Nerve growth factor is released by IL-1β and induces hyperresponsiveness of the human isolated bronchus

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ABSTRACT: Nerve growth factor (NGF) is a neurotrophic factor essential for the development and survival of neurons, and is also an important mediator of inflammation. It is released by airway cells stimulated by interleukin (IL)-1β. As IL-1β induces airway hyperresponsiveness (AHR) to the tachykinin NK-1 receptor agonist [Sar³,Met(O²)¹¹] substance P in human isolated bronchi, the aim of this study was to determine whether IL-1β was able to induce NGF release from isolated bronchi, and whether NGF might participate into IL-1β-induced AHR.

IL-1β (10 ng·mL⁻¹; 21°C; 15 h) increased the release of NGF from human isolated bronchi in vitro, and, in organ bath studies, the response of human bronchi to [Sar³,Met(O²)¹¹]-substance P (0.1 µm). A significant correlation was found between these responses. AHR induced by IL-1β was abolished by a blocking anti-human NGF antibody. Finally, NGF (1 ng·mL⁻¹; 37°C; 0.5 h) by itself induced a significant increase in [Sar³,Met(O²)¹¹]-substance P responsiveness. By contrast, it did not change the maximal contraction to acetylcholine.

In conclusion, the present study clearly demonstrated that nerve growth factor may participate in the airway hyperresponsiveness induced by interleukin-1β, which supports the neuro-immune cross-talk that may be active in the development of hyperresponsiveness in the human airways, and suggests nerve growth factor is active in the airways in asthma.

KEYWORDS: Asthma, bronchial hyperresponsiveness, inflammation, nerve growth factor, neurotrophin

Nerve growth factor (NGF) is a neurotrophic factor essential for the development and survival of neurons [1, 2]. It has recently been suggested to function as an important mediator of inflammation [3–5]. In the airways in particular, animal studies have shown NGF to contribute to the development of airway hyperresponsiveness (AHR). First, NGF blocking antibodies abolish the AHR created in sensitised and challenged mice [6]. In addition, AHR is induced by tissue-specific overexpression of NGF in the airways [7, 8]. Also, NGF by itself induces hyperresponsiveness of the guinea pig airways in vitro [9] and in vivo [10]. However, the evidence of NGF contributing to AHR in humans is lacking, although expression of NGF is increased in asthma [4, 5].

Previous studies reported that interleukin (IL)-1β induces AHR in several animal models [11–13]. In addition, BARCHASZ et al. [14] described that IL-1β causes AHR to the NK-1 tachykinin receptor agonist [Sar³,Met(O²)¹¹]-substance P (SP) in the human isolated bronchus. The mechanism of such hyperresponsiveness is unclear. In experiments performed in human airway cells in culture, IL-1β induced the release of NGF from fibroblasts [15], bronchial smooth muscle cells [16] and airway epithelial cells [17, 18]. However, whether IL-1β is able to induce release of NGF from the human bronchus ex vivo has not yet been reported.

Therefore, the aim of the present study was to determine whether: 1) IL-1β is able to induce release of NGF from the human isolated bronchus, and 2) NGF participates in AHR.

METHODS
Preparation of human bronchial tissue
Bronchial tissues were surgically removed from 42 patients with lung cancer (27 males, 15 females; mean ± sd age 61.0 ± 1.8 yrs). The protocol was approved by a local ethics committee (Comité de Protection des Personnes se Prêtant à la Recherche Biomédicale, Versailles, France). After resection, segments of human bronchi were taken and placed in oxygenated Krebs-Henseleit solution (composition: NaCl 119 mM, KCl
4.7 mM, CaCl₂ 2.5 mM, KH₂PO₄ 1.2 mM, NaHCO₃ 25 mM and glucose 11.7 mM). After removal of adhering lung parenchyma and connective tissue, rings of the same bronchus were prepared (5–7 mm length x 0.5–1 mm internal diameter), divided into paired groups and randomised [19]. The diameter solution at room temperature (21˚C) for 15 h in the presence or absence of IL-1β (10 ng·mL⁻¹) as previously reported [14, 15]. After incubation, paired bronchi were taken for contractile studies, and the supernatants kept aliquoted at -80˚C until NGF measurement. In another set of experiments, anti-human NGF blocking antibody or an irrelevant immunoglobulin (Ig) antibody (R&D Systems Europe, Lille, France) were incubated simultaneously to IL-1β for 15 h at 21˚C, before bronchi were taken for contractile studies.

The effect of exogenous NGF (0.01–1 ng·mL⁻¹) was studied on bronchial ring segments kept in Krebs-Henseleit solution at 4˚C overnight. Contractile studies were performed after NGF pre-treatment for 30 min directly in the organ baths.

**Experimental procedure**

Bronchial ring segments were suspended in 5 mL organ baths containing Krebs-Henseleit solution, gassed with 95% O₂–5% CO₂ and maintained at 37˚C. Each preparation was connected to an isometric force displacement transducer (UFI; Fiodem, Canterbury, Kent, UK) and EMKA amplifier (EMKA Technology, Les Ulis, France). Changes in tension were recorded on a polygraph. Preparations were suspended with an initial tension of 1.5 g, washed three times every 10 min, and equilibrated for another 30 min at 1–1.5 g.

The contractile response to the tachykinin NK-1 receptor agonist, [Sar⁹,Met(O²)¹¹]-SP was studied. Bronchial ring segments were washed out and acetylcholine was applied for maximal contraction. A 0.1 μM concentration of [Sar⁹,Met(O²)¹¹]-SP was selected for maximal NK-1 contractile response without interference with the tachykinin NK-2 receptor, and a 3 mM acetylcholine concentration for maximal response [20].

**Quantification of NGF protein by ELISA**

NGF was quantified in the supernatant of human isolated bronchi pre-treated for 15 h with IL-1β (10 ng·mL⁻¹) or solvent. A commercially available NGF-specific, highly sensitive, two-site ELISA kit was used following the manufacturer’s instructions (Promega, Madison, WI, USA). Briefly, 96-well immunoplates (Maxisorp™, Nunc, Roskilde, Denmark) were coated with a polyclonal goat anti-human NGF antibody in a coating buffer (25 mM carbonate buffer; pH 9.7). After overnight incubation at 4˚C, plates were washed (20 mM Tris-HCl, 150 mM NaCl with 0.05% (v/v) Tween®-20; Sigma-Aldrich, St Louis, MO, USA), and incubated in a blocking buffer for 1 h. The supernatants and the standard recombinant human NGF dilutions were incubated at 37˚C for 6 h and washed. Rat monoclonal anti-human NGF antibody (0.25 μg·mL⁻¹) was added for overnight incubation at 4˚C, and washed. Anti-rat horseradish peroxidase-conjugated IgG antibodies were incubated for 2.5 h, and the substrate (3,3′,5,5′-tetramethylbenzidine 0.02% and hydrogen peroxidase 0.01%) was added. The colorimetric reaction was stopped after 10 min by adding phosphoric acid (1 M), and the optical density was measured in duplicate at 450 nm. The detection range was 3.9–500 pg·mL⁻¹.

**Expression of results and statistical analysis**

All values are expressed as mean ± SEM. Contractile responses were expressed in g and as a percentage of the maximal contraction induced by acetylcholine (3 mM). NGF protein levels were expressed as pg of NGF·mg⁻¹·wet weight tissue. Differences between groups were analysed from raw data using an unpaired, two-tailed t-test, and a two-way ANOVA with Student-Newman-Keuls test when more than two variables were compared. Data were considered significantly different at p<0.05. The correlation between the NGF release (% increase) and contraction to [Sar⁹,Met(O²)¹¹]-SP (% increase) was evaluated using regression analysis. The coefficient of determination (r²) was determined from the regression curve.

**Drugs**

The drugs used were: [Sar⁹,Met(O²)¹¹]-SP, recombinant human IL-1β (Bachem, Bubendorf, Switzerland), recombinant NGF, anti-human NGF blocking antibody and an irrelevant IgG antibody (R&D Systems Europe). IL-1β and NGF were dissolved in distilled water at a concentration of 0.075 μM and 20 μg·mL⁻¹, respectively, and kept aliquoted at -80˚C until use. All drugs were dissolved in distilled water and further diluted in Krebs-Henseleit solution.

**RESULTS**

**Effect of IL-1β on NGF release and on AHR**

As previously reported, the human isolated bronchus incubated with IL-1β became hyperresponsive to the NK-1 receptor agonist [Sar⁹,Met(O²)¹¹]-SP (0.1 μM). Response was increased by 64.8±13.3% (p<0.001; n=24; fig. 1). In contrast, the response to acetylcholine (3 mM) was unchanged (2.12±0.18 and 2.21±0.17 g for IL-1β and saline, respectively; n=24).

**FIGURE 1.** Effect of interleukin (IL)-1β (10 ng·mL⁻¹; 15 h; 21˚C) on contractile responses induced by [Sar⁹,Met(O²)¹¹]-substance P (SP) (0.1 μM) on the human isolated bronchus. □: without IL-1β; ■: with IL-1β. n=24. *: p<0.05.
In parallel, a significant increase in the levels of NGF, although slight, was measured in the incubation medium after treatment with IL-1β (3.1 ± 0.5 and 2.5 ± 0.5 pg mg⁻¹ wet weight tissue after IL-1β and saline, respectively; n = 14; p < 0.05), i.e. a 28.8 ± 5.2% increase in NGF release. A positive correlation between NGF release and bronchial hyperresponsiveness to IL-1β was measured (r² = 0.59; p < 0.05; fig. 2).

**Effect of a NGF blocking antibody on AHR induced by IL-1β**

Pre-incubation of an anti-human NGF blocking antibody (500 ng mL⁻¹) simultaneously to IL-1β totally abolished the IL-1β-induced AHR to [Sar⁹,Met(O₂)¹¹]-SP (p < 0.05 for 50 ng mL⁻¹; fig. 3), but had no effects on the contractile response to this NK-1 receptor agonist per se. Under similar conditions, pre-incubation with an irrelevant IgG antibody had no effect on the IL-1β-induced hyperresponsiveness to [Sar⁹, Met(O₂)¹¹]-SP responses, or on the contractile response to this agonist.

**AHR to NGF**

Exogenous NGF alone had no effect on bronchial smooth muscle tone. NGF (0.01–1 ng mL⁻¹) induced a concentration-dependent increase in [Sar⁹, Met(O₂)¹¹]-SP response, with a maximal increase of 77.0 ± 31.5% at 1 ng mL⁻¹ (p < 0.05; n = 11; fig. 4). In contrast, NGF (1 ng mL⁻¹) did not change the acetylcholine (3 mM)-induced contraction (2.7 ± 6.1%, non-significant).

**DISCUSSION**

The present results show that IL-1β induces release of NGF from the human isolated bronchus, and this release is correlated with the increased response to [Sar⁹, Met(O₂)¹¹]-SP induced by IL-1β. In addition, IL-1β-induced hyperresponsiveness is abolished by pre-treatment with an anti-NGF blocking antibody. Finally, NGF by itself induces...
hyperresponsiveness of the human isolated bronchus to the tachykinin NK-1 receptor agonist [Sar⁹,Met(O²)¹¹]-SP in vitro.

This study was designed to investigate new mechanisms involved in previously reported AHR observed in inflammatory conditions created in vitro by the pro-inflammatory cytokine IL-1β [14]. In human airway cells in culture, IL-1β induced release of NGF from airway smooth muscle cells [16], epithelial cells [17, 18] and lung fibroblasts [15]. The current study demonstrated that IL-1β is also able to stimulate release of NGF from isolated ring segments from the human bronchus. Thus, overexpression of NGF may be due to increased release of NGF from bronchial structural cells, smooth muscle, epithelial cells and/or fibroblasts, upon stimulation by IL-1β. Acquisition of a secretory phenotype by the cells of human bronchi has been previously submitted as a new characteristic of airway cells actively participating in inflammation [15, 18, 20]. Additionally, increased release of NGF may also be derived from immune/inflammatory cells, such as lymphocytes, macrophages or mast cells [4] since these cells are also reported to secrete NGF [4, 21–23]. This suggests that increased NGF levels might occur in inflammatory conditions in vivo. Indeed, overexpression of NGF has been reported in humans in vivo, in situations where IL-1β is increased, particularly in asthma [24–27].

The present study also demonstrated that an anti-NGF blocking antibody abolishes the hyperresponsiveness to [Sar⁹,Met(O²)¹¹]-SP induced by IL-1β of the human bronchus in vitro, suggesting contribution of NGF to the development of AHR to IL-1β. Concordant suggestion has recently been reported in animal models, when administration of a blocking anti-NGF antibody prevents development of allergen-induced AHR to electrical field stimulation in mice [6] or attenuates the allergen-specific early airway response to ovalbumin in sensitised and challenged rats [28]. In addition, exogenous NGF has been reported to induce AHR by itself in the guinea-pig in vivo [9, 29], and tracheal hyperresponsiveness in the guinea-pig in vitro [10]. The present findings show that NGF also induces a dose-dependent AHR in the human isolated bronchus in vitro.

These results provide a potential mechanism to explain the findings of an upregulation of NGF protein in the airways of asthmatic patients, which has been associated with AHR, although no causal relationship between these events has been demonstrated. Indeed, enhanced NGF levels are reported in serum from patients with asthma, with the highest NGF levels in patients with more severe allergic asthma displaying a high degree of AHR [24]. Also, local upregulation of NGF protein was detected in bronchoalveolar lavage fluid from asthmatics, displaying AHR, as compared with control subjects [25]. These NGF levels have been further enhanced following allergen challenge in mild asthmatics [26]. In addition, previous data from Kassel et al. [27] support an upregulation of NGF in the bronchi as an early event in response to allergen in asthma, in association with an increased AHR. Indeed, increased NGF transcripts and AHR were detected in the airway tissue from mild asthmatics following exposure to allergen at a low subclinical dose [27]. All these findings suggest a close association between increased NGF levels and AHR. The present results bring support to an underlying causal mechanism since they demonstrate that exogenous NGF is responsible for an increased responsiveness of the human bronchus, and that hyperresponsiveness induced by IL-1β is abolished by anti-NGF antibodies. In contrast, no increased contraction was observed in response to a maximal acetylcholine response. This is in agreement with previous findings in human isolated bronchi that IL-1β neither modifies acetylcholine maximal response nor displaced the acetylcholine concentration-response curve [14]. This may be linked to the reported heterogeneity of AHR to contractile agonists in asthma, which is now clearly established, but remains unexplained [30].

The mechanism by which NGF induces AHR is not known. NGF is reported to activate immune/inflammatory cells, such as mast cells, lymphocytes, basophils or macrophages, as well as structural resident cells, such as fibroblasts or airway smooth muscle cells [5]. NGF is also able to sensitize neurons, and it induces an enhanced production of tachykinins in sensory neurons. This was shown by Hunter et al. [31], who demonstrated in the guinea-pig that NGF induces a phenotypic switch of airway sensory neurons 24 h after intratracheal administration, and that NGF increases SP content in neuronal cell bodies. Furthermore, Hoyle et al. [7] showed that transgenic mice overexpressing NGF in the airways develop a hyperinnervation of the airways, and an increase in SP content. However, these effects have been observed hours to days after NGF administration, involving transcriptional mechanisms that would probably not account for the rapid effect of NGF in the present experimental conditions. This evidence is further supported by the absence of sensory cell bodies within the preparation, indicating that upregulation of SP content cannot be a mechanism of NGF action in the human bronchus in these conditions in vitro. However, an early local mechanism may be proposed, as also observed in the hyperalgesia mechanism in the skin. Indeed, NGF, when applied directly to nociceptive afferents, lowers the threshold of thermal stimulation in an isolated skin-nerve preparation [32]. Similarly, NGF applied for 10 min on dorsal root ganglion neurons was able to abolish the tachyphylaxis observed in response to capsaicin or, even, to potentiate these responses [33]. Interestingly, NGF has also recently been shown in a model of inflammatory pain in the rat to have short-term effects on nociceptor sensitisation of sensory nerves of the dorsal root ganglia [34]. NGF might, therefore, contribute to an increased neuronal hyperexcitability, possibly through vanilloid receptor-1 activation without transcriptional events including their increased expression [35, 36]. These results, together with the present data, suggest that NGF is able to induce early post-transcriptional changes in neurons and cells, which may be involved in the tissue "sensitisation" [1, 37–39], and suggest parallel mechanisms in the generation of AHR and of hyperalgesia.

In conclusion, the current results clearly show that nerve growth factor released by interleukin-1β is involved in the airway hyperresponsiveness induced by this cytokine, and support the evidence of neuro-immune cross-talk in the hyperresponsiveness of the human bronchus.
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