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Upregulation of nerve growth factor expression by human airway smooth muscle cells in inflammatory conditions

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Upregulation of nerve growth factor expression by human airway smooth muscle cells in inflammatory conditions. V. Freund, F. Pons, V. Joly, E. Mathieu, N. Martinet, N. Frossard. ©ERS Journals Ltd 2002.

ABSTRACT: Recent studies have suggested that nerve growth factor (NGF) may play a role in inflammation and bronchial hyperresponsiveness in asthma. Neither the types of cells that produce NGF in the human airways nor the effect of inflammation on NGF expression are clear. The two-fold aim of the present study was to determine whether airway smooth muscle produces NGF *in vitro*, and, if so, whether the pro-inflammatory cytokine interleukin-1β (IL-1β) affects this expression.

Human airway smooth muscle cells in culture were incubated in the presence or absence of IL-1β. NGF production was measured by enzyme-linked immunosorbent assay. NGF messenger ribonucleic acid (mRNA) was measured using a specific real-time fluorescent polymerase chain reaction technique, and expressed in relation to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels.

Human airway smooth muscle cells *in vitro* expressed NGF constitutively (21.4± 7.8 pg·mL^{-1}; 14.6±5.4 pg NGF complementary deoxyribonucleic acid (cDNA)·pg GAPDH cDNA^{-1} (mean±SEM)). Stimulation with IL–1β (0.1–30 U·mL^{-1}) for 24 h induced a dose-dependent increase in NGF production (22.1 pg·mL^{-1} at 10 U·mL^{-1}; $p<0.05$). The IL-1β (10 U·mL^{-1})-induced increase in NGF expression was time-dependent. It was highest for NGF protein at 10 h (1.6-fold increase over control; $p<0.001$) and for NGF mRNA at 2.5 h (2.4-fold increase over control; $p<0.05$).

In conclusion, the present study clearly shows that the human airway smooth muscle cell is a source of nerve growth factor, the expression of which is upregulated in inflammatory conditions, mimicked *in vitro* by the addition of interleukin-1β.

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Nerve growth factor (NGF) is a well known neurotrophin whose activity is essential for nerve growth and survival (reviewed in [1, 2]). Recent reports of both animal and human studies suggest that NGF functions as an important inflammatory mediator (reviewed in [3, 4]). NGF modulates immunological and inflammatory processes by enhancing the survival and/or activation of lymphocytes [5], eosinophils [6, 7] and neutrophils [8]. Its action on the mast cell is particularly important. NGF causes an increase in the number of mast cells in peripheral tissue [2, 9], promotes mast-cell differentiation [10] and survival [11], and promotes and enhances mediator release from mast cells [12–14]. It may therefore contribute to the increase in number and degranulation of mast cells in tissue.

In addition, various animal studies suggest that NGF may contribute to the development of airway hyperresponsiveness. First, antibodies directed against NGF block the bronchial hyperresponsiveness (BHR) induced by NGF in sensitised and challenged mice [15]. In addition, BHR is induced by tissue-specific overexpression of NGF in the airways [16]. Finally, NGF by itself induces hyperresponsiveness in guinea-pig airways [17], as well as in human bronchi *in vitro* [18]. NGF may thus be suggested to play a central role in airway inflammation, through its effects on mast cells, and to participate in the increased BHR observed in asthma.

Structural cells, particularly pulmonary epithelial cells [19, 20] and fibroblasts [21], as well as vascular smooth muscle cells [22, 23], synthesise and secrete NGF. To date, however, NGF expression by airway smooth muscle cells has not been reported. For this reason, it was examined whether airway smooth muscle cell is a source of NGF in humans, and, if so, whether this expression might be regulated in inflammatory conditions, which were mimicked *in vitro* by addition of the pro-inflammatory cytokine interleukin (IL)-1β.

Material and methods

Primary culture of human airway smooth muscle cells

Human airway smooth muscle was obtained from healthy lung transplant donors after sudden death

(France-Transplant, Nancy, France). Bronchial smooth muscle was carefully dissected out, cut into small fragments and cultured in Roswell Park Memorial Institute (RPMI) 1650 medium containing L-glutamine (2 mM), penicillin (50 U·mL^{-1}) and streptomycin (50 μg·mL^{-1}), and supplemented with 10% foetal bovine serum (FBS). The muscle cells were cultured in 75 cm^2 flasks (Costar, Cambridge, MA, USA) in air containing 5% carbon dioxide in a humidified chamber at 37°C, with the medium changed twice weekly (all products supplied by GIBCO BRL, Cergy Pontoise, France). Cultures were rinsed in Hank's balanced salt solution, trypsinised (trypsin/ethylene diamine tetra-acetic acid) for 3 min, centrifuged at 200×*g* for 5 min, and cultured in Dulbecco modified Eagle medium (DMEM)/F12 supplemented with 10% FBS, nonessential amino acids (1%), L-glutamine (2 mM), penicillin (50 U·mL^{-1}), streptomycin (50 μg·mL^{-1}) (all from GIBCO BRL) and insulin (5 μg·mL^{-1}) (Lilly, St-Cloud, France). Cells were used experimentally at passage 6–8. They were characterised as smooth muscle cells morphologically (typical "hills and valleys" morphology) and immunocytochemically, using antibodies directed against vimentin and smooth muscle α-actin (Dako, Trappes, France), and showed 98% purity.

Experimental procedure

Cells were starved in low-FBS (0.3%) insulin-free DMEM/F12 for 24 h. The dose-dependent effect of IL-1β was studied by stimulating cells in the absence or presence of increasing concentrations of IL-1β (0.1–30 U·mL^{-1}) (Boehringer Mannheim, Mannheim, Germany) for 24 h. The time course of NGF expression was studied in the presence and absence of IL-1β (10 U·mL^{-1}) at 1.5, 2.5, 3.5, 5 and 10 h. Cell supernatant was collected, kept on ice, centrifuged (13,000×*g*, 5 min, 4°C), and stored at -20°C until NGF protein measurement. Cells were collected in 1 mL ribonucleic acid (RNA) extraction reagent (Tri-Reagent®; Sigma Aldrich, St-Quentin Fallavier, France) and kept at -20°C until analysis.

Quantification of nerve growth factor by enzyme-linked immunosorbent assay

A highly sensitive, commercially available, NGF-specific two-site enzyme-linked immunosorbent assay (ELISA) kit (Promega, Madison, WI, USA) was used according to the manufacturer's instructions to quantify NGF in the supernatant of control and treated smooth muscle cells. Briefly, 96-well immunoplates (Maxisorp^{TM}; Nunc, Roskilde, Denmark) were coated with polyclonal goat antihuman NGF antibody in coating buffer (25 mM carbonate buffer, pH 9.7). After overnight incubation at 4°C, the plates were washed (20 mM tris-(hydroxymethyl)-aminomethane (Tris)/hydrochloric acid (pH 7.4), 150 mM sodium chloride containing 0.05% (volume/volume) Tween®-20; Sigma Aldrich) and incubated in blocking buffer for 1 h. The supernatants and standard recombinant human NGF dilutions in the low-FBS medium were added to the wells and incubated for ⩾6 h at 37°C, and the wells were washed. Rat monoclonal anti-NGF antibody (0.25 μg·mL^{-1}) was added, overnight incubation was performed at 4°C and the wells were washed. Antirat horseradish peroxidase-conjugated immunoglobulin G was added and the wells were incubated for 2.5 h. Substrate (3,3′,5,5′-tetramethylbenzidine 0.02% and hydrogen peroxidase 0.01%) was then added and the colorimetric reaction stopped after 10 min by adding phosphoric acid (1 M). Optical density was measured in duplicate at 450 nm. The technique made it possible to detect NGF in the culture supernatant in the range 3.9–500 pg·mL^{-1} without any interference.

Extraction of total ribonucleic acid and reverse transcription

A modified guanidine thiocyanate technique, with phenol/chloroform extraction of total RNA [24], was used as previously described [25, 26]. Cells in Tri-Reagent® were mixed with chloroform (Sigma Aldrich), agitated on a vortex mixer for 15 min and incubated for 15 min at room temperature. After centrifugation (13,000×*g*, 4°C), RNA was precipitated in isopropanol (Sigma Aldrich) for 10 min at room temperature, centrifuged (13,000×*g*, 4°C) and dried at room temperature for 30 min. The isolated RNA was then diluted in ribonuclease (RNase)-free water. The RNA was extracted a second time, using the same procedure, to avoid any possible genomic deoxyribonucleic acid (DNA) contamination. Total RNA was incubated with 0.5 μg random primers for 5 min at 70°C and allowed to cool down to room temperature. The RNA was subsequently reverse-transcribed in 1×reverse transcription buffer (75 mM potassium chloride, 15 mM magnesium chloride, 10 mM dithiothreitol, 50 mM Tris, pH 8.5) containing 1 U·μL^{-1} RNase H-minus Moloney murine leukaemia virus reverse transcriptase (all reagents from Promega). The reaction was conducted for 1 h at 37°C, and the reverse transcriptase was then inactivated by heating at 99°C for 5 min. A negative control was performed without reverse transcriptase, and the complementary DNA (cDNA) product analysed by electrophoresis on a 2% agarose gel containing ethidium bromide (Euromedex, Souffelweyersheim, France) to verify the absence of genomic DNA contamination.

Quantification of nerve growth factor complementary deoxyribonucleic acid by polymerase chain reaction

The reverse transcription product (1 μL of a 1:20 dilution) was amplified by polymerase chain reaction (PCR) in 1×PCR reaction buffer: 2 μL of the reaction mix (Lightcycler-Faststart reaction Mix SYBR Green I; Roche Molecular Biochemicals, Mannheim, Germany), containing FastStart Taq DNA polymerase, deoxyribonucleoside triphosphate mix, SYBR Green I, 2.5 mM magnesium chloride, together with 0.4 μM of each primer (Invitrogen, Cergy Pontoise, France), sense 5′-CCAAGGGAGCAGCTTTCTATCCTGG-3′

and antisense 5′-GGCAGTGTCAAGGGAATGCTGA-AGT-3′, in a final volume of 20 μL for 35 cycles (15 s denaturation at 95°C, 10 s hybridisation at 58°C, 8 s elongation at 72°C) in a real-time fluorescent quantitative PCR thermocycler (Lightcycler, Roche Molecular Biochemicals). NGF cDNA was quantified on-line *via* SYBR Green fluorescence. A standard curve was obtained from amplification of a specific NGF cDNA purified after agarose extraction (Qiaex II®; Qiagen S.A., Courtaboeuf, France) and fluorescent quantification with Picogreen® (DNA Quantification reagent; Interchim, Montluçon, France). The specificity of the 189-base pair PCR product was validated using the fusion curve obtained after each reaction (Lightcycler) by electrophoresis, as well as by molecular sequencing. Results were normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression, similarly quantified (Lightcycler) using 0.4 μM of each primer, sense 5′-GGTGAAGG-TCGGAGTCAACGGA-3′ and antisense 5′-GAGG-GATCTCGCTCCTGGAAGA-3′, in a final volume of 20 μL for 45 cycles (15 s denaturation at 95°C, 10 s hybridisation at 57°C, 10 s elongation at 72°C).

Expression of results and statistical analysis

NGF levels are formulated either as picograms of NGF per millilitre of culture supernatant or as a percentage of control secretion. NGF messenger RNA (mRNA) expression is formulated either as the ratio of NGF cDNA to GAPDH cDNA, and expressed as fg NGF cDNA·pg GAPDH cDNA^{-1}, or as a percentage of control secretion. Results are presented as mean±SEM of six independent experiments performed in duplicate on airway smooth muscle cells obtained from three different donors. Differences between groups were analysed from raw data using an unpaired two-tailed t-test and two-way analysis of variance. Student-Newman-Keuls test was used to compare more than two variables. Data were considered significant at $p<0.05$.

Results

Constitutive nerve growth factor expression

Cultured airway smooth muscle cells expressed NGF constitutively (NGF protein: 21.4±7.8 pg·mL^{-1}, NGF cDNA: 14.6±5.4 fg·pg GAPDH cDNA^{-1} (mean±SEM)).

Effect of interleukin-1β on nerve growth factor expression

IL-1β (1–30 U·mL^{-1}) significantly and dose-dependently enhanced NGF protein secretion after 24 h ($p<0.05$) (fig. 1). The maximum increase in NGF production occurred at 10 U·mL^{-1} (from 21.4±7.8 to 43.5±4.8 pg·mL^{-1}), corresponding to a 2.0-fold increase over constitutive secretion levels.

The increase in NGF protein expression induced by IL-1β (10 U·mL^{-1}) was time-dependent and was greatest at 10 h (from 19.5±5.3 to 31.6±4.7 pg·mL^{-1}), *i.e.* an 11.5 pg·mL^{-1}, or 1.6-fold increase over constitutive secretion levels ($p<0.001$) (fig. 2a).

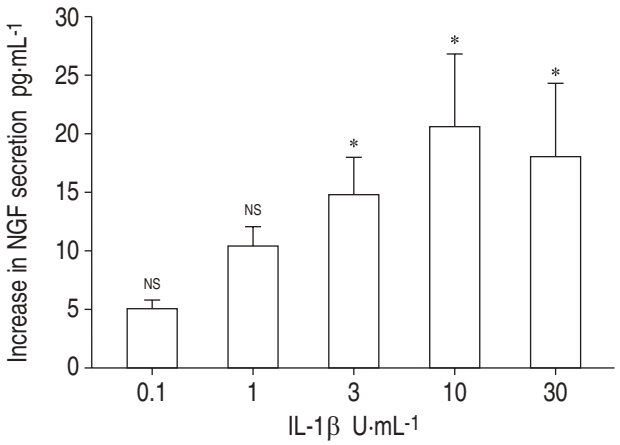

Fig. 1.–Dose-dependent effect of interleukin-1β (IL-1β) on nerve growth factor (NGF) production by human airway smooth muscle cells in culture (increase over baseline level of 21.4±7.8 pg NGF·mL supernatant^{-1}). Data are expressed as mean±SEM of six independent experiments performed in duplicate on cells from three different donors. NS: nonsignificant. *: $p<0.05$ *versus* control.

NGF mRNA levels were highest after 2.5 h of treatment (increasing from 13.2±4.3 to 31.9±5.2 pg NGF cDNA·pg GAPDH cDNA^{-1}), *i.e.* an 18.7 fg NGF cDNA·pg GAPDH cDNA^{-1}, or 2.4-fold increase over baseline levels ($p<0.05$) (fig. 2b). Levels returned progressively to baseline within 10 h.

Discussion

The present study clearly shows that airway smooth muscle cells in culture produce NGF, and that this production increases in response to the pro-inflammatory cytokine IL-1β.

The finding that human airway smooth muscle cells express NGF is consistent with and an extension of earlier reports that human smooth muscle cells of other origin, particularly vascular smooth muscle cells, express and secrete NGF [22, 23]. It also adds to knowledge regarding the cellular sources of NGF in the human lung, since other structural cells, such as fibroblasts [21] and epithelial cells [19, 20], as well as various inflammatory cells, such as mast cells [27], T-and B-cells [28, 29], eosinophils [7], and macrophages [30] (reviewed in [4]), have previously been reported to secrete NGF *in vitro*. The *in vitro* secretion of NGF by airway smooth muscle cells is also consistent with reports of NGF-positive immunolabelling of bronchial smooth muscle in bronchial biopsy samples from controls and/or asthmatic patients [31, 32]. The present data also provide evidence that NGF secretion is enhanced when airway smooth muscle cells are stimulated by IL-1β, a pro-inflammatory cytokine present in large quantities in the airways of asthmatic patients [33, 34]. Similarly, enhanced NGF secretion has been reported in other structural cells stimulated by IL-1β, including pulmonary fibroblasts [21] and

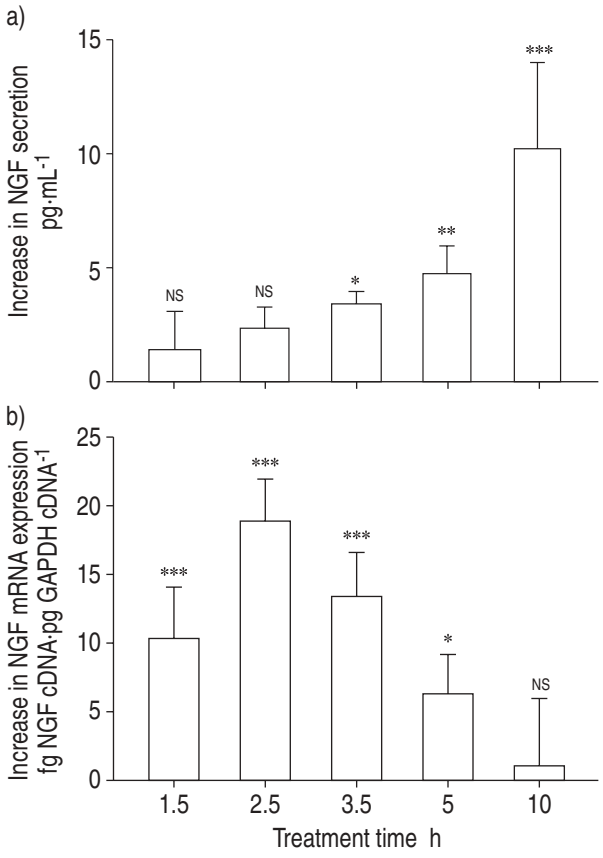

Fig. 2.–Time course of effect of interleukin-1β (10 U·mL^{-1}) on expression of a) nerve growth factor (NGF) and b) NGF messenger ribonucleic acid (mRNA). Data are presented as mean±SEM of six independent experiments performed in duplicate on cells from three different donors. cDNA: complementary deoxyribonucleic acid; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; NS: nonsignificant. *: $p<0.05$ *versus* control; **: $p<0.01$ *versus* control; ***: $p<0.001$ *versus* control.

human astrocytoma and glioblastoma cell lines [35]. This acquisition of a secretory phenotype by airway smooth muscle cells in culture after stimulation by pro-inflammatory agents, such as IL-1β or tumour necrosis factor-α, has been described as a new characteristic of airway smooth muscle [36, 37]. In particular, production of eotaxin [38] and granulocyte-macrophage colony-stimulating factor [39] are enhanced by IL-1β. Accordingly, overexpression of NGF by airway smooth muscle cells under inflammatory conditions mimicked by IL-1β *in vitro*, may be interpreted as confirmation that smooth muscle plays an active role in airway inflammation. It was also observed that the increase in NGF secretion induced by IL-1β was time-dependent and maximal after 10 h. This enhanced expression was preceded by increased NGF mRNA levels, which reached a maximum at 2.5 h. NGF transcription may be increased by IL-1β-induced activation of inflammatory transcription factors, such as activated protein-1 [40], since the activated protein-1 responsive element is present in the NGF gene promoter [41].

Excessive amounts of IL-1β have been implicated in the pathogenesis of various inflammatory conditions of the lung and airways, including asthma [33, 34]. IL-1β is produced by various cell types, particularly the infiltrated mast cells, many of which are found within the smooth muscle layer [42]. The dose of IL-1β used in the present study was within its biological range of action [43]. This suggests that increased levels of NGF might occur in inflammatory airway conditions. Indeed, enhanced NGF levels have been reported in the serum of patients with inflammatory diseases, particularly asthma [44]. Higher levels of NGF have also been reported in the bronchoalveolar [45] and nasal [46] lavage fluid of patients with asthma and rhinitis, respectively, compared with those of control subjects. Similarly, higher NGF levels have also been found in patients with asthma after allergen challenge [47]. Therefore, airway smooth muscle cells, which are capable of producing elevated levels of NGF after IL-1β stimulation, may participate in increasing NGF levels in inflamed airways. The increased NGF expression might play a role in the hyperresponsiveness observed in airway inflammatory conditions. Indeed, NGF causes hyperresponsiveness of guinea-pig [17] as well as of human [18] airways *in vitro*. In addition, NGF alone induces BHR in mice after lung-specific genetic overexpression [16]. Also, BHR induced by allergen challenge in sensitised mice *in vivo* is abolished by an antibody directed against NGF [15]. Hence, the authors propose that increased airway smooth muscle cell expression of NGF as demonstrated in the present study may play a role in BHR in asthma.

In conclusion, the present study shows that human airway smooth muscle may be an important source of nerve growth factor, and that interleukin-1β, a pro-inflammatory cytokine, may enhance this nerve growth factor secretion. This finding, together with recent reports that nerve growth factor is linked to asthma-associated symptoms in animals [15–17], suggests that airway smooth muscle, by increasing nerve growth factor secretion during airway inflammation, plays an important role in this inflammation.

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