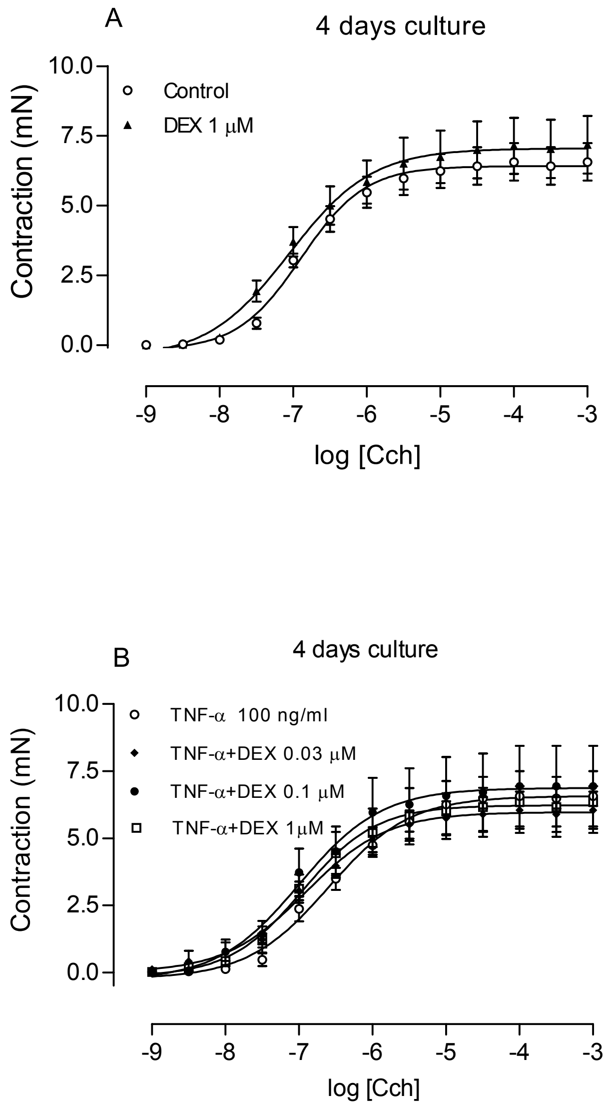

A
4 days culture
Contraction (mN)
10.0
7.5
5.0
2.5
0.0
Control
DEX 1 μM
-9
-8
-7
-6
-5
-4
-3
log [Cch]
B
4 days culture
Contraction (mN)
10.0
7.5
5.0
2.5
0.0
TNF-α 100 ng/ml
TNF-α+DEX 0.03 μM
TNF-α+DEX 0.1 μM
TNF-α+DEX 1μM
-9
-8
-7
-6
-5
-4
-3
log [Cch]

Figure 3 A-B

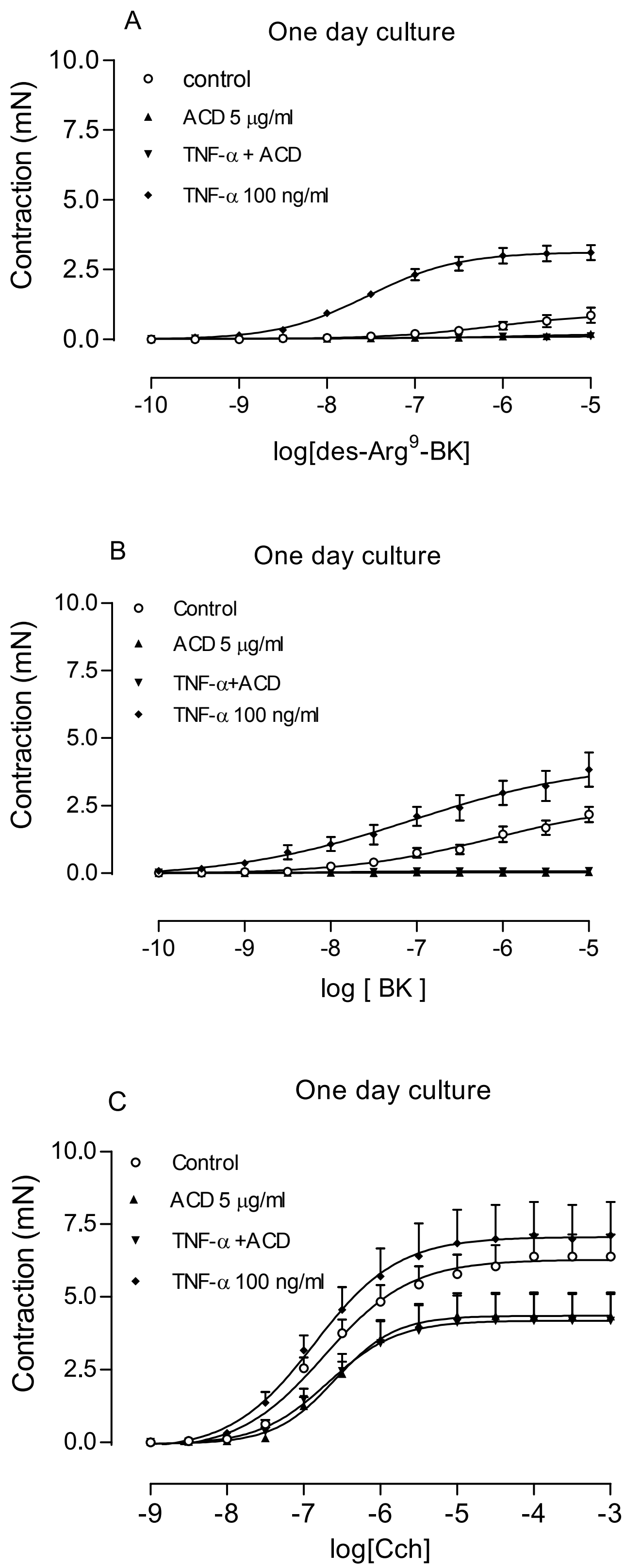
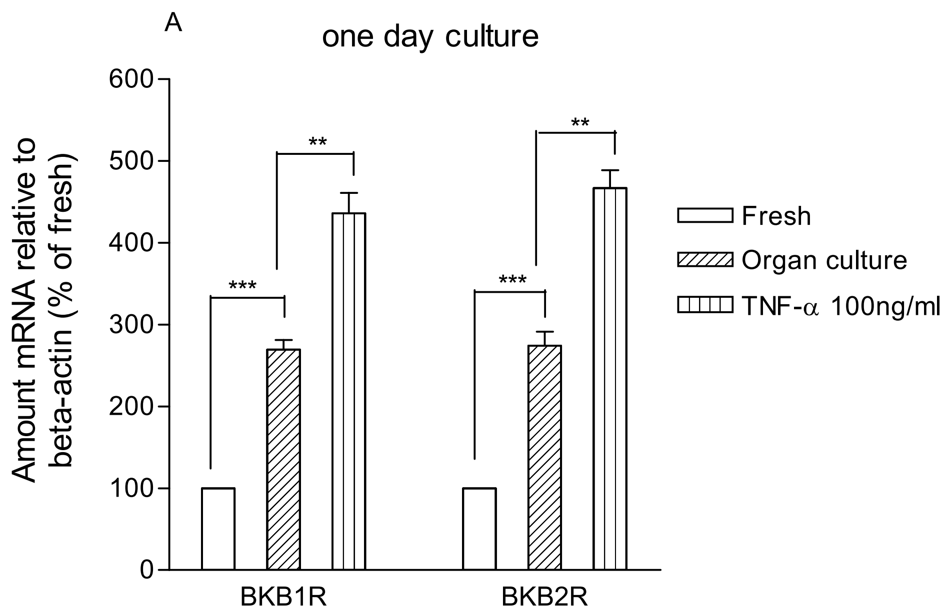

A
One day culture
control
ACD 5 μg/ml
TNF-α + ACD
TNF-α 100 ng/ml
Contraction (mN)
10.0
7.5
5.0
2.5
0.0
-10
-9
-8
-7
-6
-5
log[des-Arg9-BK]
B
One day culture
Control
ACD 5 μg/ml
TNF-α+ACD
TNF-α 100 ng/ml
Contraction (mN)
log [BK]
C
One day culture
Control
ACD 5 μg/ml
TNF-α +ACD
TNF-α 100 ng/ml
Contraction (mN)
-9
-8
-7
-6
-5
-4
-3
log[Cch]

Figure 4 A-C


A
one day culture
Amount mRNA relative to beta-actin (% of fresh)
600
500
400
300
200
100
0
***
**
***
**
BKB1R
BKB2R
Fresh
Organ culture
TNF-α 100ng/ml

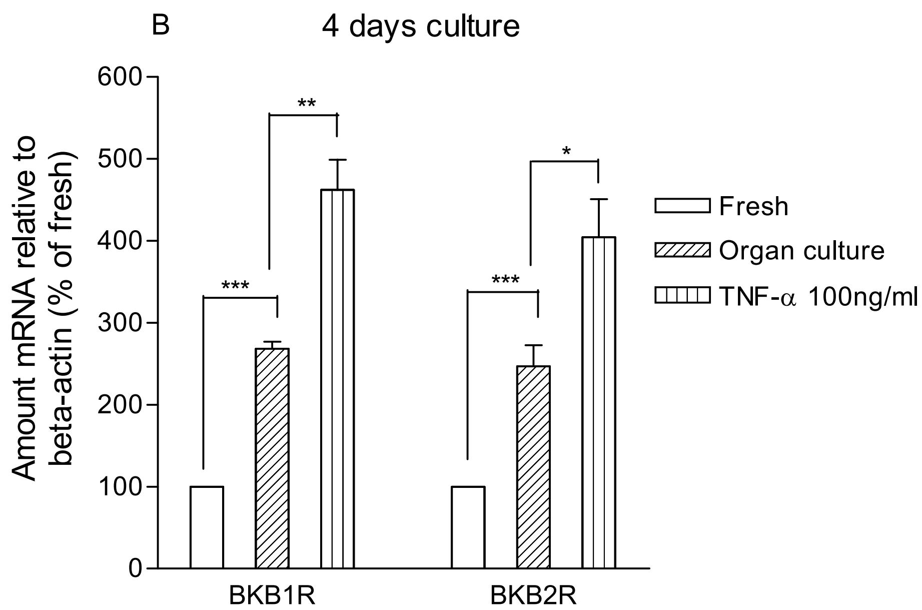

B
4 days culture
Amount mRNA relative to beta-actin (% of fresh)
600
500
400
300
200
100
0
***
**
***
*
BKB1R
BKB2R
Fresh
Organ culture
TNF-α 100ng/ml

Figure 5 A-B

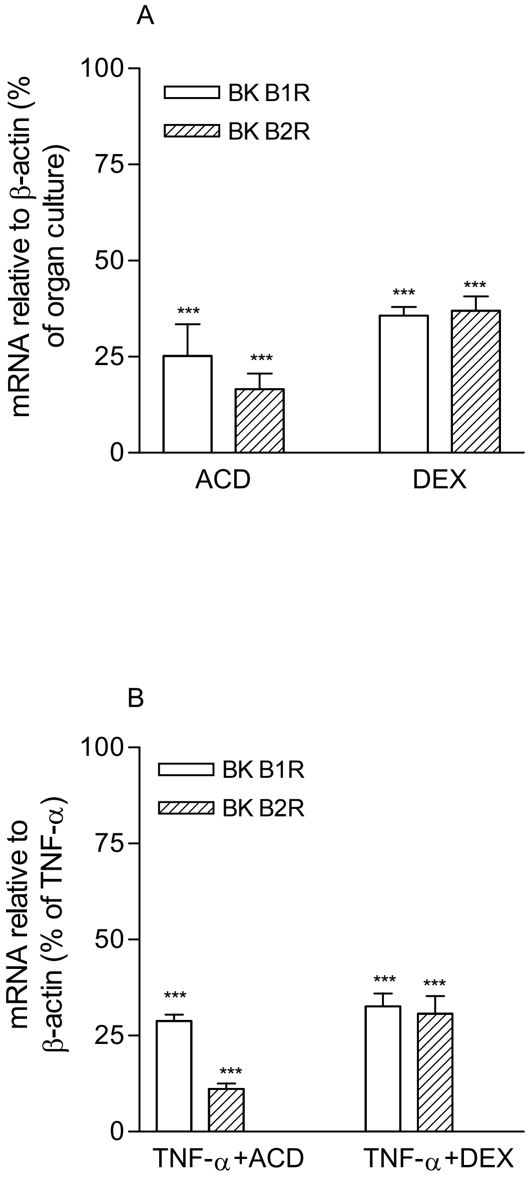

A
100
75
50
25
0
mRNA relative to β-actin (% of organ culture)
BK B1R
BK B2R
***
ACD
DEX
B
mRNA relative to β-actin (% of TNF-α)
TNF-α+ACD
TNF-α+DEX

Figure 6 A-B

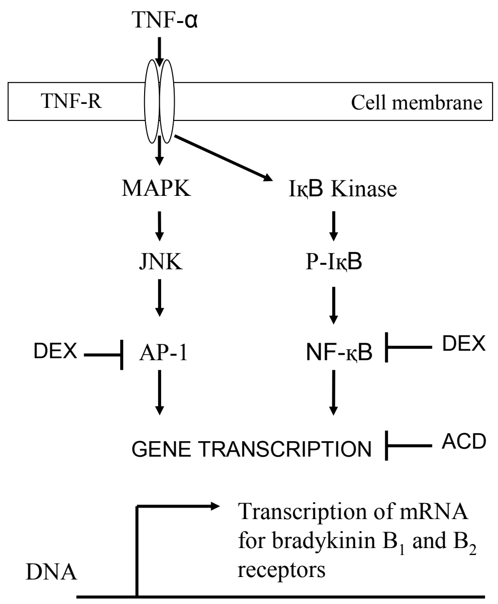

TNF-α
TNF-R
Cell membrane
MAPK
IκB Kinase
JNK
P-IκB
DEX
AP-1
NF-κB
DEX
GENE TRANSCRIPTION
ACD
Transcription of mRNA for bradykinin B1 and B2 receptors
DNA

Figure 7