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The role of sensory nerve endings in nerve growth factor-induced airway hyperresponsiveness to histamine in guinea-pigs

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1 Nerve growth factor induces an airway hyperresponsiveness *in vivo* in guinea-pigs, as we have shown previously. Since antagonizing the neurokinin-1 (NK_1) receptor can prevent this NGF-induced airway hyperresponsiveness and since sensory nerves release tachykinins, we investigated the role of sensory nerves in the NGF-induced airway hyperresponsiveness.

2 We used isolated tracheal rings from guinea-pigs to measure tracheal contractility. In these rings sensory nerve endings are present, but these endings lack any contact with their cell bodies.

3 In this *in vitro* system, NGF dose-dependently induced a tracheal hyperresponsiveness to histamine. The NK_1 receptor antagonist SR140333 could block the induction of tracheal hyperresponsiveness.

4 To further investigate the involvement of sensory nerve endings we used the cannabinoid receptor 1 (CB_1) agonist R-methanandamide to inhibit excitatory events at the nerve terminal. The CB_1 receptor agonist was capable of blocking the tracheal hyperresponsiveness to NGF in the isolated system, as well as the airway hyperresponsiveness to NGF *in vivo*.

5 This indicates that NGF can induce an increase in airway responsiveness in the absence of sensory nerve cell bodies. NGF may act by increasing substance P release from sensory nerve endings, without upregulation of substance P in the neurons. Substance P in its turn is responsible for the induction of the NGF-induced airway hyperresponsiveness.

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Abbreviations: CB, cannabinoid; NGF, nerve growth factor; NK, neurokinin; trkA, tyrosine kinase A receptor

Introduction

The neurotrophin NGF is a newly studied mediator in relation to allergic diseases (Braun *et al*., 1998; de Vries *et al*., 1999; Virchow *et al*., 1998). Circulating NGF levels are increased in humans with rhinoconjunctivitis, urticaria-angioedema or asthma; NGF serum levels were particularly high in patients with allergic asthma (Bonini *et al*., 1996). Moreover, an increase in the neurotrophins NGF, brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3) in bronchoalveolar lavage fluids after allergen challenge is reported in patients with asthma (Virchow *et al*., 1998). In patients with allergic rhinitis a very fast increase in NGF in the nasal lavage fluids is found 10 min after allergen challenge (Sanico *et al*., 2000). Furthermore, allergen challenge induced an increase in NGF levels in bronchoalveolar lavage fluid of sensitized mice (Braun *et al*., 1998).

Inflammatory mediators, including interleukin-1, interleukin-4, interleukin-5, tumour necrosis factor α, and interferon-γ have been shown to induce the release of NGF (Brodie, 1996; Brodie *et al*., 1998; Hattori *et al*., 1994; Yoshida *et al*., 1992). In addition to neurons, non-neuronal cells such as mast cells (Leon *et al*., 1994), fibroblasts (Hattori *et al*., 1994), T-cells (Lambiase *et al*., 1997; Moalem *et al*., 2000), eosinophils (Solomon *et al*., 1998) and lymphocytes (Barouch *et al*., 2000) are able to synthesize NGF. NGF shows various pathologic properties in inflammatory models, which could be interesting in relation to allergic asthma as well (reviewed in Braun *et al*., 1999; 2000). NGF affects immune cell activity, as it promotes inflammatory mediator release from basophils (Burgi *et al*., 1996), mast cells (Matsuda *et al*., 1988), T- and B-cells (Lambiase *et al*., 1997; Otten *et al*., 1989) and macrophages (Susaki *et al*., 1996). Furthermore, NGF induces antibody synthesis and secretion from B cells (Otten *et al*., 1989), attracts mast cells (Sawada *et al*., 2000), and induces differentiation of mast cells (Welker *et al*., 2000) as well as of monocytes (Ehrhard *et al*., 1993). Moreover, NGF induces a shift to the production of Th2 type cytokines in a model for multiple sclerosis (Villoslada *et al*., 2000), as well as in a mouse model for allergic asthma (Braun *et al*., 1998). Furthermore, NGF is able to sensitize neurons and it induces an enhanced production of substance P and other tachykinins in sensory nerves (Lindsay & Harmar, 1989).

Tachykinins play a role in neurogenic inflammation and in the development of airway hyperresponsiveness and

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asthma (Lundberg, 1995). We showed that administration of NGF induced an airway hyperresponsiveness in guinea-pigs (de Vries *et al*., 1999). The use of a NK_1 receptor antagonist could prevent this hyperresponsiveness. This points to a role for substance P, the preferred ligand of the NK_1 receptor (de Vries *et al*., 1999). Furthermore, Hunter *et al*. (2000) showed that NGF induces an increase in substance P in guinea-pig airway sensory neurons. The present study focuses on the role of sensory nerves in NGF-induced airway hyperresponsiveness. Isolated tracheal rings containing sensory nerve endings, without any contact with their cell bodies, were used to elucidate the involvement of sensory nerve endings as opposed to the neuronal cell body.

Methods

Materials

We used murine NGF-7S (Alomone Labs, Jerusalem, Israel), being highly conserved in different species (Rubin & Bradshaw, 1981). NGF-7S was dissolved in saline containing 0.01% bovine serum albumin. The NK_1 receptor antagonist SR140333 (Esmonds-Alt *et al*., 1993; Girard *et al*., 1997) was kindly provided by Sanofi Recherche (Montpellier, France) and was dissolved in saline containing 1% ethanol. Pilot experiments revealed that SR140333 (3×10^{-6} M) completely blocked the contraction to 10^{-7} M substance P in the isolated tracheal rings (data not shown). R-methanandamide (Tocris Cookson Ltd., Langford, U.K.) was used to activate the CB_1 receptor and was dissolved in saline containing 1% ethanol.

Animals

Male Hartley guinea-pigs (400–600 g; Harlan CPB, Zeist, The Netherlands) were used in all experiments. The Animal Care Committee of the Utrecht University approved the animal studies.

In vitro *organ bath studies*

To isolate the tracheal rings (three cartilage segments per ring), the animals were killed by an overdose of pentobarbital (Apharmo, Duiven, The Netherlands) intraperitoneally. The tracheal rings were placed in an isometric organ bath set-up, containing warmed (37°C) Krebs solution (pH 7.4), containing (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, KH_2PO_4 1.2, $MgSO_4$ 1.2 and glucose 8.3, which was gassed with 95% O_2/5% CO_2. The experiment started with four washout periods lasting 15 min each. During these washouts a tension was applied of 2000, 2000, 4000 and 2000 mg, respectively. A histamine concentration response curve (10^{-8}–10^{-3} M) was subsequently conducted to measure contractility of the tracheal rings.

NGF was applied at concentrations of 0.2, 2 and 20 ng ml^{-1} 30 min before the start of the histamine concentration response curve. SR140333 (3×10^{-6} M) and R-methanandamide (1×10^{-7} M, Abadji *et al*., 1994) were applied 10 min before addition of NGF to the organ bath.

In vivo *lung function measurement*

Guinea-pigs were anaesthetized with urethane (2 g kg^{-1} body weight intraperitoneally). Lung function was measured in spontaneously breathing guinea-pigs, essentially as described by Amdur & Mead (1958). Airflow and tidal volume were determined by cannulating and connecting the trachea *via* a Fleisch flow head (Meijnhart, The Netherlands) to a pneumotachograph. A pressure transducer (MP45-2; Validyne Engineering Corp., Northridge, CA, U.S.A.) measured the transpulmonary pressure by the difference between the tracheal cannula and an oesophageal cannula. In this way pulmonary resistance (R_L) was determined breath by breath by dividing transpulmonary pressure by airflow at isovolumetric points. R_L values were averaged per three breaths and are presented as actual value. A small polyethylene catheter (PE-50) was placed in the right jugular vein for intravenous administration of R-methanandamide, NGF and histamine.

One dose of NGF was injected 30 min before the start of the lung function measurement, preceded by the administration of R-methanandamide (0.5 μg kg^{-1} body weight) or its vehicle 10 min earlier. The airway resistance was measured in the anaesthetized guinea-pig upon intravenous administration of increasing doses of histamine. The dose response curve lasted 30–40 min.

Statistics

Means and standard error of the mean (s.e.mean) were calculated. For comparison of concentration or dose response curves a two-way ANOVA was performed. Probability values of $P<0.05$ were considered significantly different.

Results

NGF applied to the organ bath 30 min before the start of a concentration response curve induced a tracheal hyperresponsiveness to histamine (Figure 1). The increase in reactivity was dependent on the concentration of NGF. A concentration of 2 and 20 ng ml^{-1} did enhance contraction to histamine ($P<0.0001$), whereas 0.2 ng ml^{-1} did not significantly enhance the contractility of the tracheal rings. At the highest concentration of histamine (10^{-3} M), 2 ng ml^{-1} NGF increased the contraction from 23.45 ± 1.92 to 32.89 ± 5.32 mN and 20 ng ml^{-1} NGF resulted in an even higher contraction of 47.15 ± 9.12 mN.

A preincubation of 10 min with the NK_1 receptor antagonist SR140333 could inhibit this NGF-induced tracheal hyperresponsiveness (Figure 2). Preincubation of tracheal rings with SR140333 before addition of NGF reduced the maximal contraction from 42.62 ± 3.67 to 35.17 ± 3.68 mN ($P<0.0001$). The CB_1 receptor agonist R-methanandamide was capable of blocking the NGF-induced tracheal hyperresponsiveness as well (Figure 3). A 10 min preincubation with R-methanandamide reduced the maximal contraction after preincubation with NGF from 29.99 ± 2.98 to 22.18 ± 1.60 mN ($P<0.0001$). SR140333 or R-methanandamide themselves did not affect contractility of the control rings significantly (Figures 2 and 3).

Intravenous administration of NGF to guinea-pigs induces an airway hyperresponsiveness to histamine *in vivo* (Figure 4).

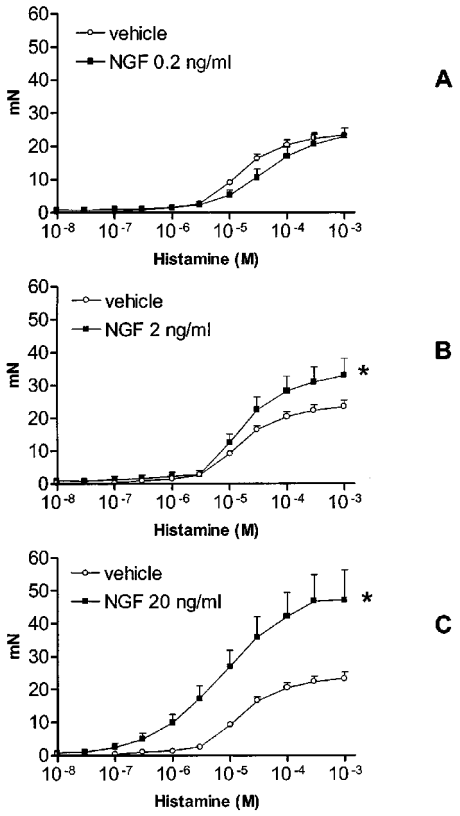

Figure 1 NGF concentration-dependently induces a hyperresponsiveness to histamine in guinea-pig tracheal rings *in vitro*. Application of 0.2 ng ml^{-1} NGF ($n=9$) 30 min before the start of a concentration response curve did not enhance contractility (A), whereas 2 ng ml^{-1} NGF ($n=6$, B) and 20 ng ml^{-1} ($n=5$, C) did induce tracheal hyperresponsiveness ($n=16$ for all vehicles). For B and C $*P<0.0001$ NGF vs vehicle.

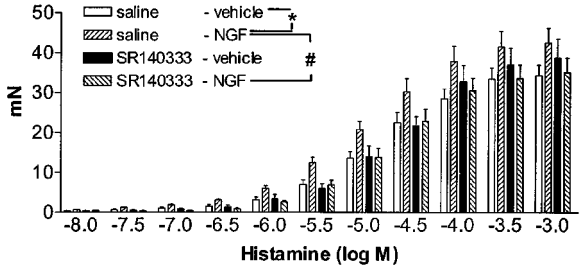

Figure 2 Addition of SR140333 (3×10^{-6} M) 40 min before start of a histamine concentration response curve in the organ bath prevents the hyperresponsiveness of the guinea-pig tracheal rings induced by NGF (2 ng ml^{-1}) added 30 min before start of the concentration response curve ($n=12$). $*P<0.0001$ saline-NGF vs saline-vehicle; $\#P<0.0001$ SR140333-NGF vs saline-NGF.

This airway hyperresponsiveness, observed after administration of 80 ng kg^{-1} body weight NGF, was completely prevented by pretreatment with R-methanandamide. Pretreatment with R-methanandamide reduced NGF-induced airway resistance at the highest dose of histamine from 4.9 ± 0.6 to 2.3 ± 0.3 cm H_2O ml^{-1} s ($P<0.0001$). Neither NGF nor R-methanandamide induced a change in basal airway resistance by themselves (data not shown).

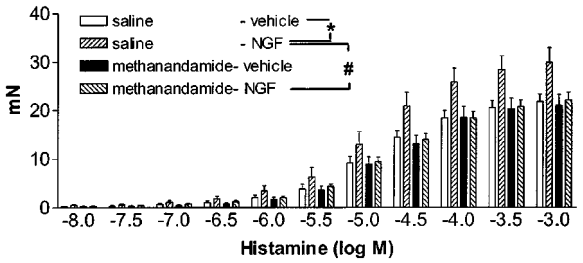

Figure 3 Addition of R-methanandamide (1×10^{-7} M) 40 min before start of a histamine concentration response curve in the organ bath prevents the hyperresponsiveness of the guinea-pig tracheal rings induced by NGF (2 ng ml^{-1}) added 30 min before start of the concentration response curve ($n=9-16$). $*P<0.0001$ saline-NGF vs saline-vehicle; $\#P<0.0001$ methanandamide-NGF vs saline-NGF.

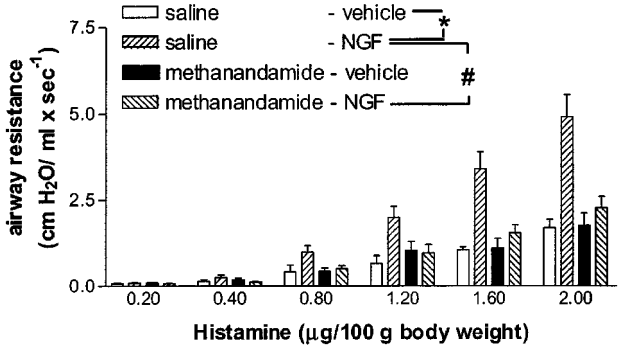

Figure 4 R-methanandamide (0.5 μg kg^{-1} body weight) when given 10 min prior to 80 ng NGF kg^{-1} body weight, completely prevents the NGF-induced airway hyperresponsiveness in guinea-pigs ($n=5-6$). $*P<0.0001$ saline-NGF vs saline-vehicle; $\#P<0.0001$ methanandamide-NGF vs saline-NGF.

Discussion

NGF induced an airway hyperresponsiveness for the contractile agonist histamine, as measured in isolated tracheal rings in an organ bath set-up (Figure 1). Interestingly, the NK_1 receptor antagonist SR140333 was able to block NGF-induced tracheal hyperresponsiveness (Figure 2). This points to a role for the preferred ligand of the NK_1 receptor, the tachykinin substance P. Substance P is predominantly released from sensory nerve endings (Lundberg, 1995). In the tracheal ring preparation sensory nerve endings are present but lack contact with their cell bodies. Our results imply that contact of the sensory nerve endings with their cell bodies is not necessary for the development of tracheal hyperresponsiveness induced by NGF.

We showed before that NGF induced an airway hyperresponsiveness 30 min after administration *in vivo*, that could be inhibited by the NK_1 receptor antagonist (de Vries *et al*., 1999). NGF is known to bind to its high affinity tyrosine kinase receptor A (trkA), after which the NGF-trkA complex is internalized and retrogradely transported to the nucleus, leading to enhanced levels of the preprotachykinin mRNA (precursor for substance P and neurokinin A) and tachykinin proteins (Lindsay & Harmar, 1989). The p75 low affinity NGF receptor is also shown to be responsible for an

upregulation in tachykinin content in sensory nerves (Lee *et al*., 1992). Hoyle *et al*. (1998) showed that transgenic mice over-expressing NGF in the airways develop a hyperinnervation of the airways and an increase in substance P. Other studies reported that long-term NGF exposure induces upregulation of substance P in sensory nerves, though this was not studied in the airways (e.g. Lindsay & Harmar, 1989). Furthermore, Hunter *et al*. (2000) showed that 24 h after intra-tracheal administration of NGF an increase in substance P content in neuronal cell bodies could be detected. It is generally agreed that protein synthesis occurs in the neuronal cell body, followed by axonal transport to the nerve endings (although studies have reported on the role of RNA in axons, van Minnen, 1994). Therefore, in the tracheal preparation in our study, an upregulation of substance P content can be excluded as the nerve endings lacked any contact with their cell bodies. Moreover, administration of NGF can induce airway hyperresponsiveness *in vivo* within 1 h, a time period we think is too short for protein synthesis, axonal transport and subsequent release of substance P (de Vries *et al*., 1999). Shortly after NGF application, presumably other mechanisms than substance P upregulation are responsible for the changes induced by NGF. This could involve a change in sensitivity of the nerve endings of the NK_1 receptor.

Alternatively, cells like monocytes, macrophages, leukocytes and T-cells are also able to synthesize substance P (de Giorgio *et al*., 1998; Ho *et al*., 1997) and might be sensitized by NGF to release it. However, a role for the sensory nerve ending in the NGF-induced effects is strengthened by our results with R-methanandamide. The CB_1 receptor agonist R-methanandamide (Abadji *et al*., 1994) prevented NGF-induced tracheal hyperresponsiveness (Figure 3). We postulate that the CB_1 receptor stimulation inhibited the release of tachykinins from the nerve endings, thereby preventing the induction of tracheal hyperresponsiveness by NGF. CB_1 receptors are almost exclusively present on nerve endings, and stimulation of these receptors inhibits neuropeptide release (Hohmann & Herkenham, 1999). Recently, Calignano *et al*. (2000) showed for the first time that CB_1 receptors are indeed present on axon terminals of airway nerves: CB_1 receptors are expressed on noradrenaline as well as on non noradrenaline expressing nerves. Capsaicin induces release of tachykinins, among which are the contractile peptides substance P and neurokinin A (Mitchell *et al*., 1995). Interestingly, a CB_1 agonist was capable of inhibiting the bronchospasm induced by capsaicin (Calignano *et al*., 2000). Furthermore, CB_1 receptor stimulation inhibited the release of bronchodilating neuropeptides when the basal constricting tone was absent. This confirms that the inhibition of neuropeptide release by CB_1 receptor stimulation is also a fact in the airways: when a basal constricting tone is present, CB_1 receptor stimulation inhibits tachykinin release. The authors suggest a bi-directional control of airway hyperresponsiveness by the endogenously released cannabinoid anandamide (Calignano *et al*., 2000). Another study on CB_1 receptors in the airways shows that alveolar Type II cells are also expressing the CB_1 receptor (Rice *et al*., 1997). However, these cells do not produce any tachykinins and therefore a role for these alveolar cells in the induction of airway hyperresponsiveness is not very likely. R-methanandamide also completely prevented the induction of airway hyperresponsiveness by NGF *in vivo* (Figure 4). The action of R-methanandamide *in vivo* could theoretically also be of a central origin, affecting responses in the brain stem or central nervous system. However, we used a very low dose ($0.5\ \mu g\ kg^{-1}$) that is unlikely to affect central CB receptors. In addition, a study by Richardson *et al*. (1998) demonstrated that not the central CB receptors but rather the peripheral CB_1 receptors mediate inhibition of inflammation induced hyperalgesia. This study on hyperalgesia suggests, like our study, that the effect of CB_1 receptor is mediated *via* the inhibition of neurosecretion from sensory nerve endings.

NGF is known to change the properties of sensory nerve endings, without affecting protein synthesis, in various ways. NGF can induce a fast accumulation of second messengers (Knipper *et al*., 1993), protein kinase C translocation to the membrane (Dupont *et al*., 2000) or phosphorylation of key transduction-related proteins or ion channels (Knipper *et al*., 1993; Woolf, 1996). NGF increases the response of the vanilloid receptor-1 to capsaicin within 10 min, at the same concentrations ($2-100\ ng\ ml^{-1}$ NGF) as used in our study (Shu & Mendell, 1999). The vanilloid receptor-1 is the receptor for the perception of heat, protons and for the agonist capsaicin. These stimuli are able to induce release of tachykinins from the sensory nerve endings (Shu & Mendell, 1999). As there is presumably no endogenous ligand present in our organ bath for the vanilloid receptor, activation of this receptor in our experiments is not very likely. Taken together, this suggests that more down-stream mechanisms in receptor signalling must be affected. In the same view, the use of the CB_1 receptor agonist, which inhibits excitatory processes at the nerve terminal (Hohmann & Herkenham, 1999) did block NGF-induced hyperresponsiveness (Figures 3 and 4). The NGF-induced neuronal sensitization could be direct, as described above, or indirect, *via* the release of sensitizing mediators from trkA-expressing inflammatory cells, e.g. mast cells and monocytes (Matsuda *et al*., 1988; Woolf, 1996).

NGF by itself did not change basal tone in the tracheal rings. This implies that NGF did not induce the release of mediators having a direct contractile effect. Instead, the release of substance P might only have been evident upon histamine application. Alternatively, a change in NK_1 receptor functioning can underlie the alterations in contractility. In favour of a change in function of sensory nerve endings, and thereby an increase in substance P release, are the findings with the CB_1 receptor agonist. Furthermore, a recent study by Zhou *et al*. (2000) showed an increase in substance P release in tracheal explants in response to NGF, thereby confirming the role of the nerve ending as opposed to an upregulation of peptide synthesis in the neuronal cell body.

In summary, we hypothesize a general threshold lowering of the sensory nerve endings due to short-term exposure to NGF, which can thereby induce the release of substance P and subsequent airway hyperresponsiveness to histamine. This is corroborated by studies in which exogenously applied substance P also resulted in an increase of airway reactivity to histamine (Boichot *et al*., 1993; Kraneveld *et al*., 1997). Interestingly, exogenous substance P not only affects airway reactivity to histamine, but also to cholinergic stimuli

(Boichot *et al*., 1993; Daoui *et al*., 2000). This suggests that NGF, through the action of substance P, may be involved in non-specific airway hyperresponsiveness as seen in asthmatics (van ˜Schoor *et al*., 2000). Indeed, in the mouse it was recently shown that NGF increases tracheal responsiveness to electrical field stimulation (Braun *et al*., 2001).

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