

The production, storage and release of the neurotrophins nerve growth factor, brain-derived neurotrophic factor and neurotrophin-3 by human peripheral eosinophils in allergics and non-allergics

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Summary

Background Recent studies have shown that neurotrophins are produced by and can act on several immune-inflammatory cells. The origin of circulating as well as local neurotrophins is unknown.

Objectives The aim of this study was to assess whether eosinophils of allergic and non-allergic donors produce, store and release the neurotrophic factors NGF, BDNF and NT-3.

Methods Eosinophils were purified by negative immunoselection (purity > 96%) from allergic asthmatics and non-allergic donors (25 to 53 years). The presence of mRNA for neurotrophic factors was evaluated by reverse transcription PCR. Specificity was demonstrated by cloning products and sequencing. Stored NGF, BDNF and NT-3 was demonstrated by Western-blotting and flow cytometry. Eosinophils were incubated and supernatants were collected for measurement of neurotrophic factors after cell stimulation with PAF. Neurotrophin content in eosinophil lysates was determined by ELISA.

Results Eosinophils demonstrate mRNA for neurotrophins. Proteins were detectable by Western blot and FACS analysis. Neurotrophins were found in the eosinophil lysates at different amounts comparing allergic and non-allergic donors. Cell stimulation with PAF (10^{-8} – 10^{-5} M) after priming with GM-CSF leads to a dose-dependant release of NGF and BDNF.

Conclusions Eosinophils store, produce and release NGF, BDNF and NT-3. They are a possible source of elevated neurotrophin levels found in allergy and asthma.

Keywords allergy, asthma, BDNF, eosinophils, neurotrophins, NGF, NT-3

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Introduction

Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (NT-3) belong to a well-described group of mediators sharing high homology. These neurotrophins play an important role in the development, function and survival of neuronal cells [1] as well as for the production of neuropeptides [2]. Responsiveness of cells to neurotrophins is modulated by the activation of their specific high affinity receptors trkA, trkB, trkC and their low affinity unspecific receptor p75 [3].

Numerous recent studies have described that neurotrophins, especially NGF, are not only essential for neurones but also represent an important inflammatory mediator. Neurotrophin receptors have been found on various immune cells like lymphocytes and mast cells [4]. The differentiation of mast cells, T and B cells and basophiles is influenced [5–8]. Besides neuronal cells and astrocytes, also immune cells like T and

B cells, mast cells and basophiles [5] but also fibroblasts as structural cells are known to produce NGF [9, 10].

The role of neurotrophins in various immune diseases is discussed [11, 12]. Elevated plasma levels were found in patients suffering from asthma, with the highest levels in allergic asthmatics [13, 14]. After segmental allergen provocation, an increase of the neurotrophins NGF and BDNF in the bronchioalveolar lavage fluid has been described in the late phase reaction (LAR) [15]. The LAR is characterized by the influx of eosinophils and activated T cells into the tissue. Eosinophils are main effector cells of allergic inflammation. In a previous study we were able to confirm that untreated allergic asthmatics demonstrated increased levels of the neurotrophins NGF, BDNF and NT-3. Circulating levels decreased after treatment with inhalative corticosteroids [16]. In animal experiments, NGF was able to induce bronchial hyper-responsiveness (BHR) [17, 18].

The production of NGF by eosinophils has been described [19]. The appearance in LAR as well as the demonstrated increased levels in allergic diseases and asthma provides a role for eosinophils as the origin of neurotrophins in allergic diseases. The aim of the presented study was to evaluate if eosinophils are a source of the neurotrophins NGF, BDNF and NT-3.

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Methods

Subjects

We studied 21 subjects, 11 patients with allergic asthma and 10 healthy controls. Allergy diagnosis was made on the basis of detailed history and skin prick test to common inhalative allergens, with at least one reaction equal to that of histamine control. Asthmatics were treated with a stable dose of inhaled corticosteroids and salbutamol as rescue medication (Table 1). Informed consent was given by all subjects.

Isolation of eosinophils

Eosinophils were purified from the peripheral blood as previously described [20, 21]. One hundred and twenty millilitres of venous blood was collected and left to sediment in 6% dextran (Sigma Chemicals, St Louis, MO, USA). Leucocytes were centrifuged on Ficoll-Paque (density 1.077) (Biochrome) for 25 min at 700 *g*. Neutrophils in the granulocyte-enriched pellet were tagged with micromagnetic beads to anti-CD16 antibodies (Miltenyi, Biotech GmbH, Bergisch Gladbach, Germany). The tagged cells were eliminated by passing them through a magnetic field (MACS). Eosinophils were collected at high purity of >96% assessed by flow cytometry using anti-CD16 PE conjugated and anti-CD 49d FITC conjugated antibodies (Coulter Immunotech, Marseille, France) and cytopins at a viability of more than 95% as assessed by flow cytometry with propidium iodide and trypan blue staining (Sigma Chemicals).

Culture of isolated eosinophils

Isolated eosinophils were cultured in RPMI 1640 medium containing 10% FCS; 5% penicillin/streptomycin on six-well plates. Stimulation experiments were performed under approved conditions in medium supplemented 50 ng/mL GM-CSF to prime cells [22]. Cells were stimulated with concentrations of 10^{-5} – 10^{-8} M platelet activating factor (PAF). After 24 h cells were centrifuged and cell culture supernatant collected.

Determination of neurotrophin content in eosinophils and culture supernatants

To determine the content of NGF, BDNF and NT-3 in eosinophils, freshly isolated eosinophils were lysated. Neurotrophic factors were detected in sandwich ELISAs according to the manufacturer's instructions for NGF, BDNF and NT-3 (R and D Systems, Minneapolis, MN, USA). All assays were performed on F-bottom 96-well plates (Nunc, Wiesbaden, Germany). Tertiary antibodies were conjugated to horseradish

peroxidase. Wells were developed with tetramethylbenzidine and measured at 450 nm. Neurotrophin content was quantified against a standard curve calibrated with known amounts of protein. The detection limits were <4 pg/mL for NGF/BDNF and <8 pg/mL for NT-3. Measurements were performed in duplicate and are expressed as means. Cross-reactivity to related neurotrophic factors was less than 3% for all three analysed neurotrophins.

Protein electrophoresis and Western immunoblotting

Briefly, 10^6 human peripheral eosinophils were homogenized on ice with 1.5% SDS, 62.5 mM Tris-HCl buffer, pH 6.8, supplemented with 1% nonidet P-40 (NP-40), 5% mercaptoethanol, 2 mM PMSF; 50 µL of proteinase inhibitors (Sigma) were added. Lysates were centrifuged at 15 000 *g* for 15 min, and supernatants were collected as total lysate. Protein content was measured at 562 nm according to manufacturers instructions (BCA, Pierce, Rockford, IL, USA). Aliquots were stored at -80°C until used. SDS-PAGE and Western blot were performed as previously described [23–25]. Samples of protein (50 µg) were loaded onto a 10 to 20% polyacrylamide gel and electrophoresed in parallel with a molecular weight marker (Bio-Rad, Hercules, CA, USA). Recombinant NGF, BDNF and NT-3 were used as standard. Proteins were blotted on supported nitrocellulose membrane (Schleicher and Schuell, Keene, NH, USA), Western immunoblotting was performed using 1:500 rabbit polyclonal anti-human NGF, BDNF or NT-3 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and 1:10000 horseradish peroxidase-linked donkey anti-rabbit secondary antibody (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). The blot was analysed using a chemiluminescence system (ECL, Amersham Life Sciences, Uppsala, Sweden). For absorption control experiments, the membrane was stripped of bound antibody, and 1° antibody was incubated with blocking peptide overnight before reprobing as previously described.

RNA isolation and reverse transcription PCR amplification

Total RNA was extracted from approximately 10^6 (highly pure) freshly prepared eosinophils by using the commercial RNeasy kit (Qiagen). First-strand cDNA synthesis reaction was catalysed by AMV reverse transcriptase and oligo(dT) primer according to the manufacturer's instructions (Boehringer, Indianapolis, IN, USA). The generated complementary DNA was amplified in a total volume of 25 µL with 1.25 U of Taq DNA polymerase and 0.2 mmol/L dNTP mixture, 0.4 µmol/L of neurotrophin primers [26, 27] (Table 2). Neurotrophin mRNA has been shown to be present in HMC-1 cells [28]. cDNA of this mast cell line was used as a positive control. The thermocycler (MWG Biotech, Ebersberg, Germany) was used for the PCR amplifications with the following settings: 36 cycles, 94°C for 1 min, 60°C for 1 min, and 74°C for 1 min. The PCR-products were size-fractionated on 1.5% agarose gels, stained with ethidium bromide, and visualized under UV-light. The PCR-products for NGF (167 bp), BDNF (296 bp) and NT-3 (176 bp) were ligated in the pGEM®-T-Vector (Promega, Mannheim, Germany) and transformed in *E. coli* (JM109, Promega). The resulting clones identified by blue/white screening were sequenced. The identity with the published sequences (GenBank:

Table 1. Baseline characteristics of the study subjects

Characteristics	Asthmatics (<i>n</i> = 11)	Non-allergic (<i>n</i> = 10)
Female	6	4
Male	5	6
Age (yr)*	28 (22–50)	27 (25–33)
FEV ₁ (% predicted)*	83 (60.8–99)	100 (87.5–115)
BDP (µg/day)*	653 (200–2000)	0
IgE (IU/L)*	354 (120–2000)	11.7 (4–42.4)

*Values are median (range).

Table 2. Primer

NT	Upstream (5'-3')	Downstream (5'-3')	Size (bp)
NGF	TAA AAA GCG GCG ACT CCG TT3	ATT CGC CCC TGT GGA AGA TG3	167 bp
BDNF	AGC CTC CTC TGC TCT TTC TGC TGG A	CTT TTG TCT ATG CCC CTG CAG CCT T	296 bp
NT-3	TTT CTC GCT TAT CTC CGT GGC ATC C	GGC AGG GTG CTC TGG TAA TTT TCC T	176 bp

XM 058005 NGF; XM 006027 BDNF; NM 002527 NT-3) was confirmed.

Intracellular staining and flow cytometry

Approximately 10^5 cells were fixed with paraformaldehyde (PFA) and permeabilized with 0.025% saponin. Cells were incubated for 1 h in 50 μ L AB serum containing either 20 μ L antibody to either NGF, BDNF or NT-3 or antibody pre-incubated overnight with specific blocking peptide (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Cells were washed with PBS supplemented with 2% FCS and 0.1% saponin, followed by incubation with a secondary anti-rabbit FITC conjugated antibody for 1 h. Isolated eosinophils were washed again with PBS containing 4% PFA. Fluorescence was analysed by flow cytometry (Epic XL, Coulter, Brea, CA, USA).

Statistical analysis

Results are presented as mean values + SEM. Values were compared by using the non-parametric test for independent (Whitney-Mann *U*-test) or dependant (Wilcoxon signed rank test) values with SPSS Version 10 (SPSS Inc., Chicago, IL, USA). *P*-values < 0.05 were regarded as significant.

Results

RNA isolation and reverse transcription PCR amplification

Each cDNA sample from isolated eosinophils or cultured HMC-1 cells demonstrated GAPDH amplification products of the expected size (167 bp). There was no evidence of genomic amplification product. During PCR amplification, control reactions were negative for amplification products, demonstrating that the PCR method and reagents used yielded specific amplified products only when a cDNA source was included. Specific bands of RNA encoding for NGF, BDNF as well as NT-3 were detectable in all samples after reverse transcription (Fig. 1). Sequence analysis demonstrated the specificity of the PCR reaction (data not shown).

Protein electrophoresis and Western immunoblotting

To confirm the expression of the neurotrophins NGF, BDNF and NT-3 at the protein level, soluble extracts from isolated eosinophils and recombinant proteins as controls were immunoblotted using specific antibodies. As shown in Fig. 2, specific bands were detectable for the neurotrophins NGF, BDNF and NT-3. The specificity was confirmed by the fact that the staining was absorbed by pre-incubation of antibodies with excess amount of blocking peptide.

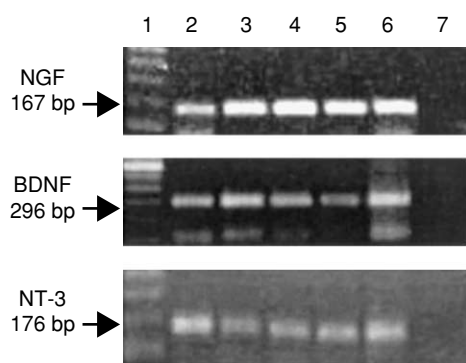


Fig. 1. RT-PCR amplificates of the neurotrophins NGF, BDNF and NT-3. Lane 1: 100 bp ladder. Lanes 2 to 5: samples. Lane 6: positive control. Lane 7: negative control. Positive control was performed with cDNA from HMC-1 cells. Negative control was performed by adding isolated RNA. Lanes 2 and 3 represent samples of non-allergic donors, while 4 and 5 were isolated from allergic asthmatics.

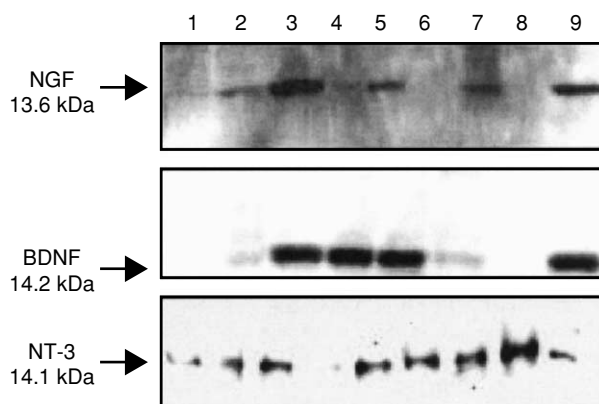


Fig. 2. Western blot analysis of NGF, BDNF and NT-3 protein. Lanes 1 to 8: samples. Lane 9: positive control. Recombinant proteins were used as positive control. Lanes 1 to 4 represent protein samples isolated from asthmatic donors, while 5 to 8 are from non-allergic origin.

Determination of neurotrophin content in eosinophils and culture supernatants

The neurotrophins NGF, BDNF and NT-3 were detectable in cell lysates and cell culture supernatants in different amounts between different individuals. To avoid differences in cell counts in the lysates neurotrophin content was divided by protein content. To avoid changes by difference in cell count values were calculated to a number of 10^6 cells.

Evaluating the values of cellular neurotrophin content allergic asthmatics demonstrated a significant lower amount of BDNF (allergic asthmatics, 0.3 ± 0.06 pg/ μ g protein; non-allergics, 1.2 ± 0.3 pg/ μ g protein; $P < 0.05$). NGF demonstrated constantly higher levels without reaching statistical significance. No difference in NT-3 content was detectable (Fig. 3).

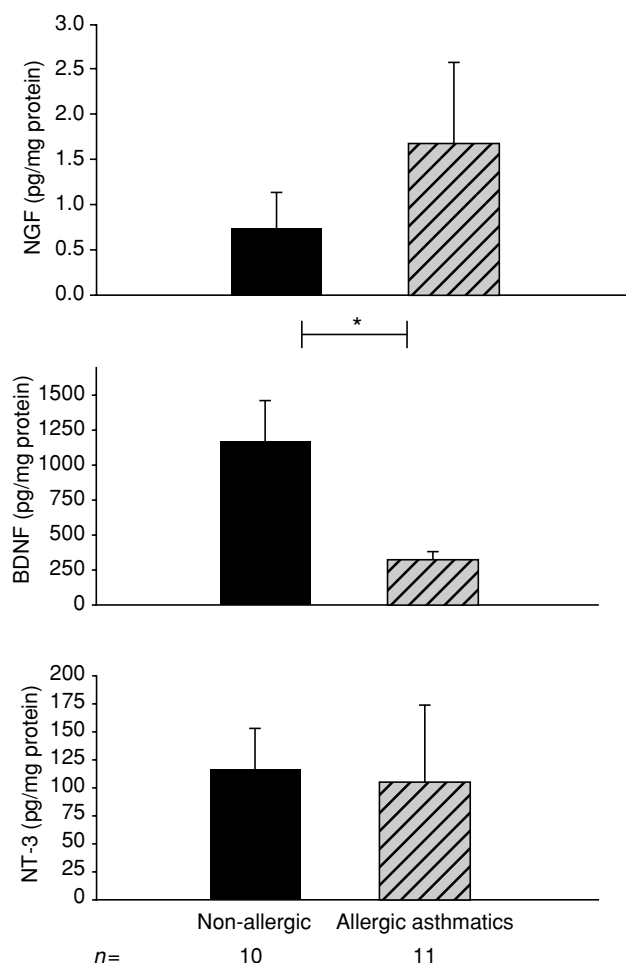


Fig. 3. Neurotrophin content of eosinophil lysates. Differences between allergics and non-allergics. The neurotrophin content was referred on total protein content of lysates. Data are presented in pg/mg protein. * $P < 0.05$.

Cell stimulation with PAF leads to a dose-dependent release of the neurotrophins NGF and BDNF (Fig. 4), while NT-3 was rarely detectable in supernatants and stimulation did not demonstrate constant release. Comparing allergic vs. healthy persons, allergic asthmatics released a higher amount of NGF and a lower amount of BDNF without reaching statistical significance.

Intracellular staining and flowcytometry

To demonstrate the content of the neurotrophins in intact cells intracellular staining was performed. FACS analysis showed positive results for NGF and BDNF as well as NT-3 for 60% of cells in three repeated experiments with cells of asthmatics (Fig. 5).

Discussion

This study shows that human eosinophils are able to produce, store and release the neurotrophins NGF, BDNF and NT-3. These cells characteristic for the cellular phenotype of allergic asthma are a source of locally and probably also circulating

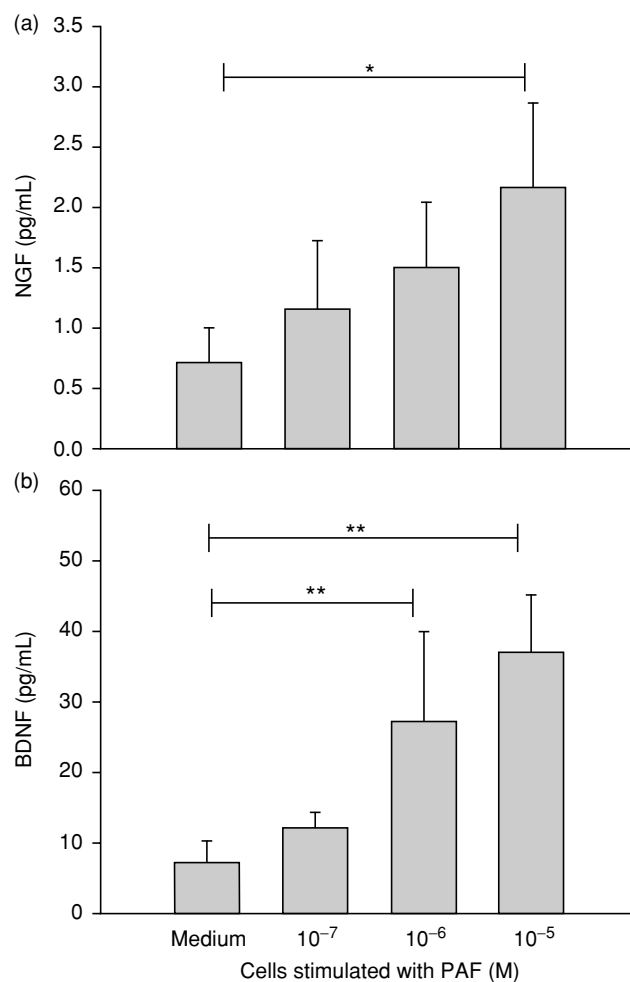


Fig. 4. Determination of NGF and BDNF in cell culture supernatants after 24 h stimulation with PAF. Mean values calculated on 10^6 cells. * $P < 0.05$, ** $P < 0.01$.

neurotrophins. Increased neurotrophin serum levels have been reported in allergic diseases, with the highest circulating levels in allergic asthmatics [14]. Increased levels in BAL fluid after segmental allergen provocation in LAR have also been reported [15]. In this study we have been able to demonstrate the production of neurotrophins on RNA as well as on protein level. Produced neurotrophins are released in response to a stimulus such as PAF. Therefore we conclude that eosinophils are a source of both circulating and local neurotrophins. The content of NGF in the isolated eosinophils of allergic asthmatics is higher than for our non-allergic group, while BDNF content is lower. This imbalance, demonstrated for the first time, might be an indicator of different physiological functions of neurotrophins in the regulation of inflammatory processes. Different roles are well known with respect to their influence on neuronal cells where BDNF is suggested to demonstrate tissue protecting abilities for inflammatory brain lesions [10]. Imbalance of pro- and anti-inflammatory mediators might lead to local inflammation causing airway hyper-responsiveness. In a previous study we reported increased neurotrophin levels in untreated allergic asthmatics, which decreased after treatment with inhaled corticosteroids [16]. With respect to the demonstrated lower content of BDNF in eosinophils the elevated number of

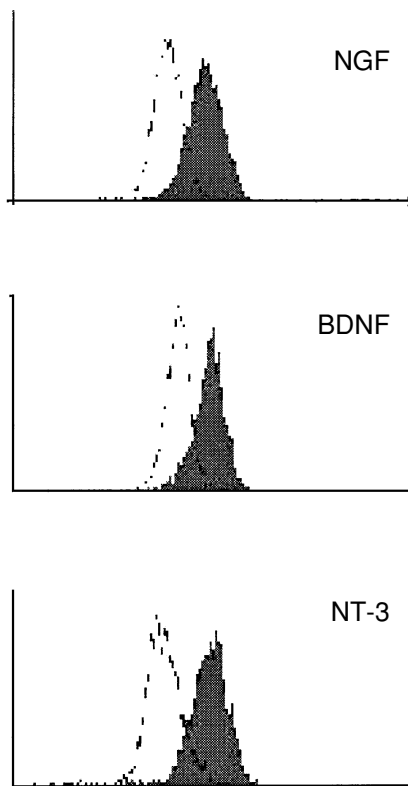


Fig. 5. FACS analysis of intracellular staining of NGF, BDNF and NT-3 with and without blocking of the antibodies with excess amount of blocking peptide. Single experiment out of three with eosinophils isolated from asthmatic donors.

these cells in allergic diseases and the production of neurotrophins by other immune cells and also structural cells have to be important for circulating levels.

An important pathway triggering airway inflammation is neurogenic inflammation. Substance P is increased in lavage fluids of allergics [29]. Activated eosinophils increase the amount of SP. Known activators except MBP failed to increase neuropeptides in dorsal root ganglion cells [30]. The expression of neurotrophins, activators of neuropeptide synthesis, might be an explanation [31]. NGF, as the best known NT, not only increases SP expression in airway neurones but changes the neuronal phenotype [32]. The increased level of neuropeptides also influences eosinophil function. Substance P is a chemoattractor for eosinophils.

Neurotrophin receptors are expressed on various immune cells [7, 33]. Allergic inflammation with the migration of eosinophils and the release of these mediators therefore influences inflammatory cells. This paracrine pathway influences activation as well as differentiation of immune cells in inflamed tissue.

Neurotrophins also influence eosinophil function. NGF activates these cells. The influence on survival is controversial [19, 34]. The production of the neurotrophins NGF, BDNF and NT-3 might influence eosinophil function by an autocrine pathway. In view of this, the difference in cellular content of NGF and BDNF might influence the appearance of allergic inflammation in the airways of asthmatics.

Production, storage and release of neurotrophins by eosinophils provides a novel aspect to understand the role of eosinophils in the pathogenesis of allergic inflammation.

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