

Regulation of NGF and BDNF by dexamethasone and theophylline in human peripheral eosinophils in allergics and non-allergics

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Abstract

Background: Recent studies have shown that the neurotrophins NGF and BDNF are produced by eosinophils. The influence of neurotrophins in allergic diseases including asthma has been described. The regulation by pharmacological substance remains unclear.

Objectives: The aim of this study was to assess whether approved pharmacological substances in the treatment of asthma such as corticosteroids or theophylline regulate neurotrophins on a cellular level.

Methods: Eosinophils were purified by negative immunoselection from allergics and non-allergic donors. Eosinophils were incubated with dexamethasone and theophylline and supernatants were collected for measurement of neurotrophic factors. The content of neurotrophins in eosinophil lysates was determined by ELISA. Regulation of stored NGF and BDNF was demonstrated by Western-blotting and flow cytometry while influence on transcription level was demonstrated by RT-PCR.

Results: Eosinophils produce and release the neurotrophins NGF and BDNF at different levels in allergics and non-allergics. Dexamethasone lead to a significant downregulation of NGF in eosinophils of allergics. The levels of BDNF were not significantly reduced. Theophylline did not influence the levels of NGF nor BDNF significantly.

Conclusions: The production of the neurotrophin NGF was downregulated by an established substance such as dexamethasone. This might further contribute to the pharmacological potential of corticosteroids in allergic asthma.

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1. Introduction

The involvement of the neurotrophins Nerve Growth Factor (NGF) and Brain-Derived Neurotrophic Factor (BDNF) in allergic diseases including asthma has become evident in the recent years. Several studies described the potential to modulate hyperresponsiveness and inflammation in animal models. An involvement of neurotrophins in the immediate and late allergic reaction [1–3] has been demonstrated. Neurotrophic factors such as NGF and BDNF belong to a well described family of neurotrophins with high structural homo-

logy sharing an outstanding role in the development, function and survival of neuronal cells [4,5].

In recent years there is growing evidence of a role in allergic diseases and asthma also in humans. The circulating level of NGF has been found to be increased in allergic diseases with the highest levels in allergic asthmatics [6]. After segmental allergen provocation neurotrophins were increased in the late phase reaction [7]. Our group was able to confirm elevated plasma levels for NGF and demonstrate this finding also for BDNF and NT-3. Furthermore it was turned out that treatment with inhaled corticosteroids (ICS) lead to a normalisation of circulating neurotrophin levels in allergic asthmatics [8]. Recently, a correlation between platelet derived BDNF and pulmonary function has been described [9].

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The exact cellular source of the increased level of circulating neurotrophins has not been determined in detail, but a variety of cells involved in allergic reactions have been found capable of producing and releasing neurotrophins [10,11]. Numerous identified cellular sources play major roles in allergic inflammation. Mast cells—as a key cell of the immediate reaction—are a source of neurotrophins [12]. Further cells like lymphocytes or monocytes [13,14] have been identified as additional sources of neurotrophins in allergic reactions. But also structural cells like fibroblasts [15] and epithelial cells [16] have the potential to produce and release neurotrophins upon inflammatory stimuli. Recently we were able to demonstrate the production, storage and release of neurotrophins by eosinophils as cells of the late allergic reaction. Furthermore a difference between allergies and non-allergics in the cellular neurotrophin content was detectable. This contributes to an imbalance of neurotrophins in allergic diseases [17].

The release of neurotrophins activates inflammatory cells. An increased secretion of IL-4 by eosinophils has been described after stimulation with NGF [18]. Mast cells differentiate and degranulate besides a TH2 type reaction is induced [19].

The appearance in LAR as well as the demonstrated increased levels in allergic diseases and asthma provides a role of eosinophils as important origin of neurotrophins. The aim of the presented study was to evaluate if approved pharmacological substances such as dexamethasone and theophylline have the potential to regulate the production and release of the neurotrophins NGF and BDNF in allergies and non-allergics.

2. Methods

2.1. Subjects

We studied 25 subjects. 13 were allergic patients, all suffering from allergic rhinitis, 4 also from allergic asthma and 12 healthy controls. No anti-allergic medication was allowed 4 weeks prior blood sampling. 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 those of histamine control (Table 1). Informed consent was given by all subjects.

2.2. Isolation of eosinophils

Eosinophils were purified from the peripheral blood by negative immunoselection. 100 mL of venous blood was

collected and left to sediment in 6% dextran (Sigma Chemicals, St. Louis, MO, USA). Leukocytes 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, Biotec GmbH, Bergisch Gladbach, Germany). The tagged cells were eliminated by passing them through a magnetic field. The purity of the isolated eosinophils was assessed by flow cytometry using anti CD16 PE conjugated combined with anti CD 49d FITC conjugated antibodies (Coulter Immunotech, Marseille, France) and cytopins. The viability was determined by flow cytometry with propidium iodide and trypan blue staining (Sigma Chemicals).

2.3. Culture of isolated eosinophils

Isolated eosinophils were cultured in RPMI 1640 medium containing 10% FCS; 5% penicillin/streptomycin on 6 well plates. Stimulation experiments were performed with addition of 0.1 µM dexamethasone or 10 µM theophylline for 24 h. Controls were performed with medium alone. After 24 h cells were harvested and cell culture supernatant was collected.

2.4. Protein electrophoresis and Western immunoblotting

Approximately 10⁶ 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) was added. Lysates were centrifuged at 15,000 rpm for 15 min, and supernatants were collected as total lysate. Protein content was measured at 562 nm according to the manufacturer's instructions (BCA, Pierce, Rockford, IL, USA). Samples were stored at –80 °C until use. 50 µg of protein were loaded on a 7.5% polyacrylamide gel and electrophoresed in parallel with a molecular weight marker (Bio Rad, Hercules, CA, USA). Recombinant NGF or BDNF were used as positive control. 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 or BDNF antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and 1:10,000 horseradish peroxidase-linked secondary antibody (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA). The blot was analyzed using a chemiluminescence system (ECL, Amersham Life Sciences, Uppsala, Sweden).

2.5. RNA isolation and reverse transcription PCR amplification

Total RNA was extracted from approximately 10⁶ eosinophils by using the 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

Table 1
Baseline characteristics of the study subjects

Characteristics	Allergics (n = 13)	Non-allergics (n = 12)
Female	7	7
Male	6	5
Age [years] ^a	39.4 (20–63)	35.5 (18–60)
IgE [IU/l] ^a	320 (135–1800)	18 (4–53)

^a Values are median (range).

μL with 1.25 U of Taq DNA polymerase and 0.2 mmol/L dNTP mixture, 0.4 $\mu\text{mol/L}$ of neurotrophin primers. Primer sequences were: NGF Upstream (5'–3') TAA AAA GCG GCG ACT CCG TT3; Downstream (5'–3') ATT CGC CCC TGT GGA AGA TG3 with a product size of 167 bp. BDNF Upstream (5'–3') AGC CTC CTC TGC TCT TTC TGC TGG A; Downstream (5'–3') CTT TTG TCT ATG CCC CTG CAG CCT T with a product size of 296 bp. Neurotrophin mRNA has been shown to be present in HMC-1 cells [20]. 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. Amplified products were electrophoresed on 1.5% agarose gel, stained with ethidium bromide, and photographed under UV light. The PCR-products were size-fractionated on 1.5% agarose gels, stained with ethidium bromide, and visualized under UV-light. Specificity of the resulting products has been demonstrated by sequence analysis previously [17].

2.6. Intracellular staining and flow cytometry

The cells were fixed with paraformaldehyde (PFA) and permeabilised with 0.025% saponin and incubated for 1 h in 50 μL AB serum containing either 20 μL antibody to NGF or BDNF or antibody preincubated over night 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).

2.7. Determination of neurotrophins in eosinophils and cell culture supernatants

To determine the content of NGF and BDNF in eosinophils, cells were harvested and eosinophils were lysated. Neurotrophins were detected in sandwich ELISAs according to the manufacturer's instructions for NGF and BDNF (R&D Systems, Minneapolis, MN, USA). The assays were performed on F-bottom 96-well plates (Nunc, Wiesbaden, Germany). Tertiary antibodies were conjugated to horseradish peroxidase. Development was performed 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 and BDNF. Measurements were performed in duplicate and expressed as means. Cross reactivity to related neurotrophic factors were less than 3% for the analysed neurotrophins.

2.8. Statistical analysis

Results are presented as mean values+S.E.M. Values were compared by using the nonparametric test for independent

(Whitney–Mann *U*-test) values with SPSS Version 10 (SPSS, Inc., Chicago, IL, USA). *P* values <0.05 were regarded as significant.

3. Results

3.1. Subjects

25 subjects were included in the study (Table 1). Baseline characteristics were well balanced between the groups. Isolation of eosinophils resulted in highly pure eosinophil preparations with a purity of 96% or more, as assessed by FACS analysis using CD 16 and CD 49d antibodies. The viability of the isolated eosinophils was at least 95%.

3.2. RNA isolation and reverse transcription PCR amplification

Each cDNA sample from eosinophils regardless of stimulation or controls demonstrated GAPDH amplification products of the expected size (197 bp) and comparable intensity. 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 and BDNF were detectable in controls. Incubation with dexamethasone or theophylline lead to a decrease of band intensity for NGF, while no difference for BDNF was detectable (Fig. 1).

3.3. Protein electrophoresis and Western immunoblotting

To confirm the expression and regulation of the neurotrophins NGF and BDNF soluble extracts from isolated eosinophils and recombinant proteins as controls were immunoblotted using specific antibodies. Specific bands were detectable for the neurotrophins NGF and BDNF. Incubation in the presence of dexamethasone stimulation lead to an decrease of band intensity for NGF and BDNF while theophylline decreased the

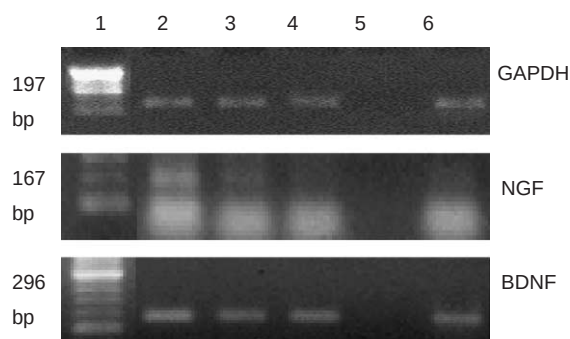


Fig. 1. RT-PCR amplificates of the neurotrophins NGF and BDNF. Lane 1: 100 bp ladder, lane 2: control, lane 3: eosinophils stimulated 24 h with 0.1 μM dexamethasone, lane 4: eosinophils stimulated with theophylline, lane 5: negative control, lane 6: positive control. Positive control was performed with cDNA from HMC-1 cells. Negative control was performed by adding isolated RNA.

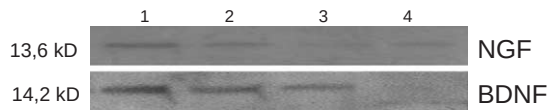


Fig. 2. Western Blot analysis of NGF and BDNF. Lanes 2–4: Samples (lane 2: unstimulated, lane 3: dexamethasone, lane 4: theophylline). Lane 1: Positive control. Recombinant proteins were used as positive control.

band intensity for BDNF, while no difference compared to unstimulated eosinophils was detectable (Fig. 2).

3.4. Intracellular staining and flow cytometry

FACS analysis showed positive results for NGF and BDNF. Dexamethasone decreased the fluorescence intensity for NGF, while no changes after incubation with theophylline was observed (Fig. 3).

3.5. Determination of neurotrophins in eosinophils and cell culture supernatants

The neurotrophins NGF and BDNF were detectable in cell lysates and cell culture supernatants in different amounts between different individuals. NGF is elevated in eosinophil lysates of allergics and BDNF is decreased. Incubation with dexamethasone leads to a significant ($p < 0.05$) decreased level of NGF in cell lysates of allergic patients. In non-allergic patients and evaluating all patients this effect is not significant. Incubation in the presence of theophylline does not lead to significant changes. Neurotrophin levels in cell culture supernatants differed after stimulation with dexamethasone and also a significant ($p < 0.05$) decrease of NGF was demonstrated in allergic patients. Theophylline did not demonstrate significant differences between the examined groups (Fig. 4).

4. Discussion

This study indicates that the production and release of NGF by eosinophils isolated from allergic subjects is influenced by dexamethasone. This effect is detectable intracellular on the transcriptional and on the protein level. Furthermore also the release of NGF is significantly decreased as detected in cell culture supernatants. BDNF was not influenced significantly. Theophylline did not lead to significant changes. Therefore we conclude that a downregulation of circulating neurotrophins after treatment with inhaled corticosteroids is due at least partly to a regulation of cellular production and release of NGF. The decrease of NGF after treatment of eosinophils with dexamethasone is limited to allergics while no significant regulation on cells of healthy donors was observable an increased production of neurotrophins in allergic diseases has to be assumed. The effect on eosinophils is potentiated throughout the induction of apoptosis of eosinophils by steroids [21]. The decrease of the local neurotrophin level in the inflamed tissue might therefore further be potentiated. A variety of other cells known to be involved in the production of neurotrophins might

be influenced by steroids also, with the consequence of a decreased or normalised amount of neurotrophins released. The normalisation of circulating neurotrophin levels after treatment of allergic asthmatics with inhaled corticosteroids [8] therefore seems to be the result of a decreased cellular production of migrated or residential pulmonary cells in combination with an decreased activation of these cells.

Differences in neurotrophin levels with increased cellular NGF levels and decreased BDNF levels have been reported in eosinophils and monocytes of allergics compared to cells of healthy subjects [16,17]. In this report the mentioned difference was reproducible. A normalisation of this described imbal-

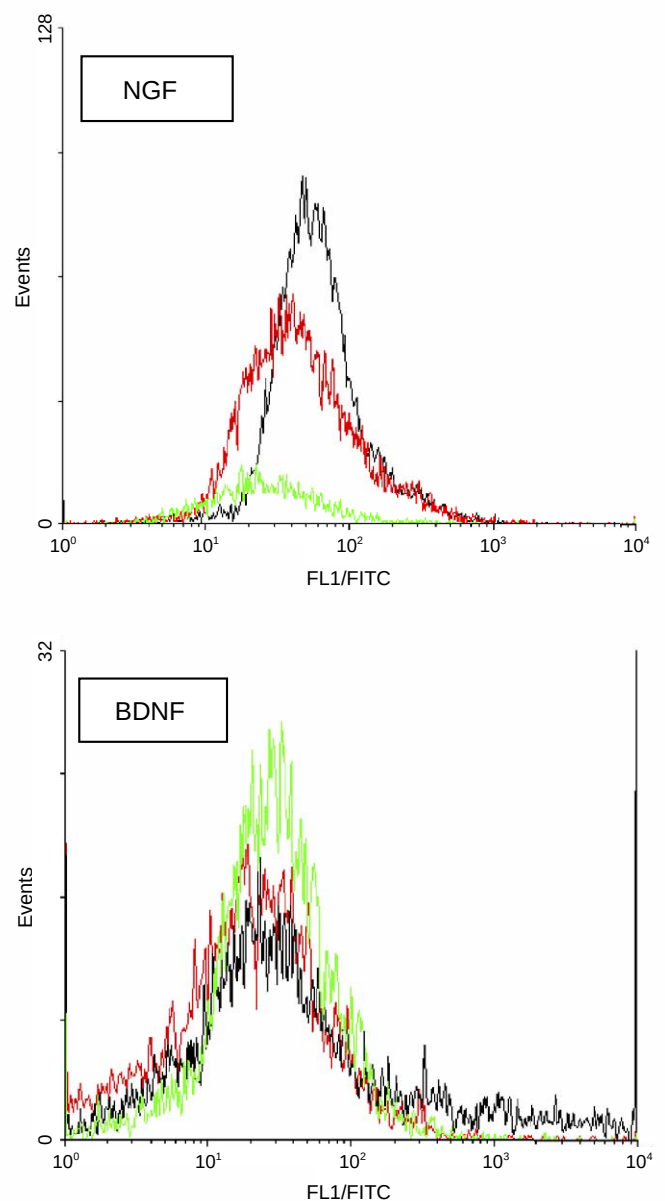


Fig. 3. FACS analysis of intracellular staining of NGF and BDNF. Control (black), dexamethasone (red), theophylline (green). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

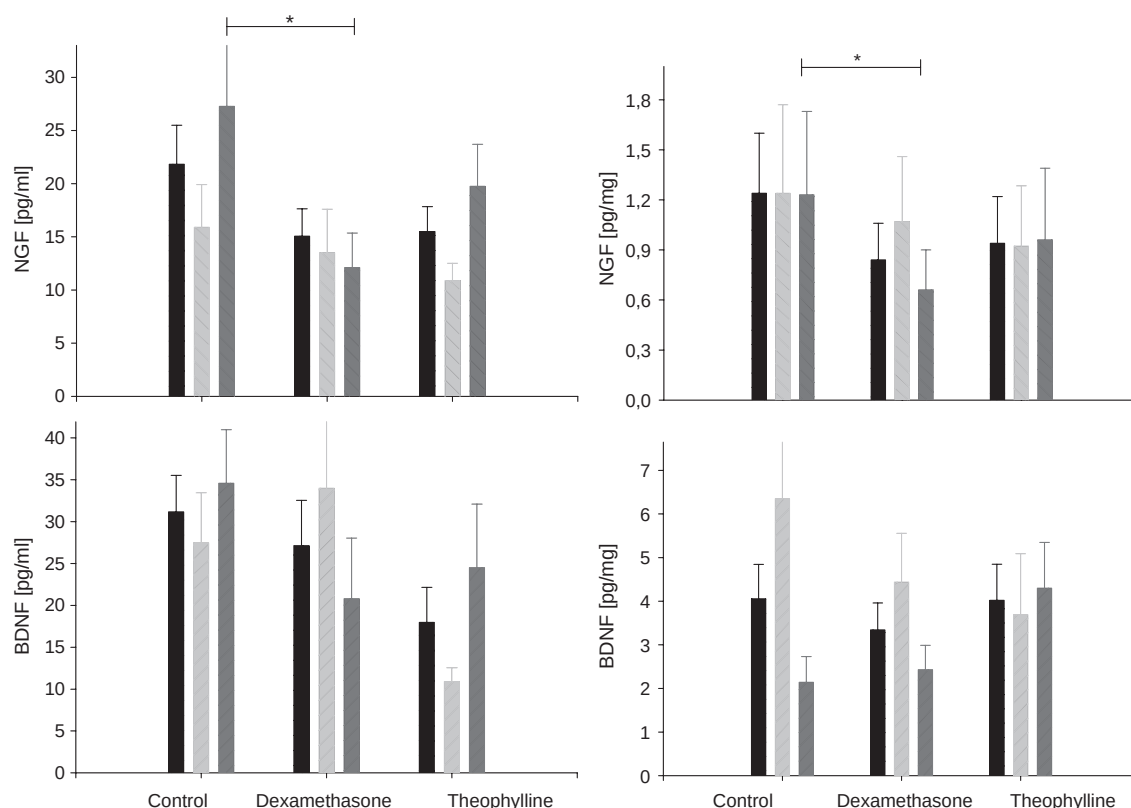


Fig. 4. Neurotrophin detection in cell culture supernatants and eosinophil lysates. Supernatants left part of the graph. Lysates right (the neurotrophin content were referred on total protein content of lysates; data are presented in pg/mg protein). Black: all subjects, light grey: non-allergics, dark grey: allergics. * $p < 0.05$.

ance might contribute to the outstanding effect of steroids as anti-inflammatory agent in asthma. The development of ICS represents a hallmark in the improvement of asthma therapy during the last decades [22]. Their high anti-inflammatory potential with all the consequences of the migration and activation of cells is well-described. Corticosteroids are able to regulate numerous inflammatory mediators by downregulation of pro-inflammatory or upregulation of anti-inflammatory mediators [23]. Early diagnosis and treatment might also protect against neuronal changes reported in the airways of mice overexpressing NGF [24]. This might be a key mechanism to influence airway hyperreactivity to influence the change of NGF induced phenotypic switch in airway sensory neurons [25]. The influence of neurotrophins in the regulation of neurogenic inflammation by neurotrophins has been described [26,27] as neurotrophins are the main inducers of substance P synthesis in neurons [28].

A decreased level of neurotrophins additionally influences the activation and influx of a variety of inflammatory cells [29–31].

Neurotrophins are survival and activation factors for eosinophils after migration in the lung [32]. An increased secretion of IL-4 as an important TH2 cytokine has been demonstrated to be regulated by stimulation with NGF [18]. Regulating neurotrophic factors provides an additional element in the clarification of the outstanding pharmacological potential of corticosteroids. Prevention of neurotrophin mediated inflammatory and probably remodeling processes provides an

additional role in the development of novel corticosteroids or anti-inflammatory drugs in allergic asthma.

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