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TNFα reduces tachykinin, PGE_2-dependent, relaxation of the cultured mouse trachea by increasing the activity of COX-2

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1 Chronic inflammation is a central feature of asthma. The inflammatory cytokine tumour necrosis factor α (TNFα) has been implicated in this disease, and is known to alter airway smooth muscle functionally.

2 The aim of this study was to investigate the influence of TNFα on tachykinin-induced airway relaxation. Mouse tracheae were cultured in the absence and presence of TNFα for 1 or 4 days.

3 In the absence of TNFα, substance P (SP) and neurokinin A (NKA) induced comparable levels of relaxation in fresh and cultured segments. Functional studies with selective antagonists/inhibitors indicated that the relaxation was mediated by the NK_1 receptor coupled to cyclooxygenase (COX)-2 activation and subsequent release of prostaglandin E_2 (PGE_2). TNFα attenuated SP- and NKA-induced relaxation in a time- and concentration-dependent manner, decreasing the ability of PGE_2 to relax tissues.

4 Further studies indicated that TNFα elevated COX-2 activity and that concomitant inhibition of COX-2 reversed TNFα-attenuated PGE_2 relaxation. Culture with PGE_2 decreased SP- and PGE_2-mediated relaxation, further implicating the activity of COX-2 in the attenuation of tachykinin signalling.

5 Gene expression analysis demonstrated that TNFα increased the expression of smooth muscle COX-2, PGE_2 synthase and EP_2 receptor mRNA, and decreased the expression of the EP_4 receptor.

6 Overall, these results show that NK_1 receptor-mediated relaxation induced by PGE_2 is attenuated by prolonged TNFα stimulation. Increased COX-2 activity induced by TNFα appears to be central to this process.

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Abbreviations: COX, cyclooxygenase; DMEM, Dulbecco's modified Eagle's medium; GAPD, glyceraldehyde-3-phosphate dehydrogenase; MAS, microarray suite; NK, neurokinin; PBS, phosphate-buffered saline; PGE_2, prostaglandin E_2; PTGES, prostaglandin E_2 synthase|SAM, significance analysis of microarrays; SP, substance P; TNFα, tumour necrosis factor α

Introduction

Airway hyper-responsiveness is a central feature of asthma that is considered to be induced by the release of agents during chronic inflammation (Wardlaw *et al*., 2002). Hyper-responsiveness is closely associated with changes in the performance of airway smooth muscle; specifically, the condition is characterized by an enhancement of agonist-induced contraction and a parallel decrease in agonist-induced relaxation (Wardlaw *et al*., 2002). Models for studying such smooth muscle changes are limited (Szelenyi, 2000), with most *in vivo* models being too complex to allow long-term effects of inflammatory mediators to be evaluated (Rytila *et al*., 2000). To address this issue, we recently developed an *in vitro* model to study chronic inflammation (Adner *et al*., 2002). Our studies showed that mouse tracheal segments in organ culture maintain their original morphological structure and their contractility for at least a month. Thus, functional and gene expression changes can be assessed in these tissues following culture with inflammatory mediators.

Tumour necrosis factor (TNF)α, an inflammatory cytokine, is suggested to play an important role in the pathogenesis of asthma (Amrani *et al*., 2000). In several studies, elevated levels of TNFα have been found in bronchial lavage isolated from asthmatic patients (Broide *et al*., 1992; Tonnel *et al*., 2001), and increased levels of TNFα are associated with bronchial hyper-responsiveness (Halasz *et al*., 2003). Administration of TNFα to rats induces hyper-responsiveness to bronchial constrictors (Kips *et al*., 1992), and treatment of isolated guinea-pig airway tissue *in vitro* with TNFα reduces

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isoproterenol-mediated relaxation (Wills-Karp *et al*., 1993). In addition, we have shown that the culture of tracheal segments with TNFα enhances 5-hydroxytryptamine 2A receptor-mediated contractile responses (Adner *et al*., 2002).

Tachykinin neuropeptides are released from sensory nerve endings in the respiratory tract and potently relax airway smooth muscle. These peptides activate airway epithelial neurokinin 1 (NK_1) receptors (Szarek *et al*., 1998; Tournoy *et al*., 2003), which subsequently induce airway relaxation by stimulating the release of prostaglandin E_2 (PGE_2), a product of cyclooxygenase (COX) enzymes (Szarek *et al*., 1998; Kao *et al*., 1999; Fortner *et al*., 2001). Recent studies in mice have emphasized an important role for PGE_2 EP_2 receptors in mediating airway relaxation (Fortner *et al*., 2001). However, transgenic mouse studies suggest that relaxant effects can also be mediated by EP_4 receptors (Fortner *et al*., 2001), consistent with the fact that both EP_2 and EP_4 receptors are coupled to $G_{s\alpha}$-subunits.

The aim of the current study was to evaluate whether prolonged TNFα exposure altered tachykinin-induced relaxant responses. Isolated tracheal segments were cultured in the absence and presence of TNFα, and relaxation to the tachykinin substance P (SP) was assessed. Tissues were cultured for up to 4 days, as phenotypic changes induced by TNFα can be readily assessed after this period of time (Adner *et al*., 2002). The basis for the changes observed was investigated by assessing tachykinin responses in intact tissue and measuring changes in smooth muscle gene expression with microarrays and quantitative RT–PCR.

Methods

Tissue preparation and organ culture

BALB/cJ mice (male; 8–10 week) were killed by asphyxiation with CO_2 or cervical dislocation. Whole tracheae were rapidly removed and placed into Dulbecco's modified Eagle's medium (DMEM; 4500 mg l^{-1} D-glucose, 110 mg l^{-1} sodium pyruvate, 584 mg l^{-1} L-glutamine), supplemented with 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. Individual tracheae were thereafter dissected free from adherent tissue under sterile conditions. Tracheae were used whole for mRNA studies or divided into segments each with three cartilage rings for functional studies. Tissues were used directly after dissection (fresh) or placed individually in multiwell plates in DMEM for organ culture (300 μl–1 ml). Under culture conditions, tissues were incubated at 37°C in humidified 5% CO_2 in air for 1 or 4 days and transferred daily to a new well containing fresh media. Where appropriate, DMEM contained 1–100 ng ml^{-1} murine TNFα or 100 ng ml^{-1} human TNFα, 10 nM DuP697, 10 nM piroxicam and/or 10 μM PGE_2. Murine TNFα was used in all studies except for microarray studies. Prior to RNA extraction, epithelial cells were removed from tracheae by scraping the lumenal surface with a scalpel, and where appropriate, smooth muscle was dissected free from the rest of the trachea. Protocols were approved by the Ethical Committee of University of Lund (Lund, Sweden) and the Johnson and Johnson Pharmaceutical Research and Development (La Jolla, CA, U.S.A.) institutional animal care and use committee.

Isometric force measurement

Tracheal smooth muscle reactivity was analysed in temperature-controlled (37°C) myographs (Organ Bath Model 700MO, J.P. Trading, Aarhus, Denmark) containing Krebs–Henseleit buffer solution composed of 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 Sigma (St Louis, MO, U.S.A.). The solution was continuously equilibrated with 5% CO_2 and 95% O_2 resulting in a pH of 7.4. The tracheal segments were 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 (ADInstruments Ltd, Hastings, U.K.). The other prong was connected to a displacement device, allowing adjustment of the distance between the two parallel prongs.

In vitro *pharmacology*

Tracheal segments were placed in a myograph containing Krebs–Henseleit buffer solution and gradually stretched to a basal tension of 0.8 mN over 1 h. After equilibration at the given tension, the contractile capacity of each segment was tested by treatment with 60 mM KCl. Following a 30 min rest period, segments were contracted with 1 μM carbachol. Contractions reached a stable plateau after ~15 min, and tissues were subsequently exposed to a single concentration of SP, neurokinin A (NKA) or neurokinin B (NKB). The concentrations of SP, NKA and NKB were chosen to induce maximum possible relaxant responses. In separate experiments, segments were cumulatively treated with PGE_2. Specific antagonists/inhibitors were added 30 min prior to stimulation. Concentration–effect curves from individual segments were fitted to the Hill equation using an iterative, least-squares method (GraphPad Prism, San Diego, CA, U.S.A.) to provide estimates of maximal contraction (E_{max}) or relaxation (R_{max}). This method was also used to estimate pEC_{50}, except where concentration–effect curves were not fully defined, when it was estimated using linear regression.

RNA extraction and amplification

The RNeasy Mini Kit (Qiagen, Valencia, CA, U.S.A.) was used to prepare total RNA from tracheae and tracheal smooth muscle. The kit was used as recommended by the supplier, except that optional DNaseI (Qiagen) treatment was also performed. Prior to RT–PCR, levels of RNA extracted from tracheal smooth muscle were increased using a T7 RNA polymerase amplification technique (Van Gelder *et al*., 1990).

DNA microarray analysis

Microarray analysis was performed using U74Av2 GeneChip arrays (Affymetrix, Santa Clara, CA, U.S.A.) as recommended by the supplier, with total RNA from three tracheae being pooled for each array. Gene expression levels were calculated using microarray suite (MAS) 5.0 (Affymetrix). Genes were not analysed further if they were absent (as defined by MAS 5.0) in all samples. Differentially expressed genes were identified using significance analysis of microarrays (SAM) 1.21 (Tusher *et al*., 2001). The minimum fold-change between treatment groups was defined as 1.5-fold, and the Δ parameter

in the software was set to 0.82 to ensure that the false discovery rate was <5% (90% percentile). Data were permuted 1000 times by SAM for statistical assessments.

Quantitative RT–PCR

Two-tube quantitative real-time RT–PCR was utilized throughout the study, that is, the RT and PCR steps were performed in different tubes (Rose *et al*., 2002). RT reactions were performed using the TaqMan Reverse Transcription kit (Applied Biosystems, Foster City, CA, U.S.A.), as recommended by the supplier. Quantitative real-time PCR assays were performed using the iCycler iQ detection system (Bio-Rad, Hercules, CA, U.S.A.). Primer sequences are listed in Table 1. PCR assays contained 300 nM of both forward and reverse primers, 20 nM Fluorescein (Bio-Rad) and SYBR Green PCR Master Mix (Applied Biosystems) in a final volume of 50 μl. Reactions were incubated at 95°C for 10 min, and then incubated for 40 cycles of 95°C for 15 s and 60°C for 1 min. Samples were assayed in triplicate. Standard curves were prepared using 10-fold dilutions of PCR product generated from target cDNA. Levels of mRNA were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPD) mRNA levels.

Immunohistochemistry

The location of tracheal proteins was investigated using immunohistochemistry. Tracheae were imbedded in OCT™ Compound (Sakura Finetek, Torrance, CA, U.S.A.), sectioned (5 μm), fixed in acetone for 2 min at −20°C, air-dried for 1–2 h and stored at −80°C. Prior to the visualization of proteins, sections were thawed and rinsed in phosphate-buffered saline (PBS) three times. To quench endogenous peroxidase activity, sections were incubated in 0.03% hydrogen peroxide (in PBS) for 10 min and then rinsed in PBS. Subsequently, tissues were incubated with avidin and biotin blocking reagent (from the streptavidin–horseradish peroxide kit; R&D systems) for 15 min each. Sections were then incubated with 5% goat or donkey serum (Jackson Immuno Research, West Grove, PA, U.S.A.) in PBS for 30 min. Primary antibodies (COX-1 (M-20), COX-2 (M-19), PGE_2 synthase (PTGES) (N-15), EP_2 (H-75), EP_4 (C-18); Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) were diluted 1 : 250 in PBS and applied to sections for 2 h. Subsequently, biotin-conjugated donkey anti-goat or goat anti-rabbit secondary antibodies (Jackson Immuno Research) were diluted 1 : 1000 in PBS and then incubated with sections for 30 min, as appropriate. To visualize proteins, tissues were incubated with streptavidin–horseradish peroxide solution (R&D systems) for 30 min followed by diaminobenzidine (R&D systems). Sections were then counter-stained using haematoxylin, dehydrated with increasing concentrations of ethanol, rinsed in xylene and secured with a glass coverslip. Where not specified, reagents were obtained from Sigma or Invitrogen.

Materials

DMEM, carbachol, PGE_2, SP, NKA and NKB were obtained from Sigma (St Louis, MO, U.S.A.), penicillin and streptomycin were obtained from Life Technologies (Gaithersburg, MD, U.S.A.), recombinant murine and human TNFα were obtained from R&D Systems (Abingdon, U.K.) and Sigma, respectively, and DuP697 and piroxicam were obtained from Tocris (Bristol, U.K.).

Statistical analysis

Unless described in the sections above, Student's *t*-test or ANOVA were used to determine statistical differences. When ANOVA indicated statistical significance, *t*-tests were performed with Bonferroni correction to test for differences between groups. Microarray and RT–PCR data were logarithmically (base 2) transformed for statistical comparisons. All data are expressed as mean ± s.e.m.

Results

Effect of culture on tachykinin-induced relaxation of tracheae

Fresh tracheal segments and segments cultured for 1 and 4 days in the absence and presence of murine TNFα contracted reproducibly upon addition of carbachol (1 μM). These contractions reached a stable plateau after ∼15 min, following which relaxant experiments were performed (Figure 1a). As tachykinin-induced relaxation was transient in nature, lasting

Table 1 Quantitative real-time RT–PCR primer sequences

Target	*Name*	*Sequence (5′–3′)*
COX-1 (NM_008969)	Mouse-COX-1-F	CACTGGTGGATGCCTTCTCT
	Mouse-COX-1-R	TCTCGGGACTCCTTGATGAC
COX-2 (NM_011198)	Mouse-COX-2-F	CTCCCTGAAGCCGTACACAT
	Mouse-COX-2-R	ATGGTGCTCCAAGCTCTACC
PTGES (NM_022415)	Mouse-PTGES-F	TTTCTGCTCTGCAGCACACT
	Mouse-PTGES-R	GATTGTCTCCATGTCGTTGC
EP_2 (NM_008964)	Mouse-EP2-F	ATCACCTTCGCCATATGCTC
	Mouse-EP2-R	GGTGGCCTAAGTATGGCAAA
EP_4 (NM_008965)	Mouse-EP4-F	TCATCTTACTCATCGCCACCT
	Mouse-EP4-R	TTCACCACGTTTGGCTGATA
GAPD (NM_008084)	Rod-GAPD-F	GAGGACCAGGTTGTCTCCTG
	Rod-GAPD-R	ATGTAGGCCATGAGGTCCAC

National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) accession numbers are shown within parentheses. Forward (5′) primer names are suffixed with an F and reverse (3′) primer names are suffixed with an R. Sequences are written 5′–3′.

for ~5 min, it was not possible to obtain fully defined concentration–effect curves within a single tissue. Accordingly, a single maximal concentration (i.e. 100 nM) of each tachykinin was used to assess the tissue responsiveness. In fresh segments, both SP and NKA produced a significant relaxation in contrast to NKB. Culture for 1 or 4 days without TNFα did not significantly alter SP-induced relaxation, although NKA-induced relaxation was attenuated after 4 days of culture (Figure 1b–c). Following culture, a relatively small degree of relaxation was induced by NKB (Figure 1d).

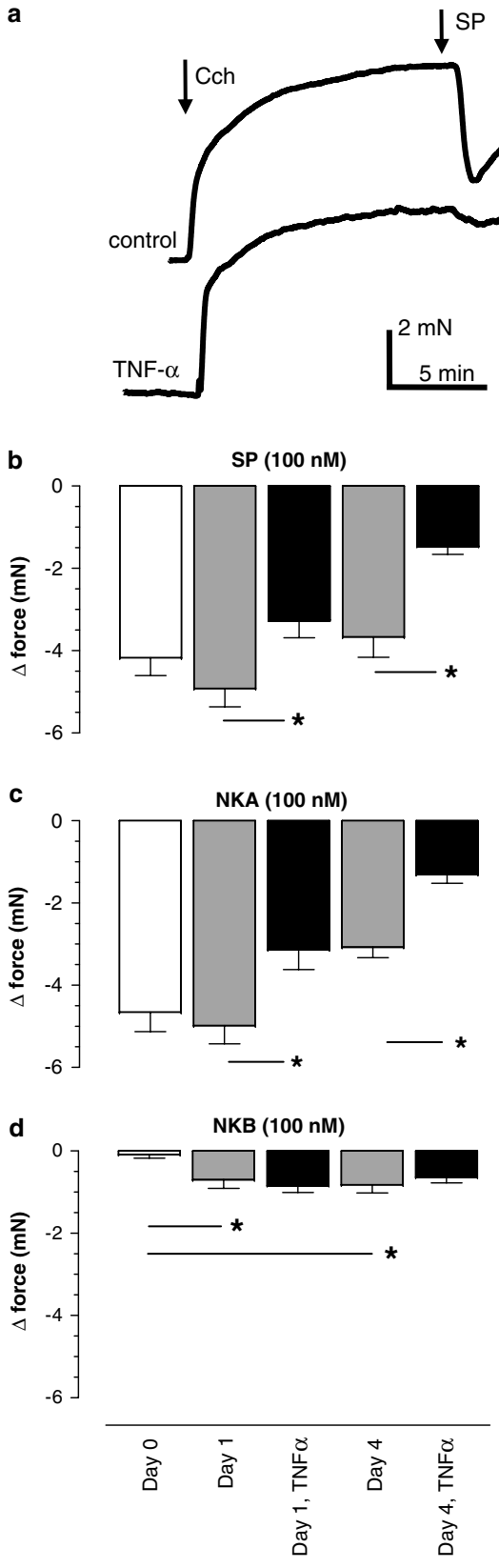

Figure 1 Relaxation induced by tachykinins. Typical experimental traces for (a) SP (cultured for 4 days in the absence and presence of 100 ng ml^{-1} TNFα), and normalized data for (b) SP, (c) NKA and (d) NKB (100 nM) are shown. Relaxation was assessed in fresh mouse tracheal segments or those cultured for 1 and 4 days in the absence and presence of TNFα (100 ng ml^{-1}). Tissues were precontracted with carbachol (1 μM). Mean data are shown with error bars representing the s.e.m. from seven to 16 animals. *$P<0.05$.

Effect of TNFα on tachykinin-induced relaxation of fresh and cultured tracheae

Prior to the analysis of the effects of TNFα on tachykinin responses, the possibility that any effects of TNFα were associated with underlying changes in the contractile behaviour of the tissue was excluded by a separate analysis of carbachol concentration–effect curves. The potency and maximal contraction in segments cultured for 4 days in the absence and presence of TNFα were not significantly different (E_{max}: 6.36±1.07 and 5.74±0.43 mN, and pEC_{50}: 6.88±0.10 and 6.64±0.09, respectively). The absence of an effect of TNFα on the maximum contractile response to carbachol was confirmed by the separate analysis of data obtained with the 1 μM doses of carbachol used to precontract tissues in the tachykinin studies (7.30±0.47 mN, control; 5.82±0.38 mN, TNFα; $n=30$).

Comparisons with time-controlled tracheal segments indicated that culture with TNFα (100 ng ml^{-1}) attenuated SP- and NKA-induced relaxation in a time- and concentration-dependent manner. Thus, SP- and NKA-induced relaxation was reduced after both 1 and 4 days of culture (Figure 1b–c). The weak NKB-induced relaxation was unaffected by TNFα treatment (Figure 1d). Treatment of segments with TNFα (1, 10 and 100 ng ml^{-1}) for 4 days caused a significant concentration-dependent reduction in SP-induced relaxation (Figure 2a). A similar pattern was observed in segments relaxed with NKA (Figure 2b), although only the effect of 100 ng ml^{-1} TNFα was significant as tested.

Tachykinin receptor characterization

To determine whether the tracheal relaxation induced by SP and NKA was mediated by NK_1 or NK_2 receptors, relaxation was investigated in the presence of antagonists of the NK_1 receptor (SR140,333; $K_i=0.04$ nM; >1000-fold selectivity vs NK_2 in the rat (Emonds-Alt *et al.*, 1993)) or the NK_2 receptor (SR48,968; $K_i=0.5$ nM; >10,000-fold selectivity vs NK_1 in the rat (Emonds-Alt *et al.*, 1992)). SR140,333 (10 nM) abolished SP- (100 nM) induced relaxation of tracheal segments cultured for 4 days in the absence and presence of 100 ng ml^{-1} TNFα, whereas SR48,968 (10 nM) did not (−2.85±0.24, −0.01±0.07 and −3.00±0.36 mN for control, SR140,333 and SR48,968, respectively, in the absence of TNFα; and −1.40±0.48, −0.04±0.03 and −1.58±0.49 mN for

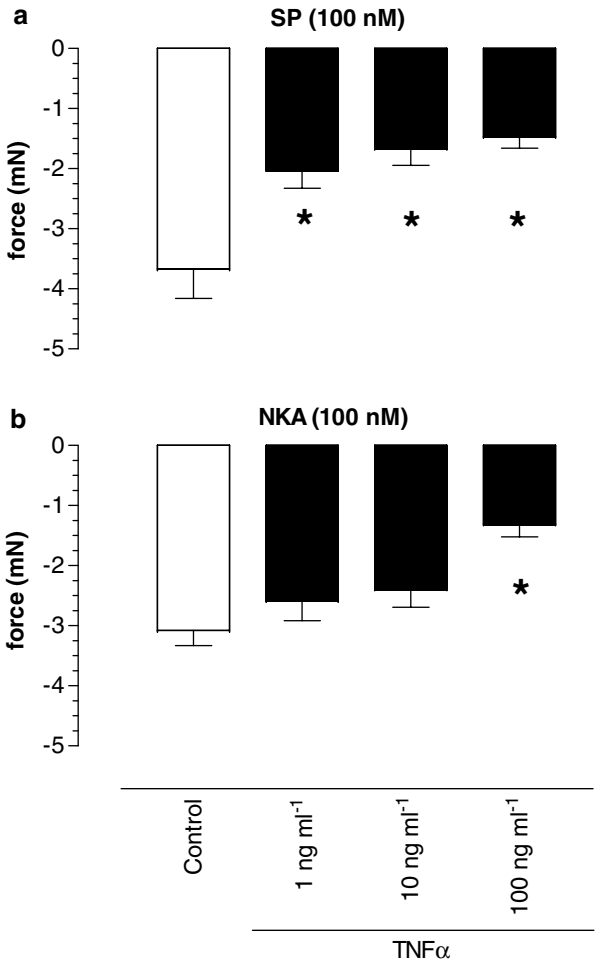

Figure 2 Relaxation induced by (a) SP and (b) NKA (100 nM) in mouse tracheal segments cultured for 4 days in the absence and presence of TNFα (1, 10 and 100 ng ml^{-1}). Tissues were precontracted with carbachol. Mean data are shown with error bars representing the s.e.m. from eight to 14 animals. $^*P<0.05$.

control, SR140,333 and SR48,968, respectively, in the presence of TNFα; $n=4$–5). Similar data were obtained with 100 nM NKA (-3.07 ± 0.12, $n=4$; -0.21, $n=2$ (individual values -0.06 and -0.36) and -3.41, $n=2$ (individual values -3.20 and -3.63) mN for control, SR140,333 and SR48,968, respectively, in the absence of TNFα; and -1.53 ± 0.57, $n=4$, -0.12, $n=2$ (individual values -0.17 and -0.07) and -1.36, $n=2$ (individual values -1.26 and -1.45) mN for control, SR140,333 and SR48,968, respectively, in the presence of TNFα; $n=2$–4). Given that the low concentrations of TNFα (1 and 10 ng ml^{-1}) did not have a significant effect on the NKA relaxation, an additional study was performed to determine if this was due to the involvement of a non-NK_1 receptor-mediated relaxatory component. SR140,333 (10 nM) abolished the response to NKA in segments treated with 1 ng ml^{-1} TNFα and 10 ng ml^{-1} TNFα, whereas SR48,968 (10 nM) had no effect (-2.42 ± 0.28, -0.04 ± 0.06 and -2.00 ± 0.41 mN for control, SR140,333 and SR48,968, respectively, in the presence of 10 ng ml^{-1} TNFα; and -2.61 ± 0.31, -0.21 ± 0.12 and -2.72 ± 0.65 mN for control, SR140,333 and SR48,968, respectively, in the presence of 1 ng ml^{-1} TNFα; $n=4$–5).

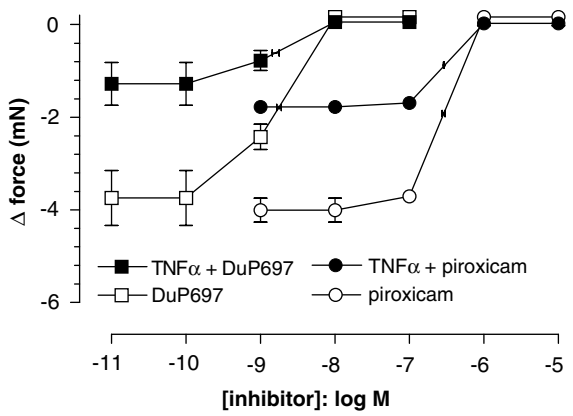

Figure 3 Concentration-dependent effects of the COX-1 inhibitor piroxicam (10 nM) and the COX-2 inhibitor DuP697 (10 nM) on relaxation induced by a single concentration of SP (100 nM). Tracheal segments were cultured for 4 days in the absence and presence of TNFα (100 ng ml^{-1}). Mean data are shown with error bars representing the s.e.m. from four animals.

COX inhibition of SP-induced relaxation

Since SP is found at higher concentrations in human airways than NKA (Joos *et al*., 2000), further studies focused on this tachykinin. Currently, it is not known which one of the two COX subtypes, the constitutively active COX-1 or the inducible COX-2, mediates SP-induced responses. In order to determine the contribution of each enzyme, segments were cultured in the absence and presence of 100 ng ml^{-1} TNFα for 4 days. Subsequently, relaxant responses to SP were obtained in the absence and presence of increasing concentrations of the COX-1 inhibitor piroxicam (reported 250-fold selectivity for COX-1; IC_{50} 2.4 nM (Frolich, 1997)) or the COX-2 inhibitor DuP697 (reported 57-fold selectivity for COX-2; IC_{50} 4.5 nM (Kargman *et al*., 1996); Figure 3). DuP697 inhibited SP-induced relaxation with a similar pEC_{50} in both control and TNFα-treated segments. Although piroxicam did inhibit SP-induced relaxation in both control segments and TNFα-treated segments, its low potency was consistent with COX-2 inhibition.

PGE_2-induced relaxation of segments cultured with TNFα

Given that tachykinins are reported to relax mouse tracheae *via* the release of PGE_2 (Szarek *et al*., 1998; Kao *et al*., 1999; Fortner *et al*., 2001), experiments were performed to investigate whether culture with TNFα modified PGE_2-induced relaxation. Segments were cultured in the absence and presence of TNFα (1, 10 and 100 ng ml^{-1}) for 4 days, and subsequently, relaxant responses induced by PGE_2 were assessed (Figure 4). TNFα induced a significant, concentration-dependent, decrease in the potency of the PGE_2-induced relaxation. The maximal relaxation was not changed by TNFα.

Effect of PGE_2 after culture with TNFα and selective COX inhibitors

As the potency of PGE_2 was reduced when tracheal segments were cultured with TNFα, it was conceivable that changes in relaxant response were governed by changes in COX-2

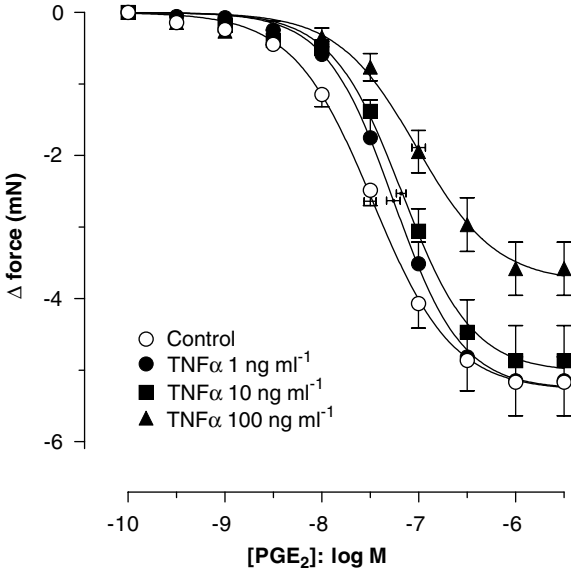

Figure 4 PGE_2 concentration–effect curves generated using mouse tracheal segments cultured for 4 days in the absence and presence of TNFα (1, 10 and 100 ng ml^{-1}). Mean data are shown with error bars representing the s.e.m. from 11 to 12 animals.

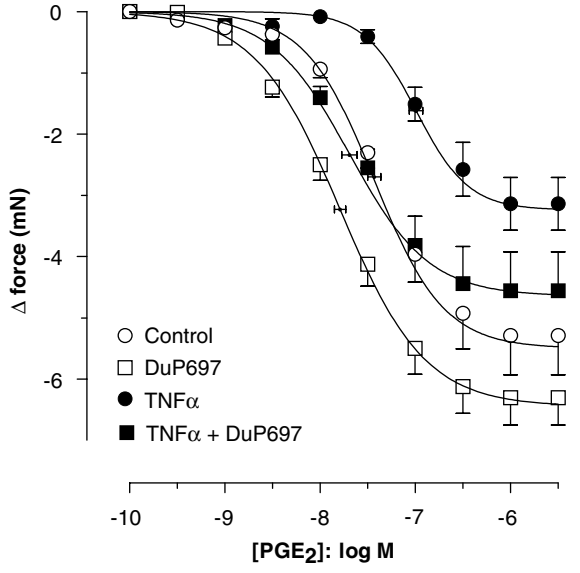

Figure 5 PGE_2 concentration–effect curves generated using mouse tracheal segments cultured for 4 days in the absence and presence of TNFα (100 ng ml^{-1}) and DuP697 (10 nM). Mean data are shown with vertical error bars and mean log EC_{50} values with horizontal error bars obtained by curve fitting representing the s.e.m. from 10 animals.

activity. To test this idea, segments were cultured with TNFα (100 ng ml^{-1}) both in the absence and presence of the selective COX-2 inhibitor DuP697 (10 nM), and relaxant responses to PGE_2 assessed. In agreement with earlier data, TNFα reduced the potency of PGE_2 relative to controls (Figure 5). Culture with both DuP697 and TNFα for 4 days reversed the changes induced by TNFα alone. In fact, culture with DuP697 (both with and without TNFα) caused a ~2-fold increase in the potency of PGE_2 compared to control segments. In this experiment, maximal relaxation induced by PGE_2 was significantly lower in TNFα-treated segments than in controls. This result is in contrast to data above (Figure 4), where a reduced R_{max} in the TNFα group did not reach significance. In similar experiments with the selective COX-1 inhibitor piroxicam (10 nM) used instead of DuP697, PGE_2-induced relaxation was unaltered in control or TNFα-treated segments (data not shown).

Effect of EP_2 receptor blockade on precontracted segments

Further experiments were performed to assess whether endogenous PGE_2 release limited tracheal contractile responses. Segments were cultured for 4 days and subsequently precontracted with carbachol. Once a stable plateau was reached, the EP_2 receptor antagonist AH6809 (3 μM; K_i 0.35 μM for EP_2 receptor with ~130-fold selectivity vs the EP_4 receptor (Kiriyama *et al*., 1997)) was administered (Figure 6). In both control and TNFα- (100 ng ml^{-1}) treated segments, a further contraction was obtained <1 min after AH6809 administration. However, the contraction in TNFα-treated segments was 3-fold greater than in control segments.

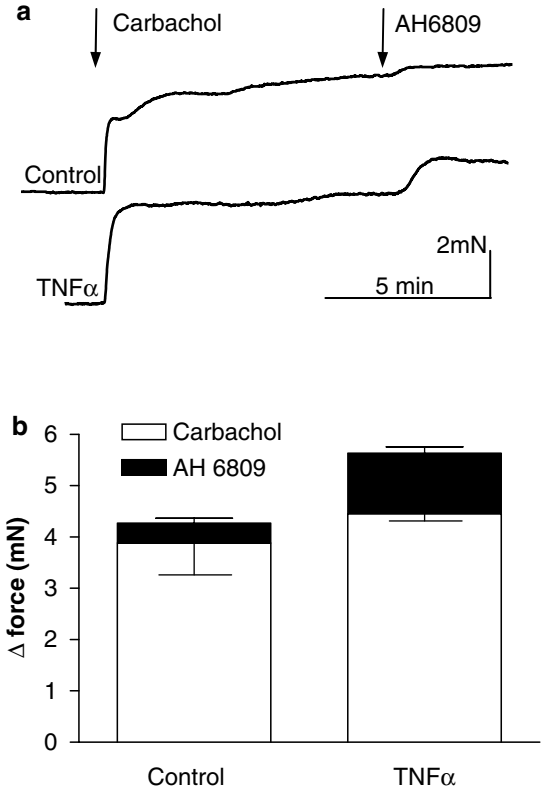

Figure 6 Effect of selective EP_2 receptor antagonist AH6809 (3 μM) on mouse tracheal segments precontracted with 1 μM carbachol. (a) Representative traces of segments cultured 4 days in the absence and presence of TNFα (100 ng ml^{-1}). (b) Mean data are shown with error bars representing the s.e.m. from four animals. $^*P<0.05$.

Culture with PGE_2

To evaluate whether elevated PGE_2 levels could account for decreased SP-mediated relaxation, experiments were performed using segments cultured for 4 days with 1 μM PGE_2 (a concentration that induced maximal relaxation; Figure 7). Following tissue washout, segments were precontracted with carbachol and then relaxed by the cumulative administration of PGE_2. Acutely, PGE_2 was 4-fold less potent in the PGE_2 pretreated segments than in control segments, although maximal relaxation was not altered (Figure 7). Relaxation induced by a single concentration of SP (100 nM) was also weaker in the PGE_2-treated segments.

Effect of EP_2 receptor blockade on SP-induced relaxation

Microarray and quantitative RT–PCR studies indicated that EP_2 receptor mRNA levels were elevated, whereas EP_4 receptor mRNA levels were decreased following TNFα treatment (see below). Therefore, an experiment was performed to determine if there was a functional consequence of these changes. Segments were cultured for 4 days in the absence and presence of TNFα. Following precontraction with carbachol, tissues were exposed to SP (100 nM) in the absence and presence of selective EP_2 receptor blockade (AH6809; 3 μM) in an attempt to expose the extent and any changes in the contribution of EP_4 receptors. In control segments, AH6809 reduced the SP-induced relaxation by 63±15% (3.16±0.41 and 1.17±0.26 mN in the absence and presence of AH6809, respectively). In TNFα-treated segments (100 ng ml^{-1}), the percentage inhibition of SP-induced relaxation by AH6809 at 75±18% was not significantly different (1.18±0.18 and 0.30±0.11 mN in the absence and presence of AH6809, respectively).

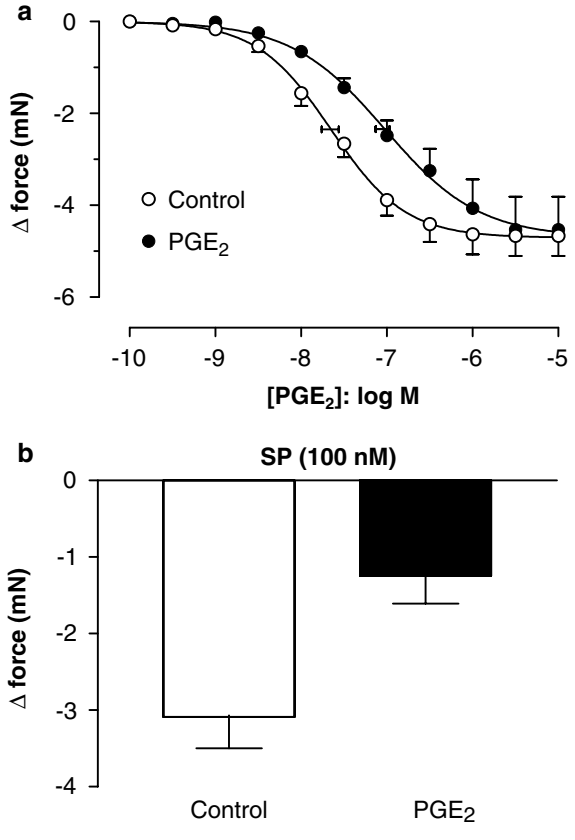

Figure 7 (a) PGE_2 concentration–effect curves, and (b) relaxation induced by SP (100 nM) in mouse tracheal segments cultured for 4 days in the absence and presence of PGE_2 (1 μM). Mean data are shown with vertical error bars and mean log EC_{50} values with horizontal error bars obtained by curve fitting representing the s.e.m. from five animals.

Gene expression analysis

In a parallel study, tracheal gene expression changes were examined using microarrays (U74Av2; Affymetrix). Mouse tracheae were cultured for 4 days in the absence and presence of 100 ng ml^{-1} human TNFα. Subsequently, epithelial cells were removed from tracheae and total RNA extracted from the remaining tissue. Human TNFα was used in this experiment as it has been reported to express selectivity for the mouse TNFα receptor (TNFR) I over the TNFRII, and the TNFRI mediates the primary effects of TNFα in airway smooth muscle cells (Amrani *et al*., 2001). Total RNA from three tracheae was pooled for each array. Differentially expressed genes were identified using the SAM algorithm. Changes in the expression of genes involved with prostaglandin synthesis and prostaglandin response were observed. Notably, COX-2, PTGES and EP_2 receptor mRNA levels were upregulated by human TNFα treatment (2.3-, 3.0- and 1.6-fold, respectively), whereas EP_4 receptor mRNA levels were downregulated (3.3-fold; Figure 8a). Although the expression of COX-1 was detected using microarrays, analysis indicated that it was not differentially expressed. These data were confirmed using quantitative real-time RT–PCR.

A further study was performed to determine whether murine TNFα induced similar changes specifically in the tracheal smooth muscle. Tissues were cultured for 4 days in the absence and presence of this cytokine. Subsequently, epithelial cells were removed from tracheae, smooth muscle strips isolated and total RNA extracted and amplified. The gene expression was assessed using quantitative RT–PCR, with data being similar to those obtained using human TNFα and whole tracheae. Thus, COX-2, PTGES and EP_2 receptor mRNA levels increased in the smooth muscle with murine TNFα treatment (4.5-, 1.6- and 1.3-fold, respectively), whereas EP_4 receptor levels decreased (1.9-fold; Figure 8b). For control purposes, the expression of COX-1 mRNA was also assessed and found to be unchanged by treatment with TNFα.

Immunohistochemistry

To confirm that COX-2, PTGES, EP_2 and EP_4 proteins could be detected in the smooth muscle, immunohistochemistry was used to identify the cellular location of prostaglandin synthesis/receptor proteins. COX-1, COX-2, PTGES, EP_2 receptor and EP_4 receptor proteins were detected in the smooth muscle of tracheae cultured for 4 days in the absence and presence of TNFα (Figure 9).

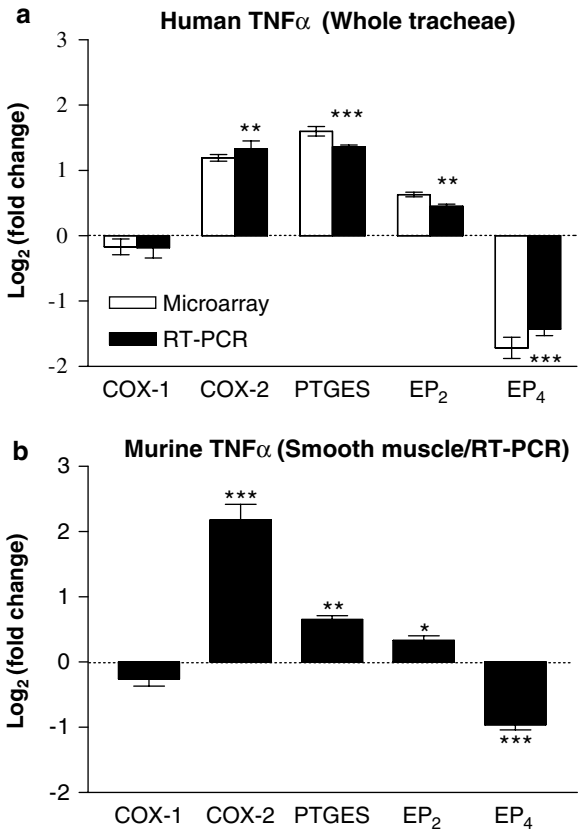

Figure 8 Gene expression measured in tracheae cultured in the presence of (a) human and (b) murine TNFα (100 ng ml^{-1}). Microarray (empty bars) and quantitative RT–PCR (filled bars) data ($\log_2$) are shown normalized to the geometric mean of control samples. (The mean of control data would lie on the dashed line.) Mean data are shown with error bars representing the s.e.m. from four pooled groups (Microarray) or 5 to 6 animals (RT–PCR). For RT–PCR data, $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.0001$ (in comparison to control samples).

Discussion

SP, as previously reported (Kao *et al*., 1999), and NKA induced reproducible transient relaxation of carbachol precontracted control mouse tracheae, in contrast to NKB that failed to relax tissues consistently. Tracheal segments cultured for 1 or 4 days maintained their ability to relax in response to SP and NKA. However, the addition of TNFα to the culture medium attenuated the SP- and NKA-induced relaxation in a time- and concentration-dependent manner. It should be noted though that the relaxant effect of NKA was significantly inhibited only in tracheal segments cultured in 100 ng ml^{-1} TNFα, and not in those cultured in 1 and 10 ng ml^{-1} TNFα. This result was in contrast to SP-induced relaxation that was significantly inhibited at all TNFα concentrations, and potentially suggested that different receptor systems mediate the effects of SP and NKA. However, NK_1 receptor antagonism completely inhibited both SP- and NKA-induced relaxation in segments cultured in the absence and presence of TNFα. Thus, the current data seem compatible with the notion that both SP- and NKA-induced relaxation is mediated exclusively by the NK_1 receptor regardless of TNFα exposure. To our knowledge, the finding that TNFα attenuates SP- and NKA-induced relaxation is novel and an attempt to determine the basis of this effect has been made in the current study.

As mentioned above, the effects of both SP and NKA were shown to be mediated by the activation of the NK_1 receptor, a result that is in agreement with studies in NK_1 receptor knockout mice (Tournoy *et al*., 2003) and other rodent studies (Szarek *et al*., 1998). Studies have also shown that the SP-induced relaxation of the mouse trachea is dependent on the release of PGE_2 from epithelial COX enzymes (Kao *et al*., 1999; Fortner *et al*., 2001). To determine whether PGE_2 release was due to the activity of COX-1 or COX-2, two selective inhibitors were evaluated pharmacologically. The COX-2 inhibitor DuP697 blocked SP-induced relaxation in the nM range (for segments cultured in the absence and presence of TNFα), strongly suggesting that COX-2 is exclusively responsible for SP-induced relaxation. Interestingly, this result is consistent with the report that protease-activated receptors also relax murine smooth muscle by stimulating the release of PGE_2 by COX-2 (Lan *et al*., 2001).

Since SP-mediated relaxation occurs *via* the release of PGE_2 (Szarek *et al*., 1998; Kao *et al*., 1999; Fortner *et al*., 2001), studies were performed to investigate whether PGE_2-induced relaxation was modified by TNFα treatment. Culture with TNFα for 4 days caused a decrease in the potency of PGE_2-induced relaxation, whereas the inclusion of DuP697 with TNFα in the culture media prevented these changes. In fact, the potency of PGE_2 was increased in segments treated with DuP697 in the absence and presence of TNFα relative to untreated segments. These results suggest that COX-2 is active during culture and that TNFα produces an additional increase in its activity that attenuates SP/PGE_2-induced responses. Enhanced COX-2 activity may cause increases in basal PGE_2 release during culture, and this notion is supported by the observation of an enhanced contraction following dosing of the EP_2 receptor antagonist, AH6809, to carbachol precontracted TNFα-treated segments.

It was conceivable that increased PGE_2 secretion during culture decreased EP receptor-mediated tissue relaxation. To test this hypothesis, segments were cultured for 4 days with PGE_2. Following culture, the potency of the exogenous PGE_2 concentration–effect curve was decreased in treated tissues relative to controls. Responses to PGE_2 following culture were more attenuated in these studies than when segments were cultured with TNFα, and this may be due to the exogenous concentration of PGE_2 utilized during culture being higher than endogenous levels. SP-induced relaxation was also attenuated in PGE_2-cultured segments. Thus, increased release of PGE_2 could potentially explain reduced tachykinin-mediated responses in TNFα-treated tissues.

The results discussed above indicated that relaxant responses mediated by EP receptors, or their associated pathways, are attenuated by TNFα-induced COX-2 activity. It is possible that this is due to EP receptor desensitization, as both EP_2 and EP_4 receptor activity is reduced after prolonged exposure of PGE_2 (Nishigaki *et al*., 1996). In an attempt to reveal any changes in the relative contribution of EP_2 and EP_4 receptors to the relaxation of control and TNFα-treated tracheae, an EP_2 receptor selective concentration of the antagonist AH6809 (Kiriyama *et al*., 1997) was used to block the SP-induced relaxation. Evidently, as judged by the similar degree of blockade of the control and TNFα-treated segment relaxatory responses, SP acts predominantly through the EP_2

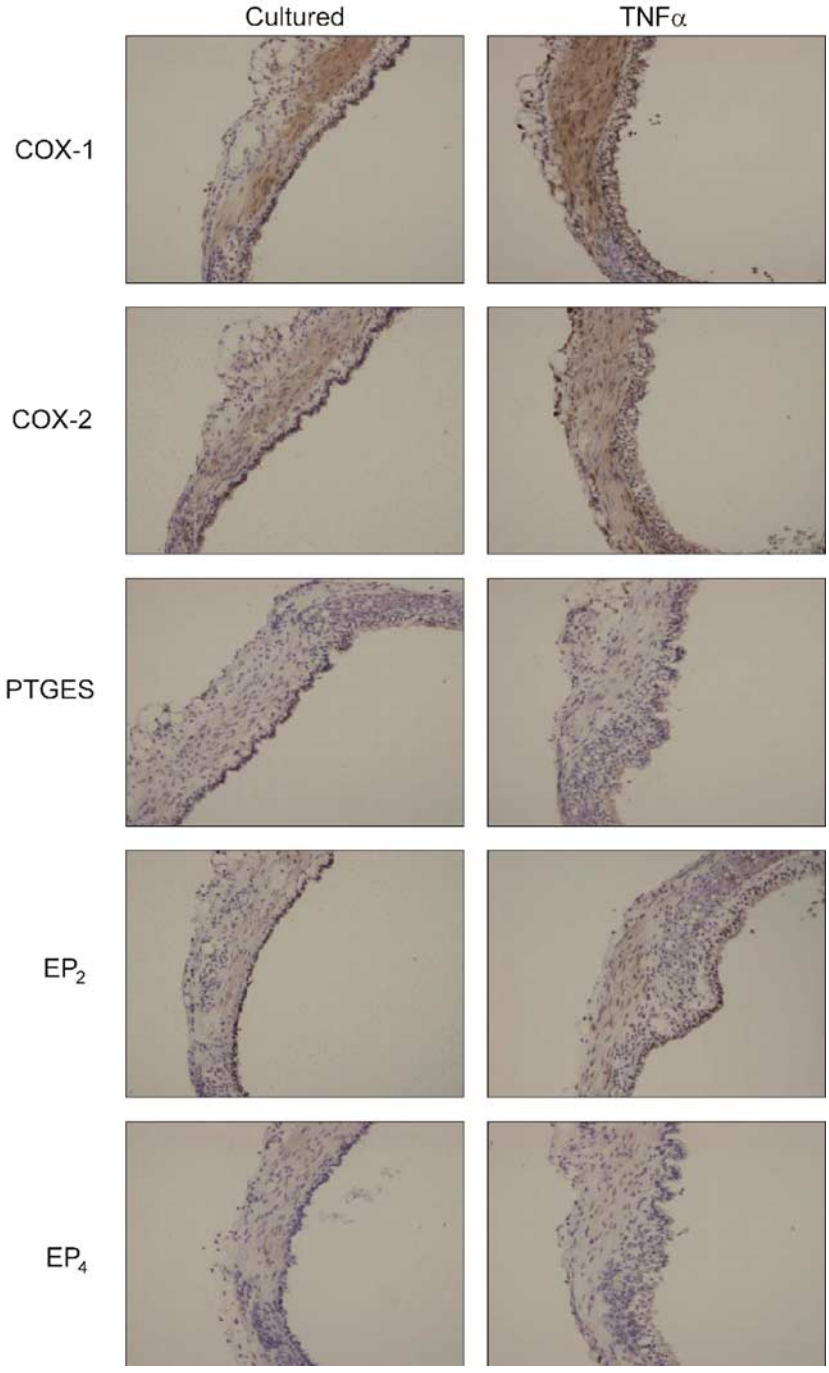

Figure 9 Representative transverse tissue sections from tracheae cultured in the absence and presence of TNFα (100 ng ml^{-1}). Proteins were localized to the smooth muscle using diaminobenzidine, which stains tissues brown. PTGES and EP_4 staining was faint. Owing to the limitations of immunohistochemistry for quantitative comparisons, it could not be definitively concluded that TNFα treatment caused changes in the levels of any protein. Epithelial staining may be aberrant, as the epithelia of control sections not incubated with primary antibody were also coloured.

receptor regardless of TNFα treatment. To delineate any contribution of EP_4 receptors in the assay would require further studies with a more selective EP_2 receptor antagonist and a selective EP_4 receptor antagonist. Owing to its limited EP_2/EP_4 receptor selectivity, a higher concentration of AH6809 could not be used to determine if the residual relaxation observed in the current experiment included an element of EP_4 receptor activation.

Microarray and quantitative RT–PCR analysis revealed alterations in the expression of a number of functionally linked genes. Levels of COX-2, PTGES and EP_2 receptor mRNA were increased in smooth muscle by murine TNFα, whereas the level of EP_4 receptor mRNA was decreased. COX-2 catalyses the formation of PGH_2 from arachidonic acid (Hla & Neilson, 1992; Jones *et al*., 1993), and PGH_2 is converted to PGE_2 by PTGES (Jakobsson *et al*., 1999). Thus, the changes in

gene expression described above may be highly significant given that TNFα induces changes in tracheal relaxant response. The finding that COX-2 expression was increased in smooth muscle is in contrast to other reports (Belvisi *et al*., 1997; Pascual *et al*., 2001), although this may be due to the extended incubation period used in the current study. TNFα has been shown to induce COX-2 and PTGES expression in a range of experimental situations, and that the expression of these enzymes appears to be coordinated (Vadas *et al*., 1996; Chen *et al*., 2000; Stichtenoth *et al*., 2001). However, we are unaware of any previous reports showing TNFα-induced alterations in EP_2 and EP_4 receptor mRNA levels. Interestingly though, bacterial lipopolysaccharide, a substance that stimulates similar transcriptional responses to TNFα, upregulates EP_2 and downregulates EP_4 receptor mRNA levels in macrophages (Ikegami *et al*., 2001), results that may be related to our findings. Immunohistochemical techniques localized prostaglandin synthesis (COX-1, COX-2 and PTGES) and prostaglandin receptor (EP_2 and EP_4) proteins to the smooth muscle of tracheae cultured in the absence and presence of TNFα. Thus, microarray and immunohistochemistry data supported a role for COX-2 and associated proteins in the alteration of tachykinin responses in TNFα-treated tracheae. However, it should be noted that changes in epithelial COX-2 expression were not assessed in the current study and it is conceivable that any change could have an effect on functional results.

In conclusion, TNFα treatment decreases the NK_1 receptor-mediated SP- and NKA-induced relaxation of cultured mouse tracheal segments. This appears to be due to an increase in COX-2 activity that augments PGE_2 release, which in turn desensitizes EP receptors. Thus, it is conceivable that long-term inflammatory stimulation may decrease the ability of PGE_2 to relax the airways, and this may explain why PGE_2 agonists are not effective in the treatment of methacholine-induced hyper-responsiveness in asthmatic patients (Pavord *et al*., 1991; 1993).

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