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**Prolonged exposure to NT-3 attenuates cholinergic nerve-mediated contractions in cultured murine airways**

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**Abstract**

Chronic airway inflammation may induce subsequent airway hyperresponsiveness (AHR) including pathological alteration of neural activity. Asthmatic airways contain elevated levels of neurotrophin-3 (NT-3) and brain-derived neurotrophic factor (BDNF) albeit; their effect on neural activity is unclear. This study evaluates the effects of NT-3 and BDNF on nerve mediated airway contractions *in-vitro*. Tracheal segments from BALB/c mice were cultured for 4 days with NT-3 or BDNF. Responsiveness to electric field stimulation (EFS) was evaluated in organ-bath and innervation patterns were examined by quantitative immunohistochemistry. In cultured segments the EFS induced contractions were inhibited by tetrodotoxin or atropine. NT-3 reduced the EFS contractions in a concentration-dependent manner whereas BDNF had no effect. The amount of nerve fibers, found in conjunction with the tracheal smooth muscle, was similar in NT-3 treated and control segments. In conclusion, NT-3 attenuates cholinergic nerve-mediated contractions of airway *in-vitro*. Considering the elevated levels of NT-3 found in asthmatic airways, the findings imply a protective role of NT-3 in AHR.

## Introduction

Airway hyperresponsiveness (AHR) can be defined as an exaggerated degree of airway narrowing in response to bronchoconstrictor stimuli. The underlying mechanisms of AHR are complex involving both acute and chronic airway inflammation (Pare et al., 1995). Human airways are innervated by post-ganglionic parasympathetic neurons that increase the airway resistance by release of acetylcholine (Barnes, 2001). Vagal activity exerts a strong effect on airway caliber that is particularly evident in acute severe asthma. Alteration of neural activity is believed to contribute to both the pathogenesis and the symptoms of asthma (Barnes, 2001; Renz, 2001), via increased neural activity and alterations of neurotransmitter release (Undem et al., 2001; Undem et al., 1999).

Augmented levels of the neurotrophins neurotrophin-3 (NT-3), brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF), have been found in the blood and airway fluid from asthmatic patients (Bonini et al., 1996; Kassel et al., 2001; Sanico et al., 2000; Undem et al., 1999; Virchow et al., 1998), and neurotrophin levels are reduced in response to corticosteroid treatment in asthmatic patients (Noga et al., 2001). A known source of NT-3 in human airways are eosinophils (Barouch et al., 2000; Kobayashi et al., 2002), whereas BDNF can be derived from macrophages, T-lymphocytes and epithelial cells (Barouch et al., 2000; Braun et al., 1999; Kerschensteiner et al., 1999). However, whether NT-3 or BDNF affects the neural activity and contribute to AHR is not clear (Renz, 2001).

We have recently developed an *in-vitro* assay for evaluation of effects induced by the prolonged presence of inflammatory mediators on endogenous cells (e.g. airway smooth muscle) of murine airways. In this model, isolated tracheal segments are cultured in the presence of different mediators and then evaluated for genetic, morphologic and functional changes (Adner et al., 2002; Johnson, 2002). Segments have been demonstrated to be viable for several weeks and to respond to pharmacological agents known to induce contractions as well as relaxations. Additionally, the model includes the evaluation of neural activity induced by electric field stimulation (EFS) (Bachar et al, 2004). EFS induced reproducible and frequency-dependent cholinergic contractions of segments cultured up to 8 days. The EFS induced contractions are blocked by tetrodotoxin (TTX) or atropine. After 4 days in culture regional differences emerge, with stronger EFS responses in the distal than in the proximal part of the trachea. The responses correlate with the immunohistochemical finding of a more densely innervated distal part. Direct administration of NGF to fresh segments did not cause

any contractions or relaxations. In contrast to direct treatment, culture with NGF for 4 days increased the innervation of proximal segments and the contractile responses to EFS were enhanced in a concentration-dependent manner (Bachar et al, 2004). The aim of the present study was to evaluate if prolonged exposure to NT-3 and BDNF affect nerve-mediated smooth muscle contractions.

## **Method**

### *Organ-culture*

Male BALB/c J mice, 10 weeks old, were sacrificed by cervical dislocation and the whole trachea was rapidly removed and placed into cold DMEM, supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml). Each trachea was dissected free from adherent tissue and divided into segments each with three cartilage rings (Adner et al., 2002). Due to regional differences within the trachea (Chiang et al., 1986) proximal and distal segments were examined separately. Individual segments were placed in a 96-well plate with 300 µl DMEM for organ culture. Under these conditions the segments were incubated at 37 °C in humidified 5% CO<sub>2</sub> in air for 4 days (Adner et al., 2002) and transferred daily to a new well containing fresh DMEM. NT-3 or BDNF were, when appropriate, added daily to the culture medium. The study was approved by the Ethics Committee of University of Lund (Lund, Sweden).

### *Isometric force measurement*

Smooth muscle reactivity of the tracheal segments was analyzed in temperature-controlled (37 °C) myographs (Organ Bath Model 700MO, J.P. Trading, Aarhus, Denmark), containing Krebs-Henseleit solution as previously described (Adner et al., 2002). The solution was continuously equilibrated with 5% CO<sub>2</sub> and 95% O<sub>2</sub>. The tracheal segments were mounted on two L-shaped metal prongs, with a resting tension of 0.8 mN. Changes in isometric tension were recorded using a Chart software (AD Instruments Ltd., Hastings, U.K.).

### *Experimental procedure*

Airway smooth muscle responsiveness to EFS was assessed in a myograph by placing electrodes at opposite sides of the tracheal segments (Current Stimulator Model CS200, J.P.

Trading, Aarhus, Denmark). Initially, each tracheal segment was given five training impulses of 4 Hz, 55 mA (approximately 10 V). Subsequently, segments were electrically stimulated with 0.2-25.6 Hz in <sup>2</sup>log-step series. Each frequency step was 1 min long consisting of 0.8 ms impulses and followed by 1.5 min of recovery. All parameters for EFS were optimized to produce stable and reproducible contractions. At the end of each stimulation series, segments were washed twice. After the initial series of EFS, segments were incubated 30 minutes with tetrodotoxin (TTX, a Na<sup>+</sup>-channel blocker; 1 μM) and the EFS series were repeated. Finally, carbachol (an acetylcholine analogue; 100 μM) was added and maximum contraction was measured after 20 minutes as a reference for the contractile capacity. In parallel experiments, TTX was replaced by atropine (a muscarinic receptor antagonist; 1 μM) and the maximal contraction was probed using U46619 (a thromboxane A<sub>2</sub> analogue; 1 μM) instead of carbachol. Additionally, tracheal relaxations induced by capsaicin (10 μM), neurokinin A (NKA; 0.1 μM) substance P (SP; 0.1 μM) and EFS, respectively, were studied in segments precontracted with carbachol (1 μM; 10 minutes).

### *Analysis*

Experimental data were expressed as the maximum point of contraction for each stimulation expressed in mN or percent of maximum tension generated by 100 μM carbachol or 1 μM U46619. Data analysis was performed using GraphPad (Prism version 3.00 for Windows, GraphPad Software, San Diego, California, U.S.A.). Individual values were compared using one-way ANOVA. If the test was significant (p < 0.05) a t-test with Bonferroni correction was performed in order to define the difference between groups.

### *Immunocytochemistry*

Tracheal segments were cultured for 4 days with NT-3 or vehicle. The sections were immersed over night 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 (10 μm) were cut in a cryostat and mounted on chrome-alum coated slides. Indirect immunocytochemistry was used for the demonstration of protein gene product 9.5 (Anti PGP 9.5 rabbit antibody, dilution of 1:200, Ultraclone, Cambridge, UK), TrkA (rabbit antibody,

dilution of 1:400, Santa Cruz Biotechnology, Santa Cruz, California, U.S.A.), TrkB (rabbit antibody, dilution of 1:600, Santa Cruz Biotechnology, Santa Cruz, California, U.S.A.) and TrkC (goat antibody, dilution of 1:300, R&D systems, Abingdon, UK). Briefly, tracheal sections were incubated with a primary antiserum overnight at 4 °C. The antigen-antibody complex was revealed by application of a secondary antibody (1:80; swine anti-rabbit IgG, DAKO, Copenhagen, Denmark) conjugated to fluorescein isothiocyanate (FITC). In the control experiments the primary antibody was omitted.

### *Biometrics*

Immunohistochemical data was analyzed using an analysis software Visiopharm Integrator System<sup>®</sup> v2.1.2 (Visiopharm, Hørsholm, Denmark). The software was trained to distinguish PGP 9.5 stained nerve fibers from the background smooth muscle layer. The nerve fiber area was expressed as percentage of the total smooth muscle area.

### *Chemicals*

Substance P and neurokinin A were purchased from Neosystem S.A., Strasbourg, France. Atropine, capsaicin, carbachol, Dulbecco's modified eagle's medium, human recombinant BDNF, human recombinant NT-3, indomethacin, N(G)-monomethyl-L-arginine (L-NMMA), and U46619 were obtained from Sigma, St. Louis, Missouri, U.S.A. Tetrodotoxin was obtained from Sankyo, Tokyo, Japan, and penicillin-streptomycin was obtained from Life Technologies, Gettysburg, Pennsylvania, U.S.A.

## **Results**

EFS of cultured tracheal segments (0.2-25.6 Hz) induced a sustained contraction reaching a maximal contraction by 12.8 or 25.6 Hz. The contraction remained stable during the stimulation period and declined rapidly when the stimulation ceased (Fig 1a). The magnitude of the contractions was frequency-dependent. TTX (1  $\mu$ M) or atropine (1  $\mu$ M) practically blocked all contractions in frequencies lower than 3.2 Hz and substantially reduced EFS contractions in the frequency span of 3.2-25.6 Hz (Fig 1a).



Tracheal segments were cultured for 4 days and NT-3 was present in the medium during the whole culture period. Subsequently, segments were placed in the organ-bath and stimulated with EFS. A high concentration of NT-3 (100 ng/ml) reduced EFS mediated contractions, especially at low frequencies (0.2-1.6 Hz; Fig. 1b) and this reduction was shown to be concentration-dependent (NT-3, 1-100 ng/ml; Fig 2). The decline in EFS response, induced by NT-3, was principally the same in both proximal and distal segments. Albeit, the relative force induced by EFS after 4 days culture in proximal control segments was weaker than that of distal control segments (Fig 2 a and b respectively), as earlier described for organ-cultured mouse trachea (Bachar, 2004).

In contrast, tracheal segments cultured for 4 days in presence of BDNF (50 ng/ml) did not change their response to EFS (Fig. 3). In these experiments both proximal and distal segments, exhibited strong and reproducible contractions, similar to contractions observed following culture with vehicle only.

Carbachol (100  $\mu$ M) and U46619 (1  $\mu$ M; not shown) induced strong contraction of tracheal segments cultured for 4 days, reaching maximum after approximately 20 minutes. These contractions ( $7.0 \pm 0.6$  mN,  $n=8$ ) were not affected by the presence of NT-3 (100 ng/ml;  $7.3 \pm 0.9$  mN;  $n=5$ ) or BDNF (50 ng/ml;  $8.2 \pm 0.9$  mN;  $n=7$ ) in the culture medium.

Additionally, the concentration-response curve for carbachol-induced contractions exhibited similar  $pEC_{50}$  values for segments cultured in presence of NT-3 (100 ng/ml) and vehicle ( $6.7 \pm 0.2$  and  $6.5 \pm 0.1$ , respectively;  $n=4$ ). No difference was observed between segments derived from the proximal and the distal part of the trachea (not shown).

Segments cultured in presence of NT-3 and BDNF did not contract in response to capsaicin (10  $\mu$ M;  $n=4$ ; not shown). Neither did segments cultured with vehicle only. Additionally, segments pre-contracted with carbachol (1  $\mu$ M) did not relax in response to capsaicin ( $n=4$ ). In contrast, SP (100 nM) and NKA (100 nM) induced strong relaxations in these segments ( $43.5 \pm 2.7$  % and  $38.3 \pm 1.3$  %, respectively;  $n=4$ ; not shown). These relaxations were inhibited by indomethacin (3  $\mu$ M), administered to the organ-baths 30 min before the neurokinins.

In separate experiments, segments cultured for 4 days, were stimulated with EFS (25.6 Hz), before and after treatment with the nitric oxide synthase (NOS) inhibitor L-NMMA (100  $\mu$ M). The EFS responsiveness of control segments before and after treatment with L-NMMA was similar ( $48.8 \pm 11.0$  % and  $50.3 \pm 10.4$  %, respectively;  $n=3$ ). In segments cultured for 4 days

in presence of NT-3 (100 ng/ml) no difference was observed when comparing the responses of the segments before and after treatment with L-NMMA ( $20.4 \pm 3.9 \%$  and  $19.6 \pm 4.8 \%$ , respectively; n=3, (not shown).

In order to further evaluate the effect of NT-3 on nerve-mediated relaxation, segments cultured for 4 days with NT-3 (100 ng/ml) or vehicle were precontracted with carbachol (1  $\mu$ M). After 10 minutes a stable level of pre-contraction was reached and segments were stimulated with EFS (4 Hz, Fig. 4 a-c). EFS induced an additional contraction on top of the pre-contraction. A rapid relaxation was observed when the EFS was discontinued. The contractions of the segments cultured in presence of vehicle were more marked than the contractions seen in the segments cultured with NT-3 ( $17.6 \pm 2.9 \%$  and  $7.4 \pm 1.7 \%$ , respectively; n=4). In contrast, no difference was observed in the relaxation phase ( $-10.1 \pm 1.4\%$  and  $-10.3 \pm 2.2\%$ , respectively; n=4).

#### *Immunohistochemistry*

Tracheal segments were cultured with NT-3 (100 ng/ml) or vehicle during 4 days. The neural marker PGP 9.5 was used to visualize the amount of nerve fibers in conjunction with the airway smooth muscle. Following culture with vehicle, only a few nerve fibers were visible in proximal segments (n=5) whereas distal segments were richly innervated (n=4; Fig. 6a). Culture in presence of NT-3 did not change the amount of nerve fibers observed in distal segments (n=4; Fig. 6b). The expression of TrkA, B and C in the cultured mouse trachea was investigated using immunohistochemistry. In 4 days cultured segments TrkA reactivity was detected exclusively in the epithelium (Fig. 5a), TrkB reactivity was not seen (Fig. 5b) and TrkC reactivity was detected in smooth muscle and in nerve cell bodies (Fig. 5c).

Quantitative analysis comparing innervation of NT-3 treated and control segments was performed. Tracheal segments were cultured for 4 days in the absence and presence of NT-3, fixed and immunostained for the detection of PGP 9.5 immunoreactivity. Innervation was estimated as percentage of PGP 9.5 stained nerve area of the total smooth muscle area (Fig. 7; control:  $10.0 \pm 1.7\%$  N=7; NT-3 treated:  $8.2 \pm 1.0\%$  N=12, no significant difference was detected between the NT-3 treated and control groups).

## Discussion

The present study demonstrates that prolonged exposure of cultured mouse tracheal segments to NT-3 markedly reduced nerve-mediated acetylcholine dependent contraction to EFS.

BDNF did not affect the EFS responsiveness in the present experimental set-up.

Immunohistochemical analysis using PGP 9.5 revealed no apparent alteration of the innervation density following treatment with NT-3.

In EFS experiments the strength of contraction was expressed as percentage of maximal carbachol or U46619 contraction in an attempt to standardize the reactivity of individual segments to be less dependent of variations in smooth muscle mass. These maximal contractions were unchanged by culture with NT-3 or BDNF, indicating that differences observed in EFS segments were not dependent on altered reactivity to carbachol or U46619.

Previous studies of mouse tracheal airways have revealed regional differences in the neural distribution with a more dense innervation of the distal portion (Chiang et al., 1986).

Therefore, proximal and distal portions of the trachea were analyzed separately. In cultured tracheal segments, the distal portion responded with stronger contractions to EFS than the proximal portion (Bachar, 2004). Inhibitory effects of TTX and atropine on both portions indicated that cholinergic nerves are the main mediators of EFS evoked contractions.

Administration of capsaicin is reported to induce relaxation of pre-contracted tracheal segments via the release of neurokinins from nociceptive nerves (van Heuven-Nolsen et al., 1997). However, capsaicin administration to the cultured segments had no effect, despite cyclooxygenase-dependent relaxations by the neuropeptides NKA and SP. The lack of capsaicin reactivity is probably due to the absence of sensory nerve cell bodies in the cultured tracheal preparation. Thus, in the present organ culture assay EFS responsiveness in the mouse tracheal segments is essentially cholinergic in accordance with the lack of response to vagal stimulation in muscarinic M3 receptor-deficient mice (M3<sup>-/-</sup> mice) (Fisher et al. 2004).

Secretion of neurotrophins from airway epithelium and inflammatory cells is suggested to modulate the activity of the airway neurons in patients with chronic airway inflammation. (Nockher et al., 2003). However, little is known concerning the effects of NT-3 and BDNF on neural function in the airways. In the present experiments, treatment with NT-3 reduced EFS contractions in a concentration-dependent manner. NT-3 is reported to mediate its effect via

TrkC and p75<sup>NTR</sup> receptors (Lamballe et al., 1991) and to be involved in the induction of apoptosis in neurons (Barrett, 2000; Kim et al., 1999). Thus, the reduced contractile response seen following NT-3 treatment could be related to increased apoptosis among tracheal neurons. The immunohistochemical finding of intact nerve fibers after NT-3 treatment makes this explanation less likely.

Only TrkA and TrkC were detected in the cultured trachea. This observation is in line with the findings that only NGF and NT-3 had an effect on the contractions of cultured segments. BDNF, a ligand to TrkB and p75<sup>NTR</sup> receptors (Squinto et al., 1991), has been reported to promote growth and enhance acetylcholine release from CNS neurons in rats (Auld et al., 2001; Kishino et al., 1997). However, treatment with BDNF did not have functional effects in the present experimental step-up. Hence, the effects of NT-3 on cultured mouse trachea are likely to be mediated by TrkC receptors. The effective concentrations used in the present experiments are similar to concentrations described for the binding of NT-3 to TrkC (Lamballe et al., 1991). Interestingly, NT-3 was reported to reduce neuronal precursor proliferation and inhibit neuronal maturation. These effects of NT-3 maintain homeostasis between neuronal populations within the olfactory epithelium in mice (Simpson et al., 2003). These findings are consistent with our results and imply a protective role for NT-3 in the context of inflammation-induced neural remodeling in the airways.

Considering the possibility that the decreased EFS responsiveness after NT-3 treatment was due to altered relaxatory properties, additional experiments were performed on pre-contracted segments. In pre-contracted segments EFS did not induce any relaxations during stimulation. Instead an additional contraction was observed, which was lower in the NT-3 treated segments. As soon as EFS was discontinued the segments relaxed. The relaxation was similar in NT-3 cultured and control segments, suggesting that NT-3 reduced the contractions rather than enhanced a possible EFS induced relaxation. EFS is reported to induce nitric oxide dependent relaxation of the mouse trachea (Mhanna et al., 2001). In the present set-up L-NMMA, a NOS inhibitor, did not alter the effect of NT-3 treated or control segments albeit, the extension and methods of the investigation were not sufficient to rule-out an effect of nitric oxide.

In the present study, prolonged exposure to NT-3 attenuated EFS induced contraction of isolated murine airways. This effect of contractile cholinergic nerve-activity could not be

explained by an increase of counteracting dilatory nerve activity. Neither could it be the product of a changed innervation pattern, overall suggesting an alteration of cholinergic nerve phenotype. Increased levels of NT-3 have been found in bronchial lavage fluids of asthmatic patients after allergen challenge (Virchow et al., 1998). This rendered the conclusion that asthma is associated with a deregulation of the complex interactions between the nervous and immune systems and that NT-3 up-regulation might have relevance for human bronchial asthma. In reference to the development of asthma the role of NT-3 might be beneficial or detrimental; the present findings suggest that NT-3 might have a protective effect in inflammatory airways.

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**Figure Legends**

## Figure 1

Representative experimental charts. Distal segments were cultured for 4 days with NT-3 (100 ng/ml) and subsequently placed in an organ-bath. Control (a) and NT-3 treated (b) segments were stimulated with EFS in <sup>2</sup>log steps series 0.2-25.6 Hz in absence and presence of TTX (1 μM) and finally treated with carbachol (100 μM). Duration of electric and pharmacological stimuli is marked in the bottom of the figure with a line.

## Figure 2

EFS of cultured tracheal segments. Segments were cultured for 4 days with NT-3 (1, 10 or 100 ng/ml). Proximal (A) and distal (B) segments were subsequently placed in an organ-bath and stimulated with EFS <sup>2</sup>log steps series 0.2-25.6 Hz. All data are expressed as percentage of maximal 100 μM carbachol contraction, \*p < 0.05 for individual stimulation frequencies compared to control; n= 5-8 segments.

## Figure 3

EFS of cultured tracheal segments. Segments were cultured for 4 days with BDNF (50 ng/ml). Proximal (A) and distal (B) segments were subsequently placed in an organ-bath and stimulated with EFS <sup>2</sup>log steps series 0.2-25.6 Hz. All data are expressed as percentage of maximal 1 μM U46619 contraction, p>0.05 for all individual stimulation frequencies compared to control; n= 5-8 segments.

## Figure 4

EFS of precontracted cultured trachea. Distal segments were cultured for 4 days with NT-3 (100 ng/ml), subsequently placed in an organ-bath, precontracted with carbachol (1 μM) for 10 minutes and stimulated by EFS (4 Hz). Representative experimental charts of NT-3 treated (a) and control (b) segments. Duration of pharmacological and electric stimuli is marked in the bottom of the figure with a line. The relative EFS induced contraction and relaxation are illustrated as well (c). Data are expressed as percentage of maximal 1 μM carbachol contraction, \*p < 0.05, n = 4 segments.

## Figure 5

Distal tracheal segments were cultured for 4 days and treated with antisera directed against TrkA and TrkC. The epithelium layer was stained positive for TrkA (a). Nerve cell bodies and smooth muscles were stained positive for TrkC (b).

Figure 6

Distal tracheal segments were cultured for 4 days and treated with antisera directed against the neuronal marker PGP 9.5. Control segments were richly innervated (a). Treatment with NT-3 (100 ng/ml) during culture did not attenuate the density of nerve fibers visible (b).

Figure 7

Tracheal segments were cultured for 4 days in the presence and absence of NT-3 (100 ng/ml) and treated with an antiserum directed against the neuronal marker PGP 9.5.

Immunohistochemical images of the segments were analyzed by a biometrics software. Nerve area is expressed as percentage of total area,  $n=7-12$ ,  $p=0.32$  (ns).

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Fig 1ab

