

Serum and sputum neurotrophin levels in chronic persistent cough

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Summary

Background Neurotrophins (NTs) are a family of growth factors, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin3 (NT-3) that are involved in inflammation. Serum and induced sputum NT levels are increased in asthma and in cough because of idiopathic pulmonary fibrosis, respectively. Neurogenic inflammation is implicated in the pathogenesis of chronic cough in individuals with normal chest radiography, but the role of NTs in this condition is unknown.

Objective To assess if NT levels are elevated in the serum and airways in subjects with chronic persistent cough.

Methods Eighty-one subjects with chronic cough persistent for over 1 year; with normal chest radiography and spirometry were included. Thirty healthy subjects were controls. Serum NGF, BDNF and NT-3 were measured by enzyme immunoassay. In a subset, NGF was measured in induced sputum. Sputum cell counts and allergen-specific serum IgE were measured and all patients received specific sequential treatment trials to achieve a final diagnosis for the cough.

Results There was no significant difference either in the levels of serum or sputum NTs in chronic cough subjects compared with controls or between the most common causes of cough: post-nasal drip syndrome, gastro-oesophageal reflux disease, asthma and bronchiectasis. The median (inter-quartile range) for sputum NGF (pg/mL) was 516 (296–772) in healthy controls and 580 (312–880) in subjects with chronic cough ($P = 0.284$). There was no correlation between NT levels and sputum cell counts. Sputum NGF levels correlated with duration of cough ($r = 0.34$, $P = 0.002$).

Conclusion: NTs are not elevated in induced sputum or serum of subjects with chronic persistent cough. This implies that NTs do not have a central role in perpetuating airway inflammation in chronic persistent cough.

Keywords chronic cough, nerve growth factor, neurotrophins

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Introduction

Chronic cough is a common clinical problem with a reported prevalence of between 14% and 23%. It often persists for over a year [1, 2] and has adverse effects on quality of life. In non-smokers with a normal chest radiograph, the most frequent causes are post-nasal drip syndrome (PNDS), gastro-oesophageal reflux disease (GORD) and cough variant asthma (CVA) [3–6]. Although chronic cough can be caused by various different conditions, underlying airway inflammation appears to be a common factor [7–11].

There is evidence that chronic cough is associated with neurogenic inflammation. The afferent sensory receptors and nerves (the myelinated rapidly adapting receptors and the unmyelinated C fibres) are considered important components

of the cough reflex [12]. Up-regulation in sensory nerve function in the afferent portion of the cough reflex appears fundamental to the pathophysiology which underlies chronic cough [13]. It is believed that activation of C-fibre receptors in the airway releases sensory neuropeptides, which cause neurogenic inflammation and may activate rapidly adapting receptors to cause cough [12]. Inhaled substance P induces cough in humans with an upper respiratory tract infection or idiopathic pulmonary fibrosis and increased levels have been found in nasal lavage fluid from patients with chronic productive cough [14]. The neuropeptide calcitonin gene-related peptide induces histamine release from bronchoalveolar lavage mast cells obtained from asthmatic and non-asthmatic subjects with chronic cough [13].

Neurotrophins (NTs) are a family of structurally related growth factors, which include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and NT-4/5. The physiological role of NTs includes regulating the development, differentiation, survival and function of distinct neurons. In addition, there is growing

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evidence for their involvement in inflammation. T cells, B cells, macrophages and mast cells may be sources of neurotrophic factors during the process of inflammation [15]. Mast cells are able to produce, store and release NGF. *In vitro* stimulation with NGF leads to degranulation with the release of pre-formed and newly generated mediators such as histamine, leukotrienes and cytokines, all of which are known to be increased in cough [8, 16]. NGF is a known regulator of neuropeptide synthesis in sensory nerves, which triggers neurogenic inflammation [17]. In a mouse model of allergic inflammation and in NGF transgenic mice, NTs altered sensory innervation, enhanced neuropeptide production and induced airway hyper-responsiveness [18, 19]. Elevated levels of NGF and BDNF have been demonstrated in plasma from patients with untreated asthma [20, 21], which were reduced by corticosteroid treatment [21] and in the sputum of patients with idiopathic pulmonary fibrosis [22].

The aim of the present study was to assess the role of NTs in chronic persistent cough by measuring the levels of NGF, BDNF and NT-3 in serum/induced sputum of subjects with this condition.

Materials and methods

Subjects

Adult subjects with a cough persistent for at least 1 year were recruited from hospitals, respiratory clinics and by advertisement in a local newspaper. No subject had evidence of any other lung disease based on history, clinical examination, spirometry and chest radiography. Exclusion criteria were treatment with inhaled/oral corticosteroids within 3 weeks of inclusion, upper respiratory tract infection within 6 weeks of inclusion, angiotensin converting enzyme inhibitor treatment and smoking within the past year. Healthy volunteers with no history of recent upper or lower respiratory tract infection, were recruited as controls.

The study was approved by the West Glasgow University Hospitals Ethics Committee and informed consent was obtained from all subjects.

Measurements

Pre- and post-inhaled salbutamol forced expiratory volume in 1 s (FEV₁) were measured according to American Thoracic Society guidelines [23]. Patients recorded their cough severity on a 10 cm linear visual analogue scale (0 = no cough, 10 = maximum cough). Total serum IgE and IgE to common allergens (house dust mite, grass pollen and cat dander) were measured by enzyme immunoassay (EIA) (Unicap System, Pharmacia UK Ltd, Milton Keynes, UK). Total IgE > 120 IU/L and allergen-specific IgE > 0.35 IU/L were considered positive. Atopy was defined as the presence of specific IgE positive to one or more common allergens. Bronchial challenge testing was undertaken using Cockcroft's technique [24] to identify those with hyper-reactivity and aid the final diagnosis of the cough. Bronchial hyper-reactivity to methacholine was defined as a PC₂₀ FEV₁ of ≤ 8 mg/mL. Sputum induction with 3% hypertonic saline was performed using a modification of the method described by Pin et al. [25, 26]. The sputum was processed according to the technique

described by Popov et al. [27]. Sputum samples were rejected if there were no sputum plugs visible when examined macroscopically. Processed samples were included only if they had enough cells ($> 0.5 \times 10^6$ cells) and adequate viability ($> 50\%$). The inflammatory cells proportion was listed after exclusion of epithelial cells. The supernatant was frozen at -70°C for NGF measurements.

Measurement of nerve growth factor, brain-derived neurotrophic factor and neurotrophin-3

Serum NGF, BDNF and NT-3 immuno-reactivity was measured by EIA according to the manufacturers' instructions (Promega UK Ltd, Southampton, UK). For NGF the performance characteristics of the assay were the following: intra-assay co-efficient of variation less than 4.3%, sensitivity 15.6 pg/mL, and less than 3% cross-reactivity with other neurotrophic factors. For BDNF, the performance characteristics were the following: intra-assay CV less than 8.9%, sensitivity 15.6 pg/mL, and less than 3% cross-reactivity with other neurotrophic factors. For NT-3 the performance characteristics were: intra-assay CV less than 5.3%, sensitivity 10 pg/mL, and less than 3% cross-reactivity with other NTs. As the reducing agent dithiothreitol (DTT) used to homogenize the specimens can affect measurements, we performed an extra test for assay performance and found full recovery of NGF immunoreactivity in normal serum samples spiked with exogenous NGF (Sigma Ltd, Poole, UK) as well as from samples of whole blood and sputum.

Final diagnosis of the cough

To achieve a final diagnosis for the cause of the cough, all subjects were offered sequential trials of specific therapy depending on the likely clinical diagnoses for their cough. Final diagnosis or diagnoses of the cause of the cough were based on defined pre-treatment criteria [28] and accepted only when the cough significantly improved or disappeared with specific therapy [5]. In particular, a diagnosis of cough variant asthma was made when there was a history of cough with a positive methacholine challenge test and a response to 2 months of treatment with inhaled corticosteroids (400 mcg budesonide b.i.d.).

The subjects who participated in this study overlapped with another study where we recorded the effect of inhaled corticosteroids on cough severity and inflammatory mediators [8].

Statistical methods

The associations between demographic variables, sputum cell counts and NTs were assessed by Spearman rank correlations. Differences between groups of patients were examined by Wilcoxon tests. A *P*-value of < 0.05 was considered significant. All analyses were carried out with SAS version 8.02 software.

Results

Baseline subject characteristics

We screened 110 subjects with cough, 81 of whom met all the inclusion criteria. Of these, 49 were recruited from the

community and 32 were referred from hospital clinics. A final diagnosis for the cough was obtained in 73/81 (90%) of subjects; 73% had a single diagnosis, 25% had two diagnoses and 3% had three diagnoses. The pattern of primary diagnoses was PNDS (29), GORD (16), CVA (12), bronchiectasis (9), eosinophilic bronchitis (5), habitual cough (1) and bronchitis (1). In eight subjects, the cough was termed idiopathic. 20/81 subjects had evidence of bronchial hyper-reactivity based on the methacholine challenge test. Thirty-one percent of cough subjects were atopic (allergen-specific IgE positive) and the median (inter-quartile range (IQR)) total serum IgE levels were 28.5 (16.5–72.5).

Cough subjects were significantly older than the control group; median (IQR) age 63 (51.3–68.4) years compared with 31 (26–32) years for controls, $P < 0.001$. Thirty cough subjects were males (51 females) and 16 controls were males (14 females). Median (IQR) FEV₁% predicted was 96 (83–106) in cough subjects and 100 (89–105) in controls ($P = 0.314$).

Subjects with cough variant asthma had a mean (SD) age of 47.3 (17.4) years. All had bronchial hyper-reactivity and 3/12 were atopic.

Serum NGF, BDNF and NT-3 were not elevated in persistent cough subjects ($n = 77$) compared with healthy controls ($n = 30$) (Table 1). NGF was measured in induced sputum samples of 46 subjects with persistent cough and 17 healthy controls and there was no significant difference between the two groups (Table 1).

Induced sputum cell counts showed a significantly higher total cell count and neutrophil proportion in cough subjects compared with controls (Table 1). Neutrophils in induced sputum did not correlate with the duration of cough or age of the patient. The cell viability was $79 \pm 9\%$ and the epithelial cell proportion was less than 17%.

There was no correlation between NT levels in serum/sputum with age of the patient, gender, baseline VAS cough severity scale, FEV₁, FEV₁% predicted, total IgE level, atopy to common allergens, total and differential sputum cell counts, blood cell counts, presence of lower airway hyper-reactivity, previous smoking history or whether the patient was recruited from the community or hospital clinics.

Table 1. Baseline neurotrophin levels (pg/mL) and induced sputum cell counts in chronic cough subjects compared to controls

	Controls	Persistent cough
Induced sputum cell counts		
Total cell count (%)	2.7 (2.0–3.4)	7.3 (4.5–15.9)**
Neutrophil	32.1 (10.1–53.1)	67.3 (44.0–80.5)**
Eosinophil	0.0 (0.0–0.2)	0.2 (0.0–1.0)
Lymphocyte	0.35 (0.0–0.7)	0.0 (0.0–0.2)*
Macrophage	67.1 (46.0–89.5)	30.8 (18.3–52.3)**
Neurotrophins		
Sputum NGF	516 (296–772)	580 (312–880)
Serum BDNF	1157 (1068–1367)	1064 (838–1290)
Serum NGF	116.1 (0–547.4)	44.3 (1.7–541.2)
Serum NT-3	1464.5 (183–9562)	211 (129–1195)

Data entered as median (IQR);

* $P = 0.005$, ** $P < 0.001$

NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; NT-3, neurotrophin-3; IQR, inter-quartile range.

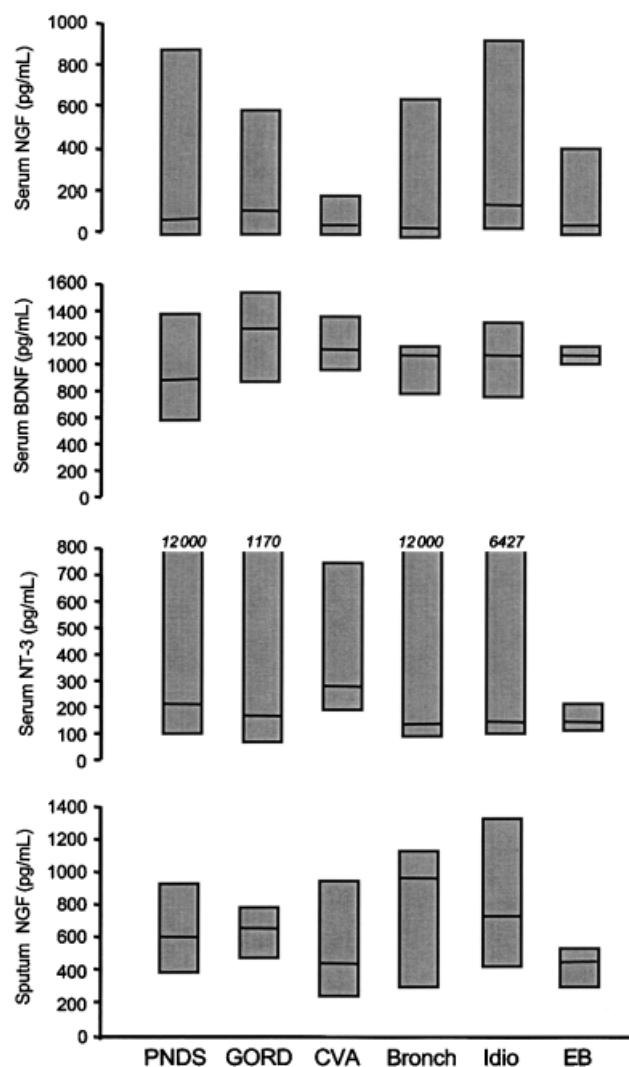


Fig. 1. Serum nerve growth factor (NGF), brain-derived neurotrophic factor, Neurotrophin-3 and induced sputum NGF in different causes of chronic persistent cough. The box represents the inter-quartile range and the line in the box is the median value. PNDS, post nasal drip syndrome; GORD, gastro-oesophageal reflux disease; CVA, cough variant asthma; Bronch, bronchiectasis; Idio, idiopathic cough; EB, eosinophilic bronchitis.

There was no major difference in the NT levels based on the final diagnosis of the cough (Table 2, Fig. 1). Individual data for the smaller diagnostic subgroups are not depicted because the numbers were too small for meaningful analysis. There was a significant inverse correlation between serum BDNF and serum NGF ($r = -0.24$, $P = 0.032$), but no correlation between the other NTs. Serum NGF levels were related to the duration of cough ($r = 0.34$, $P = 0.002$).

Discussion

NGF, BDNF and NT-3 are the common NTS associated with airway inflammation and atopy. This is the first study measuring the levels of these NTs in serum/induced sputum in subjects with chronic cough. We found no increase in NT levels in cough patients compared with healthy controls, despite the presence of airway inflammation.

Table 2. Neurotrophin levels (pg/mL) in different causes of chronic persistent cough

	PNDS	GORD	CVA	Bronch	Idio	EB
Serum	(n = 28)	(n = 16)	(n = 11)	(n = 7)	(n = 8)	(n = 5)
BDNF	887 (636–1322)	1265 (929–1483)	1102 (996–1290)	1057* (829–1069)	1074 (804–1255)	1072 (1054–1080)
NT-3	217 (126–12000)	172 (95–1170)	278 (211–717)	132 (124–12000)	150.5 (128–6427)	156* (140–193)
NGF	52 (16–827)	145 (16–535)	15.6 (16–146)	16 (16–590)	118.0 (40–873)	15.6 (16–351)
Induced sputum	(n = 11)	(n = 9)	(n = 8)	(n = 8)	(n = 4)	(n = 4)
NGF	584 (432–880)	656 (520–736)	444 (288–900)	964 (344–1096)	728 (468–1292)	444 (348–492)

Data depicted as median (IQR); statistical significance:

* $P = 0.050$.

PNDS, post-nasal drip syndrome; GORD gastro-oesophageal reflux disease; CVA cough variant asthma; Bronch bronchiectasis; Idio, idiopathic cough; EB, eosinophilic bronch.

There are various factors that could account for the normal NT levels in patients with chronic cough. Firstly, the technique of separation of sputum involves the addition of DTT which has been shown to reduce the levels of certain mediators [29]. This is unlikely to account for the normal levels of NGF in our study as the technique was similar in patients and controls and we found full recovery of immunoreactivity of exogenous NGF when added to normal serum. Furthermore, Hope-Gill et al. [22] have demonstrated that NGF levels in sputum were unaffected by DTT. Secondly, inhaled corticosteroids can suppress levels of NGF and BDNF in serum in subjects with asthma [21]. This cannot explain the normal levels we observed, as cough subjects were excluded if they had received inhaled corticosteroids for 3 weeks or oral corticosteroids for 6 weeks prior to inclusion. The third consideration was whether the study was adequately powered to demonstrate a difference between the groups. We believe it was, as studies in asthma and pulmonary fibrosis have demonstrated differences with smaller group sizes [20–22]. Results could vary in cough of a shorter duration, but we found a positive correlation with serum NGF and the duration of cough. There was no correlation between age/gender and the levels of any of the NTs, therefore, it was unlikely that any difference between the study groups was important.

We noted a wide variation in control values of serum and sputum NGF in our study compared with other studies [20–22]. The difference could arise from technical difference in the methodology of measurement or the numbers of subjects included.

We chose to measure only the NGF in sputum as it has been measurable in previous works [22] whereas NT-3 has not and levels of BDNF would have been affected by DTT we used in our method of sputum processing [22]. Besides, NGF is the NT that is most expressed in pulmonary tissues, followed by BDNF and then NT-3 as revealed by microdensitometric analysis of bands of immunoreactivity [30] and is usually representative of the other NTs.

NGF increased eosinophilic airway inflammation and augmented IL-5 production in an animal model of airway inflammation [31]. The major inflammatory cells in our cough subjects was the neutrophil, in keeping with the findings of others [7]. A significant increase in NTs has been demonstrated in allergic conditions [20, 32]. Only 31% of the cough subjects were atopic and there was no difference in NT levels based on total or allergen-specific IgE levels. It is possible that NTs are less involved in inflammation associated with

neutrophils as compared with eosinophilic inflammation in asthma and allergic conditions.

The main causes of cough in our subjects were post-nasal drip syndrome, gastro-oesophageal reflux disease, cough variant asthma, bronchiectasis and eosinophilic bronchitis; similar to other studies on chronic cough [3–5, 16]. We found no significant difference in NTs in any of the causes of cough, including cough variant asthma. However, the number of subjects in individual diagnostic groups was small and a true increase in any subgroup could be missed because of this.

It is possible that rather than absolute concentrations of NTs in the body fluids, it is their expression at receptor level that is important. It has been speculated that NGF affects airway nerves through the vanilloid receptor (VR1) in allergic asthma [33]. This receptor, originally known as the capsaicin receptor, has been proposed as a receptor for cough [34]. Further studies are indicated to determine the effect of NGF at the receptor level in chronic cough.

In conclusion, we have demonstrated that NGF, BDNF and NT-3 are not elevated in serum/sputum of subjects with chronic cough, regardless of the aetiology of cough. This implies that these NTs do not have a central role in perpetuating airway inflammation in chronic persistent cough.

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