

Nerve growth factor enhances cholinergic innervation and contractile response to electric field stimulation in a murine *in vitro* model of chronic asthma

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Summary

Background Asthma is a chronic inflammatory disease characterized by airway hyper-responsiveness. Alterations in the neurogenic control are believed to contribute to the pathogenesis. Yet, the long-term interaction between nerves and inflammatory mediators, such as the neurotrophin nerve growth factor (NGF), are not fully understood much due to the absence of appropriate experimental assays.

Objective To develop an *ex vivo* mouse organ culture assay and to investigate the effects of NGF on nerve-mediated airway contractions.

Method Mouse tracheal segments were cultured in periods of up to 16 days. Their contractile responses to electric field stimulation (EFS) were investigated. In addition, the effect of 4 days of NGF treatment was analysed using EFS and immunohistochemistry.

Results EFS (0.2–25.6 Hz) induced reproducible and frequency-dependent cholinergic contractions of both fresh and cultured tracheal segments. The main part of the EFS response was blocked by tetrodotoxin or atropine. After 4 days in culture, regional differences appeared, with stronger EFS responses in distal than in proximal segments. More nerve fibres were seen in distal segments than in proximal segments. Treatment with NGF during 4 days of culture increased the innervation of the proximal segments, at the same time as the cholinergic contractile responses to EFS were enhanced dose-dependently.

Conclusion Culture of tracheal segments appears to be a suitable assay for the examination of long-term effects induced by inflammatory mediators on neurally mediated airway contractions. NGF treatment enhanced the cholinergic, nerve-dependent contractions and increased the amount of nerve fibres seen in the murine tracheal segments, suggesting a role for NGF in the development of airway hyper-responsiveness.

Keywords asthma, depolarization, inflammation, murine, protein gene product 9.5, tetrodotoxin

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Introduction

Asthma is a heterogeneous disease characterized by airway obstruction and hyper-responsiveness. The pathological process is linked to chronic inflammation of the airways [1]. This inflammation results in structural alterations of the lower airways, such as thickening of airway smooth muscle, connective tissue deposition and shedding of epithelium cells, typically observed in patients with severe longstanding asthma [2, 3]. Alterations in the neurogenic control are believed to contribute both to the pathogenesis and the symptoms of the disease [4]. Chronic inflammation of the airways is reported to increase neural activity and to change neurotransmitter release in the guinea-pig trachea [5]. However, the mechanisms by which inflammation leads to perturbation of neuronal control are not fully understood.

Nerve growth factor (NGF) is produced by human pulmonary fibroblasts [6], pulmonary epithelium [7] and eosinophils [8] in response to various inflammatory mediators [9]. In accordance, elevated levels of NGF have been detected in the blood circulation and airways of allergic and asthmatic patients [9–13]. In asthmatics, NGF levels are decreased by corticosteroid treatment [14]. NGF has been demonstrated to enhance nerve-mediated airway hyper-responsiveness in mice [15, 16] and guinea-pigs [17–19]. However, the long-term effect of NGF on the cholinergic reactivity of the airways is not clear. This is partly attributed to lack of models where the effects of long-term exposure to inflammatory mediators could be characterized in relation to neuronal activity and airway reactivity.

Organ culture can be used as an alternative to cell culture in the study of pathological processes [20, 21]. In the cultured tissue, contact between endogenous structures such as extracellular matrix, epithelium, smooth muscle and nerve cells is maintained, reducing the phenotypic alteration of the cells [21, 22]. In accordance, 2 days of culture of guinea-pig trachea demonstrated an intact cholinergic transmission [22].

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We have recently developed a novel organ culture assay for the evaluation of long-term effects of inflammatory mediator on isolated murine airways [23]. Tracheal segments were demonstrated to be viable after up to 32 days of culture. In the cultured segments, the maximum contractile responses to KCl and carbachol changed in parallel during culture. These changes were relatively small, with a slight increase in the response during the first 2 days, followed by a decrease towards a stable level at day 8. The maximal relaxations induced by isoprenaline were not affected by the culture procedure. The potency of KCl and isoprenaline also increased with culture time. DNA microarray data revealed that culture induced limited changes of the global gene expression. However, the changes that took place were found to be more pronounced when tissues were introduced to culture than during the culture periods *per se*. The morphology of smooth muscle cells appeared to remain unchanged throughout the culture periods [23].

The aim of the present study was to examine whether the tracheal organ culture also is a suitable assay for the investigations of inflammatory mediator long-term effects on neural activity. Moreover, we attempted to use this improved assay to study the effects of NGF on cholinergic nerve transmission in relation to the smooth muscle contractility of the airways.

Method

Tissue preparation and organ culture

Approximately 10-week-old, male BALB/c J mice were killed by cervical dislocation and the whole trachea was rapidly removed and placed into cold DMEM containing D-glucose (4500 mg/L), sodium pyruvate (110 mg/L) and L-glutamine (584 mg/L), supplemented with penicillin (100 U/mL) and streptomycin (100 µg/mL). Each trachea was dissected free from adherent tissue under sterile conditions and divided into three or four segments each with three cartilage rings. The segments were either used directly after dissection or placed individually in a 24-well plate with 1 mL DMEM for organ culture. During culture, the tracheal segments were incubated at 37 °C in humidified 5% CO₂ in air for 1, 2, 4, 8 and 16 days [23]. The wells were inspected daily; infected segments were immediately removed and the medium was changed every day. Separate experiments for treatment with NGF were conducted in 96-well plates with 300 µL DMEM. The segments were transferred daily to a new well containing fresh DMEM. The protocol was approved by the Ethical Committee of the University of Lund (Lund, Sweden).

Isometric force measurement

Smooth muscle reactivity of the tracheal segments 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 Ca²⁺, 2.5 mM Mg²⁺, 128 mM Cl⁻, 1.2 mM H₂PO₄⁻, 1.2 mM SO₄²⁻, 25 mM HCO₃⁻ and 10 mM D-glucose. The solution was continuously equilibrated with 5% CO₂ and 95% O₂ resulting in a pH of 7.4. The tracheal segments were mounted on two L-shaped

metal prongs, with a resting tension of 0.8 mN [23]. One prong was connected to a force–displacement transducer for continuous recording of isometric tension by the Chart software (ADInstruments Ltd, Hastings, UK). The other prong was connected to a displacement device, allowing adjustment of the distance between the two parallel prongs.

Experimental procedure

Airway smooth muscle responsiveness was assessed by electric field stimulation (EFS) in the myograph, by placing electrodes delivering electrical stimulation at opposite sides of the tracheal segments (Current Stimulator Model CS200, J.P. Trading, Aarhus, Denmark). Initially, the segments were given five training impulses of 4 Hz, 55 mA ($\approx$ 10 V). Later, segments were given a ²log EFS series of 0.2–25.6 Hz [24]. Each impulse was 0.8 ms long with a duration of 1 min, followed by 1.5 min recess. All parameters were optimized to produce a stable and reproducible contraction. In the following series of experiments, segments were washed and incubated for 30 min with 3 µM of the cyclooxygenase (COX) inhibitor indomethacin before EFS, followed by the same procedure for 1 µM hexamethonium (a nicotinic receptor blocker), 3 µM timolol (a β -adrenoceptor antagonist) and 1 µM tetrodotoxin (TTX; a Na⁺-channel blocker). Carbachol (100 µM), an acetylcholine analogue, was then added and maximum constriction was measured after 20 min. Finally, segments were incubated for 30 min with 1 µM atropine (a muscarinic receptor antagonist) and stimulated as described above. In complimentary experiments, 100 nM ω -conotoxin GVIA (CTX; a neural L-type Ca²⁺-channels blocker) was used. In experiments where segments were treated with NGF for 4 days, a shorter procedure was used. A control stimulation series of 0.2–25.6 Hz was followed by a stimulation series in the presence of 1 µM TTX, and subsequently a final maximal carbachol contraction. In separate experiments, to test the involvement of sensory nerves, capsaicin (10 µM) was administered either directly on baseline or after pre-contraction by carbachol (1 µM) for 10 min.

Immunocytochemistry

Tracheal segments were cultured for 4 days in the absence and presence of NGF. The sections were immersed overnight in a fixative consisting of 2% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 7.2. After fixation, the specimens were rinsed twice in sucrose-enriched Tyrode solution. The specimens were frozen on dry ice and stored at –70 °C. Sections were cut at 10 µm thickness in a cryostat and mounted on chrome-alum-coated slides. Indirect immunocytochemistry was used for the demonstration of protein gene product 9.5 (PGP 9.5, dilution of 1:1600, Ultraclone, Cambridge, UK), calcitonin gene-related peptide (CGRP, dilution of 1:1280, Eurodiagnostica AB, Sweden) and substance P (SP, dilution of 1:800, Eurodiagnostica AB, Sweden). Briefly, the sections were incubated with the primary anti-serum overnight at +4 °C. The site of the antigen–antibody complex was revealed by application of a secondary antibody (1:80; swine anti-rabbit IgG, DAKO, Copenhagen, Denmark) conjugated to fluorescein isothiocy-

nate (FITC). In the control experiments the primary antibody was omitted.

Analysis

Experimental data were expressed as the maximum point of contraction for each stimulation frequency in mN or percentage of maximum tension generated by 100 μ M carbachol. Data analysis was performed using GraphPad Prism (version 3.00 for Windows, GraphPad Software, San Diego, CA, USA). Individual EFS responsiveness values (E) of each trachea were analysed separately in regression analysis to fit the following equation:

$$E = \text{bottom} + \frac{(\text{top} - \text{bottom}) \times \text{frequency}^n}{ES_{50}^n + \text{frequency}^n}$$

Bottom and top denote the lowest and highest contraction, respectively, of the contractions induced by EFS. ES_{50} denotes the frequency imposing half of the maximum contraction and n denotes the midpoint slope co-efficient. The values were calculated separately for each segment and stimulation series, and the mean values with standard error of mean were calculated. The individual values were then compared by a one-way ANOVA and if significant ($P < 0.05$), t -tests with Bonferroni's correction were performed.

Chemicals

Recombinant rat β -NGF was obtained from R&D Systems, Minneapolis, MN, USA. Atropine, capsaicin, carbachol, Dulbecco's-modified eagle's medium, hexamethonium, indomethacin, timolol and U46619 were obtained from Sigma, St Louis, MO, USA. Tetrodotoxin was obtained from Sankyo, Tokyo, Japan, and ω -conotoxin GVIA was obtained from Alomone labs, Jerusalem, Israel. Penicillin-streptomycin was obtained from Life Technologies, Gettysburg, PA, USA.

Results

Carbachol contractions following culture

The maximal contraction induced by 100 μ M carbachol was measured in fresh segments and segments cultured for 1, 2, 4, 8 and 16 days. No major differences in the attained contraction were seen comparing fresh segments (9.2 ± 0.5 mN; $n = 7$) with segments cultured for 1, 2 and 4 days (7.7 ± 0.8 , 9.3 ± 1.7 and 6.4 ± 1.0 mN, respectively; $n = 8$). After 8 and 16 days of culture, a significantly weaker maximal contraction was attained, compared with fresh segments (6.5 ± 0.6 and 5.3 ± 0.6 mN, respectively; $n = 7$ and 6). Subsequently, the maximal carbachol contractions were used as reference for EFS-stimulated contractions.

EFS following culture

EFS (0.2–25.6 Hz) induced distinct, frequency-dependent contractions in both fresh and cultured segments (Figs 1a–d). No significant difference in the maximal EFS contractions was obtained comparing fresh and cultured segments after 1, 2 and 4 days. Starting at 4 days of culture, the ability at each frequency to maintain the EFS contractions was compro-

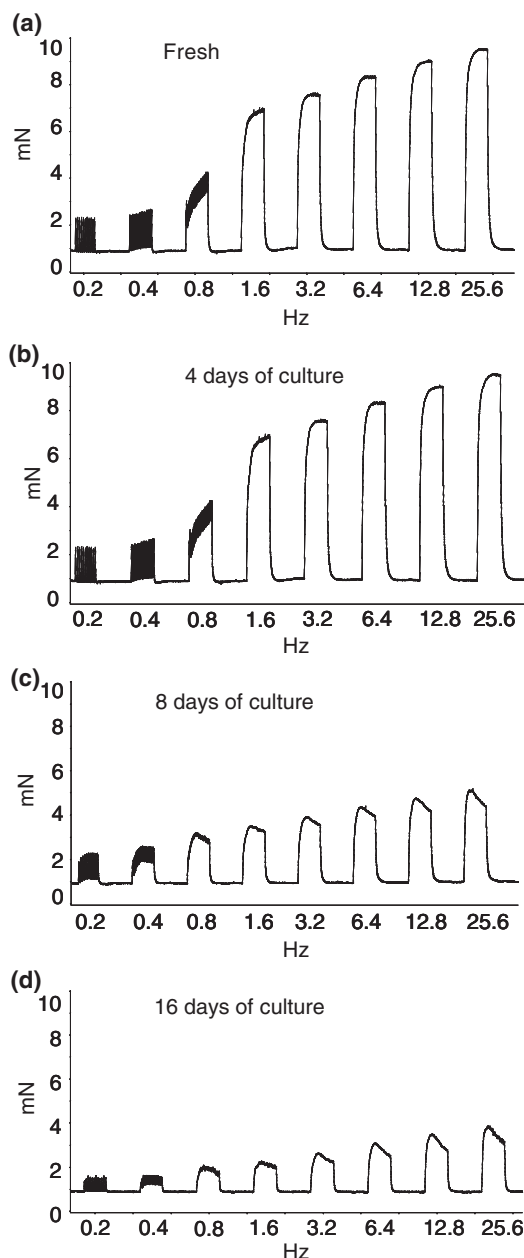


Fig. 1. Electric field stimulation (EFS) contractions of tracheal segments after 0, 4, 8 and 16 days in culture (a–d, respectively). After culture segments were stimulated by EFS (0.2–25.6 Hz). After 16 days in culture (d) only half of the segments responded to EFS.

mised and after 8 days all segments demonstrated a similar decline (Figs 1a–c). At day 16, all segments reacted weakly and only half of them contracted in response to low-frequency stimulation (0.2–0.8 Hz) (Fig. 1d). There were no differences in the bottom, top and ES_{50} values for segments cultured 0–8 days. However, the midpoint slope co-efficient was significantly ($P < 0.05$) lower after 2–8 days of culture (Table 1).

Incubation with the COX inhibitor indomethacin induced an increased responsiveness to EFS. A significant difference between fresh and cultured segments was found at day 4 of culture with a higher bottom value and a lower midpoint

Table 1. EFS of cultured tracheal segments

Days in culture	Indo.	Max. contraction (mN)	No.	EFS bottom (%)	EFS top (%)	EFS ES_{50} (Hz)	EFS midpoint slope co-efficient
0	–	8.8 ± 0.8	7	5.8 ± 0.5	51.1 ± 3.1	2.37 ± 0.17	1.68 ± 0.13
	+		7	6.7 ± 0.4	53.3 ± 2.2	2.52 ± 0.44	1.55 ± 0.09
1	–	8.6 ± 0.6	8	7.3 ± 2.0	50.6 ± 5.4	5.05 ± 1.57	1.52 ± 0.08
	+		4	14.7 ± 6.7	52.3 ± 7.1	2.87 ± 1.41	1.74 ± 0.21
2	–	8.5 ± 0.5	8	11.5 ± 1.8	67.3 ± 6.7	3.25 ± 0.75	*1.29 ± 0.07
	+		4	12.4 ± 2.0	60.1 ± 2.0	2.65 ± 0.85	1.52 ± 0.17
4	–	8.6 ± 0.6	8	10.8 ± 3.3	54.3 ± 5.1	6.87 ± 2.58	*1.21 ± 0.12
	+		8	*23.8 ± 2.1	65.7 ± 2.8	1.42 ± 0.38	*1.44 ± 0.38
8	–	6.5 ± 0.8	7	10.6 ± 3.4	48.6 ± 6.4	3.04 ± 0.54	*1.26 ± 0.05
	+		7	16.3 ± 3.8	60.8 ± 4.1	4.97 ± 2.70	1.36 ± 0.14

Tracheal segments were cultured for 0–8 days and stimulated by EFS (0.2–25.6 Hz) in the absence (–) or presence (+) of indomethacin (Indo, 3 μ M) and later treated with carbachol (0.1 mM) to induce maximal contraction. Regression analysis was performed for each segment separately. The midpoint slope co-efficient was significantly lower starting at day 2 of culture. After 4 days in culture, indomethacin treatment enhanced the bottom value and lowered the midpoint slope co-efficient (* P < 0.05). 'Bottom' and 'top' data are presented as percent of maximal carbachol contraction. EFS, electric field stimulation.

slope co-efficient value (Table 1). Incubation with the nicotinic receptor blocker hexamethonium or the β_2 -adrenoceptor antagonist timolol did not affect the EFS contractions in fresh and cultured segments. No differences in the estimated curve parameters were obtained following incubation with either compound in comparison with control experiments (data not shown). Capsaicin (10 μ M) added to segments after 2 and 4 days of culture induced no effect on segments at baseline tension or carbachol (1 μ M) pre-contracted segments (data not shown).

Difference in response to EFS between proximal and distal parts of the trachea

An increase in the standard deviation of top, bottom and ES_{50} values was observed following culture (Table 1). In order to test if these changes were related to segment position on the trachea, EFS contractions of proximal and distal segments were compared. Subsequently, cultured segments from the distal portion of the trachea reacted with stronger contractions than proximal segments (Fig. 2). In contrast, no regional differences were observed in fresh segments (data not shown). The regional differences were not observed in maximal contractions induced by carbachol (1 mM, Fig. 3). Stimulation with EFS (25.6 Hz) was weaker only in proximal segments after 4 days of culture, although a non-significant decline in the responsiveness of proximal segments to EFS was observed already after 1 day of culture.

TTX 1 μ M was added to the organ bath in order to block nerve activation and the subsequent release of acetylcholine. In freshly isolated segments, TTX practically blocked all contractions at lower frequencies (0.2–3.2 Hz), whereas a modest contraction was observed at higher frequencies (6.4–25.6 Hz, Fig. 4), predominantly in proximal segments. Following 2 days of culture, the TTX-resistant contraction became more prominent, beginning at a frequency of 1.6 Hz. The TTX-resistant component was more substantial in proximal segments and almost non-existing in distal segments. Additionally, incubation with atropine or CTX (1 μ M or 100 nM, respectively) did not block the TTX-resistant contractions (data not shown). In order to examine whether the difference in TTX-resistant contraction between proximal and distal segments was linked to smooth muscle cell

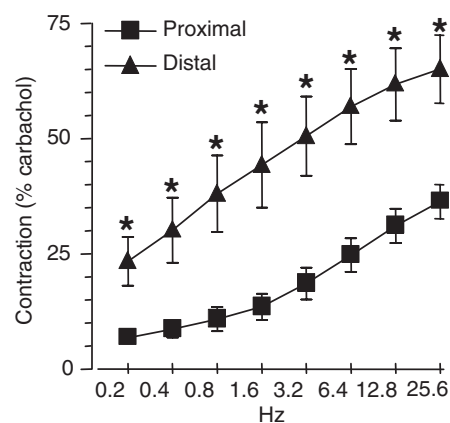


Fig. 2. Differences between proximal and distal mouse trachea to electric field stimulation (EFS)-induced contractions after 4 days in culture. Proximal and distal tracheal segments were cultured for 4 days and stimulated with EFS (0.2–25.6 Hz). Contractions obtained with distal segments were significantly stronger at all frequencies (n = 6–8 segments, * P < 0.05). Data are presented as percent of maximal carbachol contraction (100 μ M).

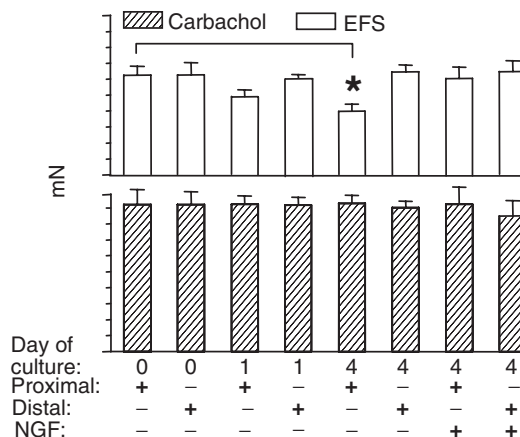


Fig. 3. Maximal carbachol and electric field stimulation (EFS) contraction of tracheal segments. Proximal and distal segments were used fresh (day 0) or cultured for 1 or 4 days. After culture, the segments were placed in an organ bath and stimulated as described with EFS (25.6 Hz) or carbachol (1 mM). Proximal segments, after 4 days of culture, responded with weaker contractions to EFS in comparison with fresh proximal segments (n = 5–7 segments, * P < 0.05).

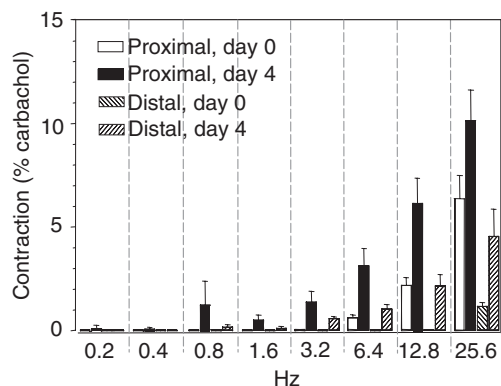


Fig. 4. Tetrodotoxin (TTX)-resistant electric field stimulation (EFS) contractions of fresh and cultured tracheal segments after a 30-min incubation with indomethacin ($3\mu\text{M}$). Fresh proximal segments reacted with stronger contractions than distal segments. The TTX-insensitive contractions were stronger after 4 days in culture and especially strong in proximal segments ($n = 5-6$ segments). Data are presented as percent of maximal $100\mu\text{M}$ carbachol contractions.

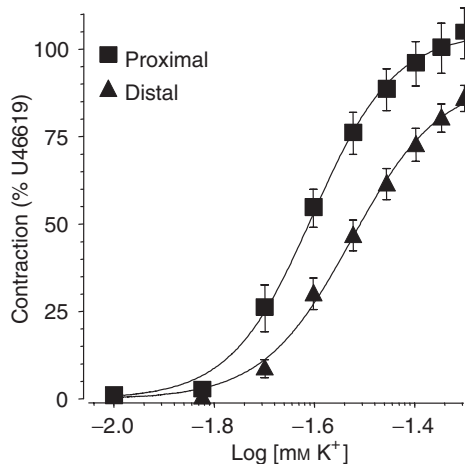


Fig. 5. Concentration-response curve of proximal and distal tracheal segments after 4 days in culture in response to K^+ . After treatment with $1\mu\text{M}$ atropine, a cumulative administration of potassium was added to the organ bath ($n = 7-8$ segments). Data are presented as percent of maximal $1\mu\text{M}$ U46619 contraction.

membrane polarity, the sensitivity of the smooth muscle was investigated by cumulative administration of KCl . Accordingly, a significant difference was obtained between the pEC_{50} value of proximal and distal segments (1.62 ± 0.02 , $n = 7$ and 1.52 ± 0.02 , $n = 6$, respectively; Fig. 5).

Incubation with NGF

Fresh tracheal segments were treated with NGF (100 ng/mL) 1 h before stimulation, and segments cultured for 1 and 4 days were treated with NGF during the whole incubation period. Segments' responsiveness to accumulative administration of carbachol was sensitized by culture as previously described (Fig. 6) [23], with no difference between proximal and distal segments. Treatment with NGF did not affect the reactivity to NGF. An increase in the absolute and relative contractile response to EFS was observed in proximal NGF-treated segments after 4 days of culture, compared with controls, but

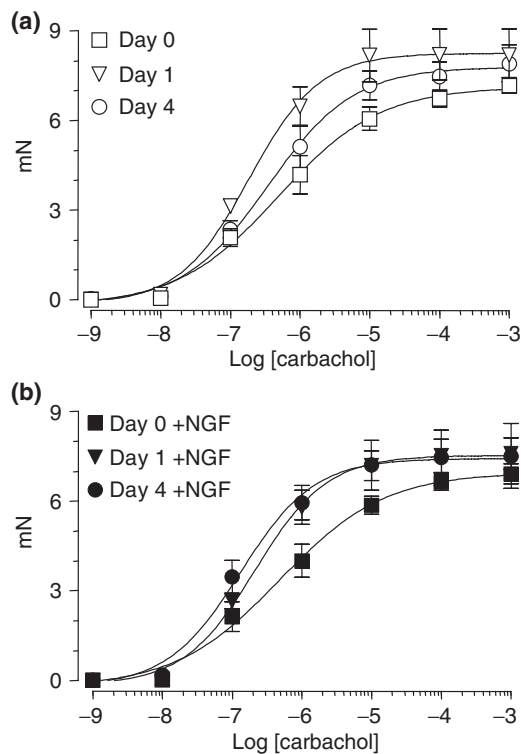


Fig. 6. Responsiveness of cultured tracheal segments to accumulative administration of carbachol. (a) Segments were used fresh (day 0) or cultured for 1 or 4 days. After culture, the segments were placed in an organ bath and stimulated with accumulative administration of carbachol. (b) In separate experiments, nerve growth factor (NGF) (100 ng/mL) was added to segments during culture ($P > 0.05$, $n = 6$ segments).

not in distal segments (Figs 3 and 7). The effect of NGF in 4 days cultured segments was concentration-dependent starting at concentrations of 1 ng/mL (Fig. 8). EFS responsiveness of proximal segments was strongly reduced by TTX (not shown). Additionally, NGF did not modify the TTX-resistant contractions. Following 4 days of treatment with NGF ($1-100\text{ ng/mL}$), capsaicin ($10\mu\text{M}$) neither induced contraction at baseline tension nor relaxation of carbachol ($1\mu\text{M}$) precontracted segments (data not shown), demonstrating lack of involvement of capsaicin-sensitive sensory nerves in cultured segments. Differences between NGF-treated and control proximal segments were maintained in the presence of indomethacin ($3\mu\text{M}$; Fig. 9).

Immunohistochemistry

The density of nerve fibres was assessed with immunohistochemistry for PGP 9.5 in both fresh and cultured trachea. During control conditions, fresh segments displayed numerous nerve fibres across the smooth muscle and subepithelial layer (Fig. 11g) and no marked difference in innervation density was seen when proximal and distal segments were compared. After 4 days of culture regional differences became apparent, with less immunostained nerve fibres in proximal than in distal sections of the trachea. Only a moderate number of nerve fibres were seen in proximal segments ($n = 5$), whereas more nerve fibres were seen in distal segments ($n = 4$).

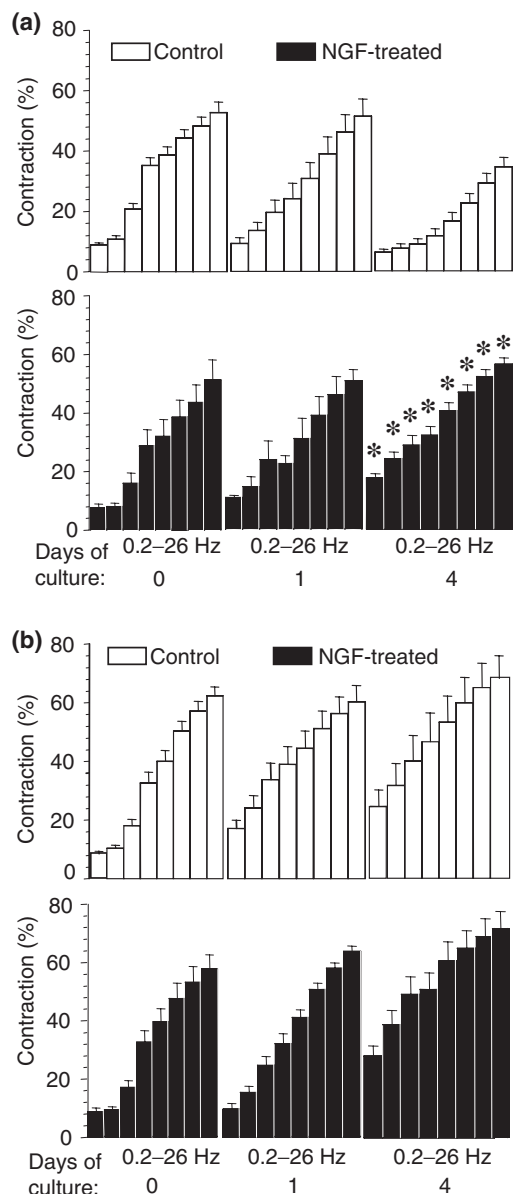


Fig. 7. Responsiveness of cultured tracheal segments to electric field stimulation (EFS). Proximal (a) and distal (b) segments were used fresh (day 0) or cultured for 1 or 4 days. After culture, the segments were placed in organ bath and stimulated with a ^{2} log series of EFS (0.2–25.6 Hz). In separate experiments, nerve growth factor (NGF) (100 ng/mL) was added to segments during culture. Proximal segment treated for 4 days with NGF responded with stronger contractions than control segments after the corresponding time of culture (* $P < 0.05$, $n = 6$ –8 segments).

The treatment of cultured segments with NGF (100 ng/mL) during 4 days did not affect the immunostaining of distal segments. The same amount of nerve fibres was seen in treated and untreated segments. Following culture in the presence of NGF, there was an increase in the amount of nerve fibres seen in proximal segments, with a marked increase in PGP 9.5 immunostaining ($n = 4$). More nerve fibres were seen in the smooth muscle layer, resulting in a picture similar to the one obtained in distal control segments (Figs 10 and 11g–i).

Fresh and 4 days cultured segments in the absence and presence of NGF (100 ng/mL) were also stained for the

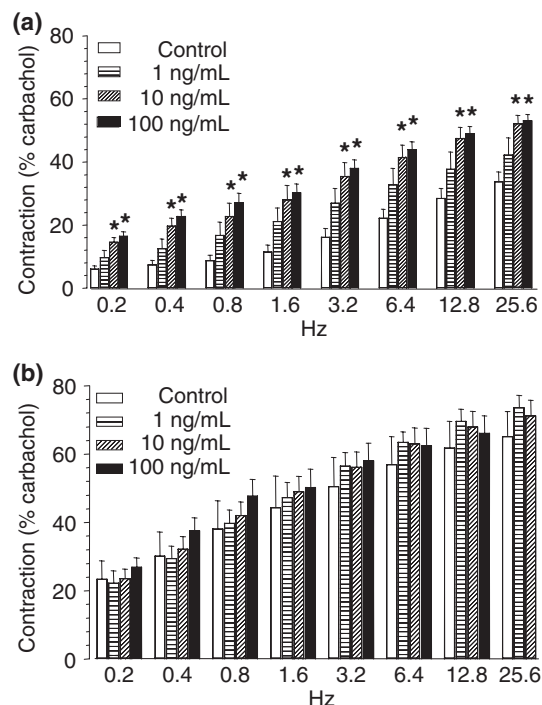


Fig. 8. Tracheal segments were cultured for 4 days with nerve growth factor (NGF) and stimulated by electric field stimulation (EFS) (0.2–25.6 Hz) in the organ bath. (a) Proximal segments reacted by stronger EFS contractions, correlating to NGF concentration. (b) Distal segments showed no significant difference when comparing NGF-treated segments with controls (* $P < 0.05$, $n = 6$ –7 segments). Data are presented as percent of maximal 100 μ M carbachol contractions.

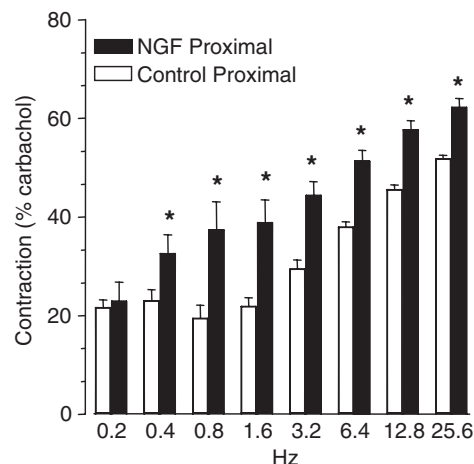


Fig. 9. Responsiveness of cultured tracheal segments to electric field stimulation (EFS). Proximal segments were cultured for 4 days. After culture the segments were placed in an organ bath treated with indomethacin (3 μ M) and stimulated with a ^{2} log series of EFS (0.2–25.6 Hz). In separate experiments, nerve growth factor (NGF) (100 ng/mL) was added to segments during culture. NGF-treated segments responded with stronger contractions than control segments of corresponding time of culture (* $P < 0.05$, $n = 4$ segments).

neuropeptides CGRP (Fig. 11a–c) and SP (Fig. 11d–f). Fresh segments demonstrated a strong, consistent CGRP- and SP-immunostaining, indicating nerve fibres mainly in the subepithelial and smooth muscle layer of proximal and distal segments. A few CGRP- and SP-positive nerve fibres were

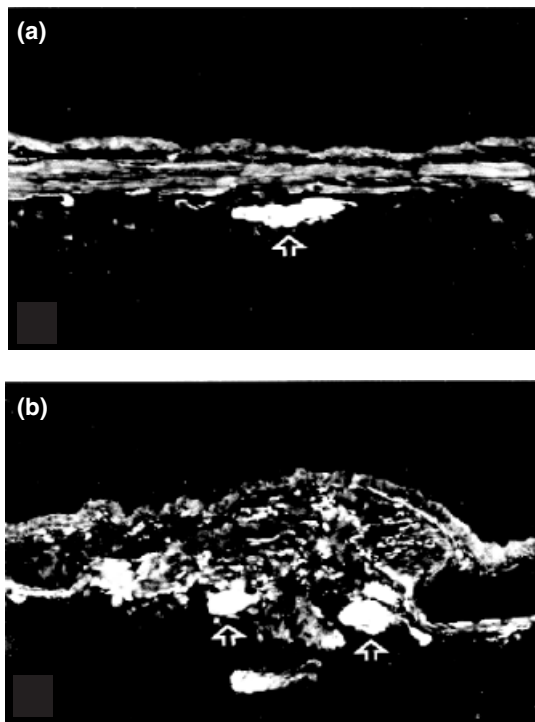


Fig. 10. Innervation of proximal trachea after 4 days of culture: tracheal sections were treated with an anti-serum directed against the neural marker protein gene product 9.5 (PGP 9.5). (a) Poorly innervated proximal tracheal segments (arrows denotes PGP 9.5 immunoreactive nerve cells bodies; $n = 4$ tracheae). (b) Proximal tracheal segments, treated with nerve growth factor (NGF) (100 ng/mL) in the culture medium ($n = 4$ tracheae).

seen to penetrate the epithelium. In cultured segments, the immunostaining for both SP and CGRP was generally weak, and no differences were seen between proximal and distal segments. NGF treatment did not affect the staining for SP and CGRP ($n = 3$).

Discussion

The present study demonstrates that culture of mouse tracheal segments can be used for evaluation of the long-term effects of inflammatory mediators (e.g. NGF) on EFS-evoked smooth muscle contractions. Cultured segments exhibited reproducible EFS contractions in relation to carbachol during culture periods of up to 16 days. The EFS contractions were frequency-dependent and diminished by nerve blockade and atropine. Regional differences in relation to EFS developed as a result of culture. Distal segments exhibited a strong, predominantly nerve-mediated contraction, whereas proximal segments exhibited weaker contractions, partly generated by direct EFS of the smooth muscle. Treatment with NGF during culture increased the responsiveness to EFS in a concentration-dependent manner in proximal segments, but did not alter the responsiveness of distal segments. In correlation, increased density of nerve fibres was observed after treatment with NGF of the proximal trachea.

The maximal contractile response to carbachol was stable during the first 4 days of culture. After 8 days of culture, the

contractions became weaker in comparison with fresh segments. This alteration in the smooth muscle contractile capacity has been previously described in detail by our group [23] and might reflect a cellular adaptation to the new *ex vivo* environment. Correspondingly, a culture-induced phenotypic shift has been described in blood vessels, caused by the decreased expression of L-type Ca^{2+} channels and smooth muscle-2 myosin heavy chains [21, 25].

The contractile responses to EFS in segments cultured for time periods longer than 4 days decreased in parallel with decreased carbachol responsiveness, in correlation to the number of days in culture. Therefore, when EFS-induced contractions were expressed as percentage of carbachol contractions, no significant difference was observed between days of culture, although the absolute contractions were weakened. These results imply that mouse tracheal nerves are capable of releasing effective concentrations of acetylcholine after several days of culture, a finding described for guinea-pig tracheal segments cultured for 2 days [22].

Following culture, there was a gradual change in the shape of the frequency-effect curve in that the midpoint slope coefficient decreased. This may be the result of increased nerve sensitivity to depolarization by EFS as observed in cultured smooth muscle cells [26]. In addition, an increase in spread of the curve parameters was observed, which initiated experiments to test if these changes were related to segment position on the trachea. In accordance with a study in guinea-pig tracheal segments [27], fresh segments did not show regional variation in EFS responsiveness. However, following 4 days of culture, a significant regional difference was observed, with distal segments responding with stronger contractions than proximal segments. This change in regional responsiveness correlated to the distribution of nerve cell bodies, which were located mainly in the distal part of the mouse trachea [28]. Nerve fibres, as displayed by PGP 9.5 staining, were predominantly located in the distal part of the trachea following 4 days of culture, although there was no clear difference in the staining of freshly isolated segments. Thus, the immunohistochemical analysis correlated with the myographic findings in segments cultured for 4 days.

TTX and atropine are reported to abolish EFS contractions in mice [29, 30] and to inhibit the main part of the contraction in guinea-pig trachea [27]. In the present study, the TTX/atropine-resistant component of EFS contraction was observed mainly as a side-effect of the organ culture in the proximal trachea and increased over time of culture. No involvement of sympathetic or non-adrenergic, non-cholinergic nerve activity was obtained since neither capsaicin nor timolol affected the EFS-induced contractions. The inability of TTX, atropine or CTX to abolish EFS contractions suggests a direct effect of EFS on the smooth muscle cells in the mouse trachea after culture. This non-neural-mediated contraction was particularly strong in segments from the proximal portion of the trachea known to hold few ganglia [28]. The results suggested that the smooth muscle in the proximal part was more sensitive to depolarization than the distal part. To test this hypothesis, cumulative administration of KCl was performed in both proximal and distal segments. In accordance, the proximal segments responded with higher sensitivity. The reason for this phenomenon may be related to the loss of contact to nerves caused by dissection, which has been

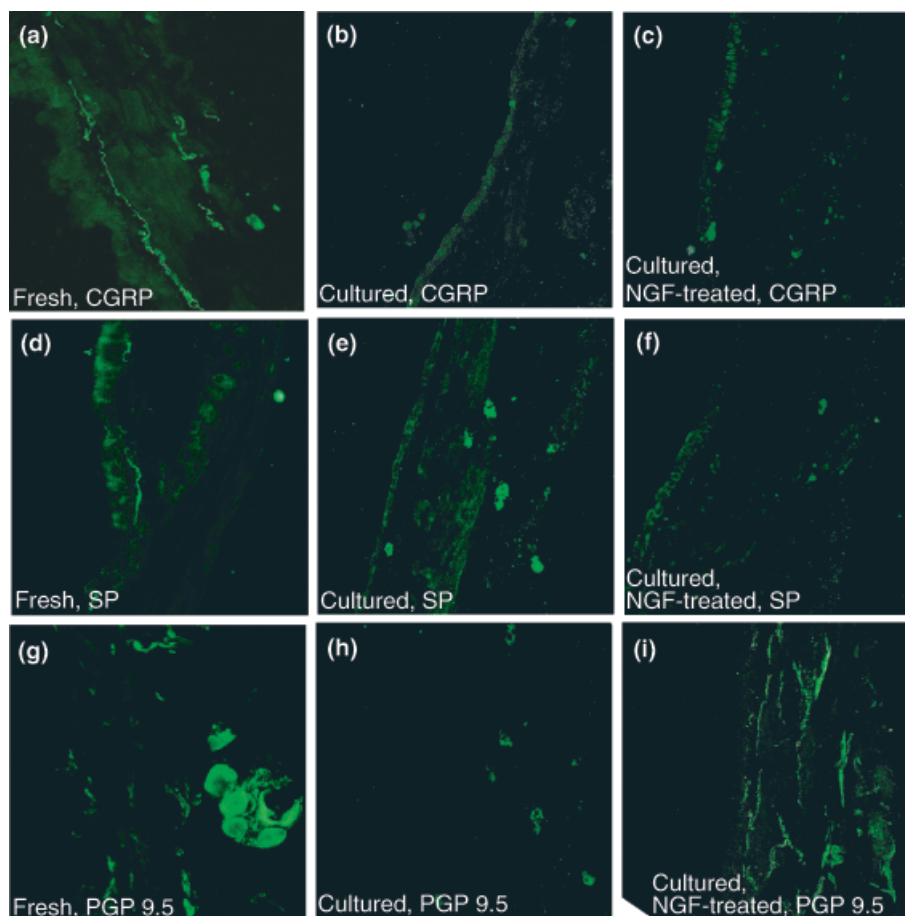


Fig. 11. Immunostaining of fresh and cultured tracheal segments, for calcitonin gene-related peptide (CGRP) (a–c), substance P (SP) (d–f) and protein gene product 9.5 (PGP 9.5) (g–i). Fresh segments were compared with segments cultured (4 days) with and without nerve growth factor (NGF) (100 ng/mL) ($n = 3$). As can be seen, the immunostaining for SP and CGRP were generally weak after culture, regardless of the NGF treatment. In contrast, the staining for PGP 9.5 increased markedly when segments were cultured with NGF.

described to decrease the membrane potential in both cultured and denervated canine colonic smooth muscle cells [26].

In the second part of the present study, tracheal segments were cultured in the presence of NGF for 0 to 4 days. This treatment did not affect the smooth muscles' reactivity to the cumulative administration of carbachol. Neither did NGF affect the responses of carbachol and EFS in fresh segments. However, NGF enhanced the contractions seen following EFS in proximal, but not distal tracheal segments. The enhancing effect of NGF was concentration dependent, starting at a concentration of 1 ng/mL. NGF binds to two receptor subtypes [31], a high-affinity receptor tyrosine kinase A (TrkA; $K_d = 0.01$ nM) and a low-affinity receptor p75^{NTR} ($K_d = 1$ nM). In this study, the highest concentration of NGF used was 3.8 nm (100 ng/mL), suggesting that the NGF effect was mainly mediated by TrkA receptors. A recent report [32] identified the uncleaved form of pro-NGF as the high-affinity ligand for p75^{NTR} with a K_d of 0.1 nM. Thus, this latter report suggested that the earlier description of NGF as a ligand of p75^{NTR} is a misconception caused by pro-NGF contamination of NGF used in early experiments [33]. Additionally, the report offers a mechanism linking NGF signalling to protease activity and inflammation [32].

NGF has been reported to elevate the number of substance P positive neurons [12, 18] in guinea-pig airways, and to

induce neurokinin-mediated hyper-responsiveness [17] in the same species. In mice, nasal installation of NGF is reported to induce hyper-responsiveness *in vitro* [16]. In the present study, NGF increased the EFS-induced contractions of proximal tracheal segments only after culture. Furthermore, capsaicin had no effect on the EFS response of controls or NGF-treated segments, indicating the absence of endogenous neurokinin release from capsaicin-sensitive sensory nerves. This observation is in line with the relatively weak immunostaining for CGRP and SP found in cultured segments. Thus, the NGF-dependent increase in EFS-induced contraction of proximal segments suggests that NGF promotes the preservation or restores cholinergic innervation. This conclusion is supported by the increase in the amount of PGP 9.5 positive nerve fibres presently noted after NGF treatment. It might be that the culture procedure *per se* reduced the number of neuropeptide-containing nerves.

The first part of the study demonstrates that the tracheal culture assay could be used for investigations of long-term effects of inflammatory mediators on neural activity. A good reproducibility was shown up to at least 8 days of culture, which opens new possibilities for investigation of neural involvement in the development of airway hyper-responsiveness due to chronic inflammation. In the second part of the study, NGF was demonstrated to enhance cholinergic, nerve-

dependent contractions and to increase the amount of nerve fibres in less densely innervated parts of the trachea. Even though these NGF data were obtained *in vitro*, it is tempting to speculate a similar role for NGF *in vivo*, as well as in relation to airway pathophysiology. Thus, NGF might induce axonal growth not only in the scarcely innervated parts of the mouse trachea but also generally in all scantily innervated regions of the airways. The human lower respiratory tract, known to be cholinergic innervated, is a clinically important example of one such region [34]. NGF has been detected in the blood circulation as well as in airways of allergic and asthmatic patients [9–13]. Thus, it is possible that NGF might contribute to airway hyper-reactivity, seen among these patients, by increasing cholinergic neural activity in the peripheral airways.

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