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Effects of terbutaline on NGF formation in allergic inflammation of the rat

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1 The aim of this study was to determine the effects of the beta adrenergic agonist terbutaline on NGF increase caused by allergic inflammation in rats.

2 Intraplantar antigen injection in sensitized rats increased paw volume and stimulated NGF biosynthesis in the skin of the injected paw as determined 3 and 6 h after injection. Treatment of rats with terbutaline (0.1–0.3 mg kg^{-1}, s.c.) had no significant effect on the NGF concentration in non-inflamed skin, but reduced oedema, and at 0.3 mg kg^{-1} also NGF mRNA and immunoreactive NGF in the skin of the inflamed paw in a propranolol-reversible manner. In carrageenan-induced inflammation, terbutaline did not significantly reduce the inflammation-induced increase of NGF in paw skin.

3 Exposure of sensitized rats to aerosolized antigen (twice, 24 h interval) increased protein content, eosinophil leukocytes, and immunoreactive NGF in the bronchoalveolar lavage fluid (BAL, obtained 16 h after the second antigen exposure). Treatment of rats with terbutaline (0.3 mg kg^{-1}, s.c. 30 min before the second antigen challenge) suppressed antigen-induced elevation of protein and eosinophil leukocytes, and reduced the concentration of NGF in BAL to values similar to those found in non-sensitized rats.

4 The present results demonstrate anti-allergic properties of terbutaline in rats that were accompanied by a marked reduction of antigen-induced NGF increase in skin and BAL, respectively. These results are compatible with the assumption that terbutaline primarily suppressed the immune response to antigen thereby attenuating the release of vasoactive mediators and the stimulation of NGF biosynthesis.

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Keywords: Nerve growth factor; allergic inflammation; terbutaline

Abbreviations: BAL, bronchoalveolar lavage; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde-3 phosphate dehydrogenase; NGF, nerve growth factor; PBS, phosphate buffered saline; RT–PCR, reverse transcription polymerase chain reaction

Introduction

Nerve growth factor (NGF) belongs to a family of structurally related neurotrophins that bind with high affinity to tyrosine kinase-coupled receptors (trk) and, with low affinity, to an additional neurotrophin receptor (p75). In the adult, it has been shown that the concentration of NGF is elevated at sites of inflammation, including allergic inflammation (see Discussion). Sources of NGF include keratinocytes (Tron *et al*., 1990), fibroblasts (Young *et al*., 1975), smooth muscle cells (Ueyama *et al*., 1991), mast cells (Leon *et al*., 1994), eosinophils (Solomon *et al*., 1998), T cells (Lambiase *et al*., 1997), B cells (Torcia *et al*., 1996), macrophages and Langerhans cells (Torii *et al*., 1997). The local increase of NGF seems to be a central factor for the development of increased sensitivity of the afferent nervous system, manifesting itself as hyperalgesia or hyperreflexia in the skin or viscera, respectively (Dmitrieva & McMahon, 1996; Dmitrieva *et al*., 1997; Shu & Mendell, 1999).

Relatively little is known about the effect of anti-inflammatory drugs on the NGF response. It has been shown that steroids and treatment of rats with high doses of indomethacin suppresses NGF increase in adjuvant-induced paw inflammation (Safieh-Garabedian *et al*., 1995). Recently, we have further investigated the effect of indomethacin and sodium salicylate on inflammatory oedema and NGF levels in the carrageenan-inflamed rat paw, and observed no significant reduction of the NGF content at doses sufficient to prevent inflammatory oedema (Amann & Schuligoi, 2000). This indicates that pharmacological suppression of inflammatory symptoms is not necessarily accompanied by inhibition of NGF formation in inflamed tissues, and thus may leave unopposed neuronal and non-neuronal consequences of increased NGF.

Given the putative role of NGF in allergic inflammation, the question of pharmacological interference with the inflammation-induced NGF increase seems of interest, in particular the question to which extent drugs used in the treatment of asthmatic disease can inhibit the NGF increase in allergic inflammation.

In the present study, we have investigated the effects of terbutaline, a beta-adrenoceptor agonist with reported anti-allergic properties (Chong *et al*., 1995; O'Connor *et al*., 1994; Svensson *et al*., 1995) on the NGF increase caused by allergic inflammation. Initial experiments designed to characterize the effect of terbutaline on oedema and NGF increase were

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conducted using a model of allergic inflammation of the rat hind paw. A second series of experiments aimed at determining whether or not terbutaline can attenuate the increase of NGF in bronchoalveolar lavage fluid (BAL) of rats exposed to aerosolized antigen.

Methods

Animals

Male Sprague-Dawley rats (200–300 g body wt.; Himberg, Austria) were used, following guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain (Zimmerman, 1983) making all efforts to minimize both the suffering and the number of animals used.

Sensitization of rats

Rats received one injection (1 ml, i.p.) of 0.1 mg ovalbumin (Grade II, Sigma, Vienna, Austria) dissolved in 0.1% aluminium hydroxide (Sigma), or 0.1% aluminium hydroxide alone (vehicle group), 2 weeks prior to the experiments (Church & Miller, 1978). In order to ascertain successful sensitization, initial experiments were conducted to determine *in vitro* antigen-induced histamine release in preparations of peritoneal cells as described previously (Schuligoi, 1998): Anaesthetized rats were exsanguinated, and peritoneal lavage was performed with Tris/HCl 25 mM, pH 7.8, containing NaCl 120 mM, KCl 5 mM, gelatine 0.1% and heparin 10 U ml^{-1}. After centrifugation (10 min, 300 g), the pellet was washed twice with Tris/HCl 25 mM, pH 7.8 (containing (mM): NaCl 120, KCl 5, gelatine 0.1%), resuspended in Tris/HCl 25 mM, pH 7.6 (containing (mM): NaCl 120, KCl 5, gelatine 0.1%). The cells were incubated for 15 min at 37°C, thereafter L-α-phosphatidyl-L-serine (10 $\mu g\ ml^{-1}$, final concentration, Sigma) together with ovalbumin (50 ng ml^{-1}) was added to the aliquots. After another 30 min incubation, samples were centrifuged (10 min, $300 \times g$) and the supernatant used for determination of histamine. To obtain the total amount of histamine, the pellet was resuspended in buffer, boiled for 5 min and centrifuged. In the supernatants histamine was determined using high pressure liquid chromatography as described previously (Skofitsch *et al*., 1981). Histamine release was expressed as a percentage of the total histamine content.

Experimental protocol

Hindpaw inflammation For intraplantar injections, rats were placed in a plastic box with gas inlet and outlet ports, and exposed to N_2O (80%, 20% O_2) for 5 min. Thereafter one paw was injected with ovalbumin (5 μg in 100 μl 0.9% NaCl) or carrageenan (Serva, Heidelberg, Germany; 1 mg in 100 μl 0.9% NaCl). This protocol prevented the appearance of behavioural signs of pain during and after the injections were made. Intraplantar subcutaneous injections were made with a Hamilton syringe and a 30-gauge hypodermic needle in volumes of 100 μl. The needle was inserted into the pad region of the glabrous skin and moved 6–8 mm proximal towards the tarsal region.

Thirty minutes before intraplantar injection of ovalbumin or carrageenan, rats were pretreated either with vehicle (0.9% NaCl, 0.1 ml kg^{-1}), or with terbutaline (0.1–0.3 mg kg^{-1}, s.c.). Additional groups were treated with propanolol (0.01–1.0 mg kg^{-1}, s.c.) or vehicle, 20 min before receiving s.c. injections of 0.3 mg kg^{-1} terbutaline. Oedema formation was determined before, and in 1 h intervals after induction of inflammation for up to 6 h. For determination of NGF immunoreactivity, paw skin samples were taken 3 or 6 h after intraplantar injections. Paw skin samples obtained 3 h after induction of inflammation were used to extract RNA.

Antigen challenge by aerosolized ovalbumin Rats were placed in a plastic box and exposed for 60 min to aerosolized ovalbumin (0.1% in 0.9% NaCl) using a pressure nebulizer (Pari-boy type 038, Pari GesmbH, Starnberg, Germany). This procedure was repeated after a 24 h interval according to Braun *et al*. (1998). Thirty minutes before the second antigen challenge rats were pretreated either with vehicle (0.1 ml kg^{-1}), or with terbutaline (0.1–0.3 mg kg^{-1}, s.c.). Bronchoalveolar lavage (BAL) was obtained 16 h after the second antigen challenge.

Determination of oedema formation

Paw volume (up to the tarsal joint) was measured using a plethysmometer (Ugo Basile, Italy). The volume difference (ml) between treated and contralateral paw was used as estimate of oedema formation.

Determination of nerve growth factor

NGF immunoreactivity was determined as described previously (Amann & Schuligoi, 2000): samples of paw tissue were homogenized in phosphate buffer (100 mM, pH 7.4) containing 0.4 M NaCl, 0.5% bovine serum albumin, 0.1 mM benzethonium chloride, 0.1 mM phenylmethylsulphonyl fluoride, 10 mM ethylenedinitrilo tetra acetic acid (EDTA), 10 U ml^{-1} aprotinin, and 0.1% Triton X-100 (all Sigma). After centrifugation ($50{,}000 \times g$), supernatants were further processed according to the manufacturer's (NGF E_{max} ImmunoAssay System™, Promega) protocol. The dry weight of samples was determined after drying pellets in a vacuum evaporator. Supernatants of BAL were diluted in 2× concentrated homogenization buffer (1 : 2) and processed as described.

Reverse transcription polymerase chain reaction (RT–PCR)

RT–PCR was performed as described previously (Amann *et al*., 2000): total RNA from rat paw tissue was extracted using Trizol (Life Technologies, Lofer, Austria). After treatment with RNase-free DNase I (Roche) to remove contaminating DNA, RNA was purified using a Nucleo-Spin Kit (Macherey and Nagel, Düren, Germany). Reverse transcription of 0.8 μg RNA was performed with avian myoblatosis virus (AMV) reverse transcriptase, and an oligo $(dT)_{15}$ primer (Promega, Mannheim, Germany). For NGF and glyceraldehyde-3 phosphate dehydrogenase (GAPDH), specific primers were used to amplify the reversed-tran-

scribed products. PCR was carried out using $MgCl_2$ 2.5 mM, deoxynucleotide triphosphates (Sigma) 0.2 mM, primers 10 pmol and Taq polymerase 0.25 U (Promega). Samples were denatured for 2 min at 95°C and specific cDNA amplified using a cycling program: 95°C for 1 min, 59°C for 1 min, and 72°C for 2 min (Stratagene RoboCycler). Final extension time was 4 min at 72°C. Thirty-three cycles were performed for NGF and 26 for GAPDH amplification. The PCR-products were separated by electrophoresis in agarose gel stained with ethidiumbromide. Specific primers for NGF (Biognostik, Göttingen, Germany), and GAPDH (Clontech, Heidelberg, Germany) yield amplification products of 267 and 450 bp, respectively. GAPDH was used as an internal reference to verify similar levels of RNA. The ethidium-bromide (Sigma) stained bands were visualized under u.v. light with a Gel Doc 2000 system (Bio-Rad, Vienna, Austria) and the optical densities were analysed with Quantity One software (Bio-Rad). The ratios of NGF/GAPDH were calculated.

Bronchoalveolar lavage (BAL)

Animals received an overdose of pentobarbitone 16 h after the second antigen challenge. Immediately thereafter, the airways were lavaged three times with 2 ml ice-cold phosphate buffered saline (PBS). The lavage fluid was collected, a sample taken for differential cell counts on a cytospin prepared slide stained with Wright Giemsa (Diff-Quick, Dade, Düdingen, Switzerland). BAL fluid was then centrifuged at $300 \times g$ for 10 min and the supernatant used for determination of protein content (Bio-Rad Protein Assay, Vienna, Austria) and immunoreactive NGF. Cells were re-suspended in 1 ml PBS and the cell number was counted using Cobas Minos Vet (Roche, Mannheim, Germany).

Drugs

Terbutaline sulphate and (−)-propranolol hydrochloride, were obtained from Sigma and dissolved in 0.9% NaCl. All doses of drugs refer to the base.

Statistical analysis

Values were calculated as means ± s.e.mean. Unless stated otherwise, statistical analysis was performed using One Way ANOVA, or Kruskal Wallis One Way Analysis of Variance, when appropriate, using Dunnett's or Dunn's post-test respectively (SigmaStat statistical software, Jandel Scientific, Erkrath, Germany). $P<0.05$ was considered statistically significant.

Results

Preliminary experiments were performed in order to ascertain successful sensitization. *In vitro* addition of ovalbumin (50 ng ml^{-1}) to peritoneal cells obtained from sensitized rats induced a significant release of histamine (40.83 ± 7.91% of the total content; $n=7$), as compared to peritoneal cells obtained from vehicle treated rats (5.0 ± 0.85% of total content; $n=7$).

Allergic inflammation of the rat paw stimulates NGF biosynthesis

Initial experiments were conducted to determine a possible influence of sensitization itself on skin NGF levels in non-injured skin: In sensitized rats, the concentration of immunoreactive NGF was 80.7 ± 5.4 ng g^{-1} dry weight ($n=12$), which was not significantly different from the value determined in non-sensitized rats (80.3 ± 4.5 ng g^{-1} dry weight; $n=12$).

In sensitized rats, intraplantar injection of ovalbumin caused oedema (Figure 1), pronounced increase of NGF mRNA expression (Figure 2a), and a more than 2 fold increase of immunoreactive NGF in the paw skin as compared to the contralateral non-injected paw (Figure 3). The concentration of NGF in the skin of the contralateral paw 3 and 6 h post injection was not significantly different from the value obtained in sensitized rats, which had received no intraplantar injection ($n=5$ each, results not shown).

In non-sensitized rats, intraplantar injection of ovalbumin caused no detectable oedema (data not shown), but seemed to produce a moderate increase in the tissue concentration of NGF, which was, however, statistically non-significant at $n=12$ (Figure 3).

Terbutaline inhibits antigen-induced oedema NGF increase

Treatment of rats with terbutaline (0.1 and 0.3 mg kg^{-1}, s.c., 30 min before antigen injection) reduced oedema formation (Figure 1) and, at 0.3 mg kg^{-1}, also attenuated the antigen-induced increase of NGF mRNA expression (Figure 2b). Terbutaline treatment also reduced the concentration of NGF in inflamed paw skin (Figure 3), while it had no significant effect on the concentration of NGF in the contralateral, non-inflamed paw skin (Figure 3).

Additional experiments were conducted using intraplantar carrageenan as an inflammatory stimulus. Three hours after carrageenan injection the volume of the injected paw was

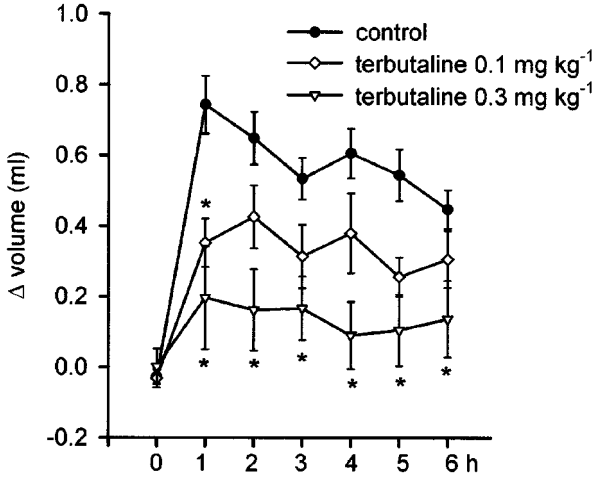

Figure 1 Difference between the volume (ml) of antigen-injected and contralateral, non-injected hind paw of sensitized rats. Measurements were taken before and after intraplantar injection in groups treated with terbutaline (0.1 mg kg^{-1}, 0.3 mg kg^{-1}; each $n=6$) or vehicle (control; $n=12$). Values are mean ± s.e.mean. *$P<0.05$ as compared to the corresponding value in the control group.

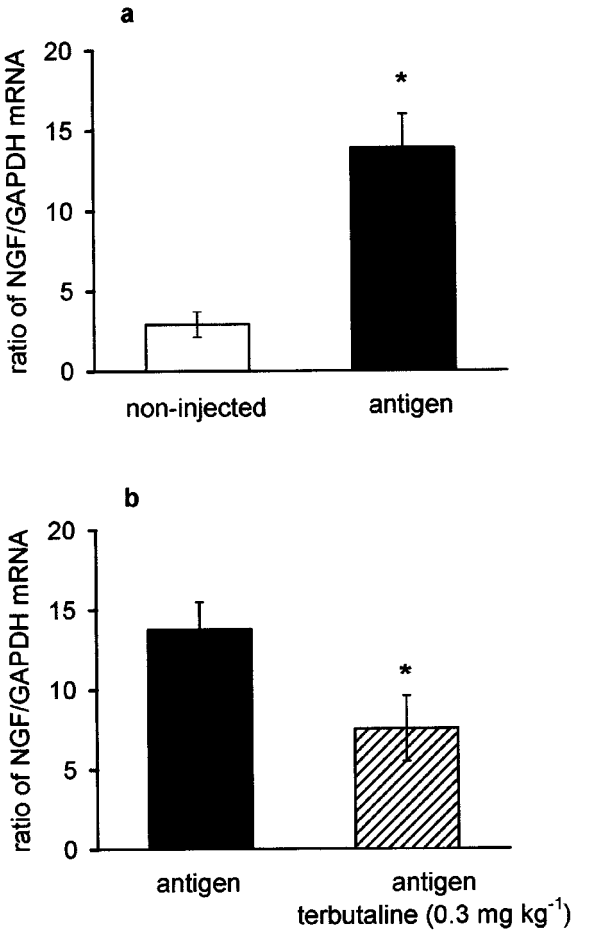

Figure 2 Expression of NGF mRNA in the raw paw skin of sensitized rats, as determined by RT–PCR. NGF mRNA levels were normalized to GAPDH to correct for variations in RNA concentration. (a) Ratio of the relative signal intensities NGF/GAPDH in the paw skin 3 h after intraplantar injection of antigen and in the skin of the non-injected contralateral paw. Values are mean ± s.e.mean; $n=6$; for statistical comparison t-test was used, $^*P<0.05$. (b) Ratio of the relative signal intensities NGF/GAPDH in paw skin 3 h after intraplantar injection of antigen. Rats received either vehicle or terbutaline (0.3 mg kg^{-1}) 30 min prior to intraplantar injection. Values are mean ± s.e.mean; $n=6$ each; for statistical comparison t-test was used, $^*P<0.05$.

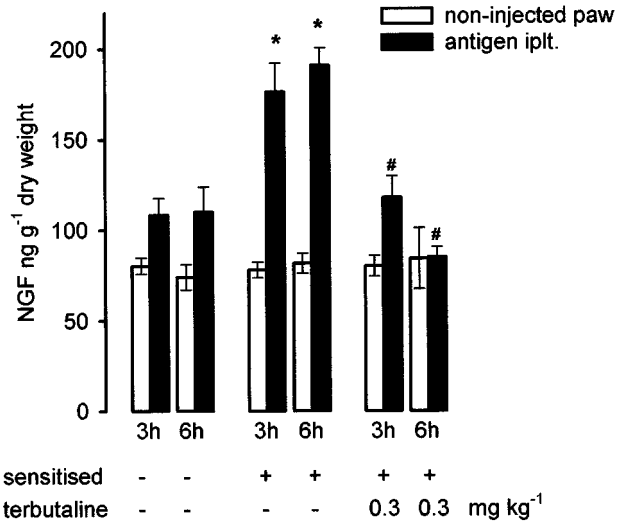

Figure 3 Concentration of NGF in paw skin 3 and 6 h after intraplantar injections of antigen in non-sensitized rats ($n=12$ each time point), and in sensitized rats that had received vehicle ($n=19$ each time point) or terbutaline 0.3 mg kg^{-1} (3 h: $n=12$; 6 h; $n=6$) 30 min before intraplantar antigen injection. Values are mean ± s.e.mean. $^*P<0.05$ as compared to corresponding values obtained in non-sensitized rats; $\#P<0.05$ as compared to corresponding values obtained in sensitized rats.

increased by 0.40 ± 0.03 ml ($n=18$); the concentration of NGF was 71.7 ± 6.6 ng g^{-1} dry weight ($n=12$) in the non-injected, and 161.2 ± 7.8 ng g^{-1} dry weight ($n=12$) in the injected paw skin. In rats that had received terbutaline (0.3 mg kg^{-1} s.c., 30 min before carrageenan) the carrageenan-induced increase of paw volume was 0.31 ± 0.03 ml ($n=18$); the concentration of NGF was 80.9 ± 13.6 ng g^{-1} dry weight ($n=12$) in the non-injected, and 157.3 ± 22.9 ng g^{-1} dry weight ($n=12$) in the injected paw skin, thus showing no significant effect ($P>0.05$) of terbutaline on oedema or NGF increase.

Terbutaline-induced inhibition of antigen-induced oedema and NGF increase is prevented by propranolol

Treatment of rats with (−)-propranolol ($\geqslant$0.1 mg kg^{-1}) 50 min before antigen injection reversed the inhibition of the antigen-induced NGF increase by terbutaline (0.3 mg kg^{-1}) (Figure 4a). At 1.0 mg kg^{-1}, (−)-propanolol also counteracted the terbutaline (0.3 mg kg^{-1})-induced inhibition of oedema formation (Figure 4b).

Antigen-induced NGF increase in the respiratory tract is attenuated by terbutaline

Preliminary experiments showed that under the present experimental conditions, reliable inflammatory responses in BAL were obtained 16 h after the second of two exposures (24 h interval) to aerosolized antigen. Using this protocol, antigen challenge increased in BAL protein content (Figure 5), the total number of cells (control: $2.2\times10^6\pm0.3\times10^6$ ml^{-1}, $n=10$; sensitized rats: $3.6\times10^6\pm0.4\times10^6$ ml^{-1}, $n=14$), eosinophil leukocytes, and immunoreactive NGF (Figure 5).

Calculation of the ratio NGF/protein in BAL of controls (non-sensitized rats exposed to aerosolized ovalbumin) and sensitized rats, showed a statistically significant ($P<0.05$) increase of NGF/protein in the sensitized group (1.28 ± 0.20 pg μg^{-1}; $n=10$ in controls, *vs* 1.91 ± 0.10 pg μg^{-1}; $n=14$ in sensitized rats), suggesting that the increase of NGF was not primarily caused by increased microvascular leakage.

Treatment of rats with terbutaline (0.3 mg kg^{-1} 30 min before the second antigen exposure) attenuated the rise in protein content, eosinophils, and NGF concentration in BAL of antigen challenged rats (Figure 5), while it had no significant effect on total cell numbers ($3.2\times10^6\pm0.4\times10^6$ ml^{-1}, $n=6$).

Discussion

The present results show that in the hind paw of sensitized rats, intraplantar antigen injection caused oedema, stimulated

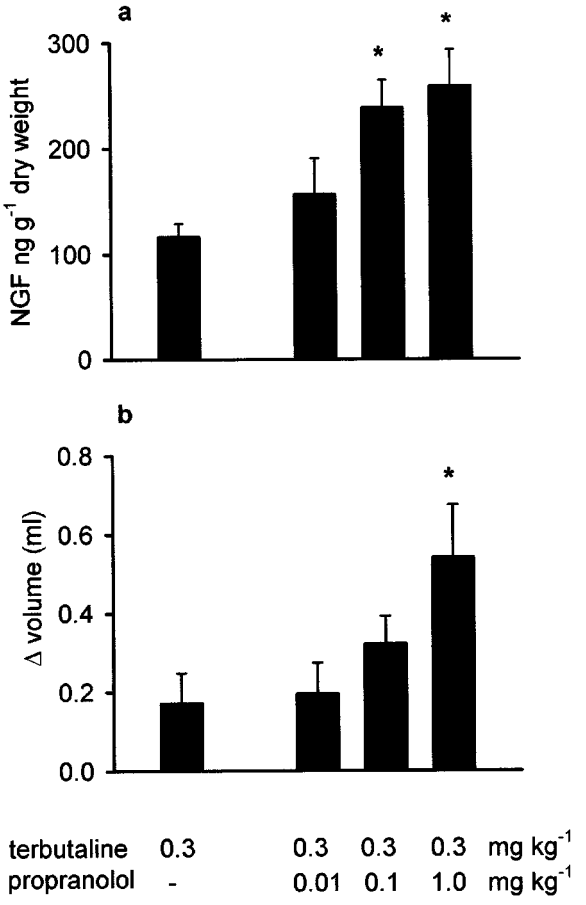

Figure 4 Effects of antigen injection in the hindpaw of sensitized rats on concentration of NGF (a) and paw volume (b). Measurements were taken 3 h after intraplantar injection in rats treated with terbutaline (0.3 mg kg^{-1}; $n=8$) alone or in combination with different doses of (−)-propranolol ($n=4-7$). Values are mean ± s.e.mean. *$P<0.05$ as compared to terbutaline treatment.

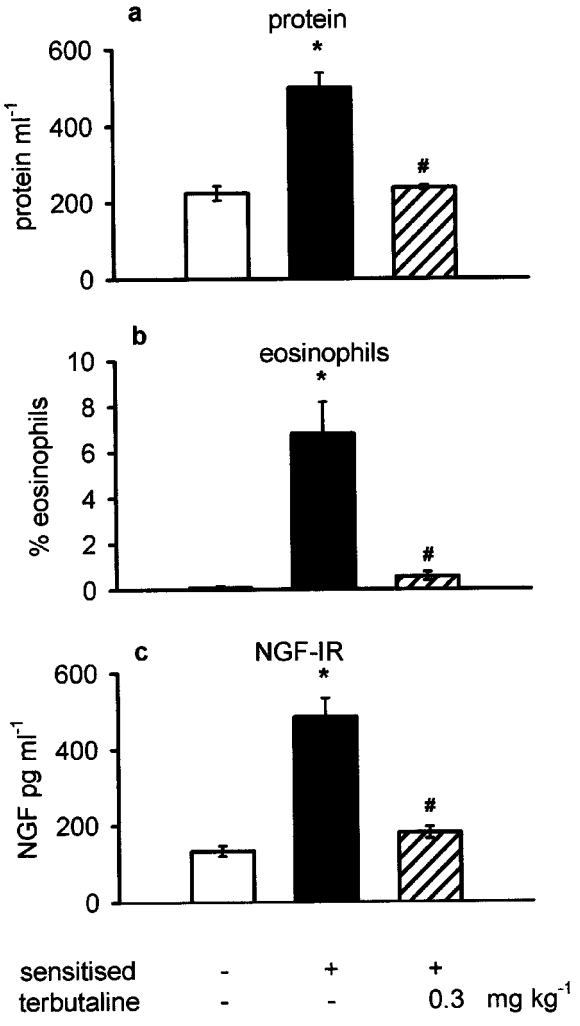

Figure 5 Concentration of protein (a), percentage of eosinophils (b), and concentration of NGF (c) in the BAL of rats exposed to aerosolised ovalbumin. Open columns: non-sensitized rats ($n=10$), rats sensitized to ovalbumin that had received treatment with vehicle (black columns; $n=14$), or terbutaline 0.3 mg kg^{-1} (hatched columns; $n=6$). Values are mean ± s.e.mean; $n=4-8$, *$P<0.05$ as compared to values obtained from non-sensitized rats; #$P<0.05$ as compared to vehicle treatment.

NGF mRNA expression, and increased the concentration of immunoreactive NGF in paw skin. Since the sensitization protocol itself had no significant effect on skin NGF levels, and intraplantar ovalbumin in non-sensitized rats caused only a moderate, statistically non-significant, increase of skin NGF, we assume that NGF biosynthesis in rat paw skin was stimulated by the immune response to antigen. This is in agreement with previous studies showing stimulation of NGF biosynthesis by allergic inflammation (Bonini *et al*., 1996; Braun *et al*., 1998; Sanico *et al*., 2000).

The present observation that terbutaline suppressed the antigen-induced oedema and NGF increase in a propranolol-reversible manner suggests that beta adrenergic receptor activation attenuated the local inflammatory reaction to antigen. We have obtained no evidence for an effect of terbutaline treatment on the concentration of NGF in the non-inflamed paw skin. This indicates that in our experimental model, beta adrenergic receptor activation did not interfere with NGF biosynthesis in general, but specifically suppressed NGF biosynthesis induced by antigen.

It is known that beta adrenergic agonists, depending on the experimental model, can inhibit mediator release from mast cells (Banner *et al*., 1996; Bissonnette & Befus, 1997; Chong *et al*., 1995; 1998) and reduce microvascular permeability (Allen & Coleman, 1995; Proud *et al*., 1998), effects that may contribute to observed anti-allergic properties of these compounds (Petersen & Skov, 1999; Svensson *et al*., 1995). However, we have observed no significant effect of terbutaline on NGF levels in carrageenan-inflamed paw skin suggesting that possible mast cell stabilizing properties of terbutaline were not sufficient on their own to produce marked anti-inflammatory effects. Therefore, it seems likely that the observed inhibition of allergic inflammation by terbutaline was mainly due to inhibition by terbutaline of the immune response itself, i.e. mast cell activation following antigen binding.

Pharmacological inhibition of NGF biosynthesis in inflamed tissue can be considered to have some bearing on the development of inflammation: NGF acts on CGRP-containing primary afferent C-fibres, where it causes peripheral sensitization, stimulates the expression of tachykinins and

CGRP, and facilitates spinal transmission of noxious input (McMahon, 1996). In the respiratory system, the neuronal effects of NGF may be analogous to hyperalgesia in the somatosensory system. Increased responsiveness of C-fibres, together with facilitation of afferent transmission in the brain stem may enhance bronchoconstrictor reflexes, contributing to bronchial hyperresponsiveness seen in asthmatic disease. Thus, it has been shown in guinea-pigs that allergen challenge as well as NGF application increases the neuropeptide content of respiratory afferents (Fischer *et al*., 1996; Hunter *et al*., 2000). Increased central release of SP and NKA, in turn, may lead to enhanced synaptic activity in the parasympathetic nervous system (Myers & Undem, 1993). In addition to these neuronal effects, NGF may also directly influence cells of the immune system expressing the trkA receptor (Aloe *et al*., 1999; Bischoff & Dahinden, 1992; Otten & Gadient, 1995).

Direct evidence for a central role of NGF in allergic airway inflammation, and its consequences on bronchial smooth muscle contractility, has been obtained by studies showing that overexpression of NGF in transgenic mice leads to bronchial smooth muscle hyperreactivity (Hoyle *et al*., 1998) and that in mice, *in vivo* immunoneutralization with anti-NGF antibody prevents the development of airway hyperreactivity after antigen exposure (Braun *et al*., 1998).

Therefore, it seems of interest that our results show that terbutaline attenuated the increase of NGF in BAL of rats challenged with aerosolized antigen. As to the probable modes of action, similar possibilities arise as addressed above: The assumption that terbutaline interfered with the immune response itself is compatible with the observation that terbutaline also inhibited the increase of protein concentration and eosinophil cell number in BAL. However, these results also provide reason to be cautious in extrapolating these findings to the situation in patients. Although there are studies showing a decrease of allergen-induced vascular permeability (Bolton *et al*., 1997; Proud *et al*., 1998) and eosinophil infiltration (Bowden *et al*., 1994; Dente *et al*., 1999), many studies find no major anti-inflammatory effects by beta adrenergic agonists (Gardiner *et al*., 1994; Roberts *et al*., 1999; Turner *et al*., 1998). It remains to be investigated, therefore, whether or not attenuation of antigen-induced NGF increase can also be observed in patients receiving beta adrenergic agonists.

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