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BDNF and DPP-IV in polyps and middle turbinates epithelial cells*

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SUMMARY

Hypothesis: *Neuropeptides released from sensory nerves may contribute to airway inflammation, particularly if their metabolism is impaired through defective inactivation and/or increased production. In the airways, neuropeptides are subjected to degradation by enzymes such as dipeptidyl peptidase (DPP-IV), and are upregulated by neurotrophins such as brain derived neurotrophic factor (BDNF). We therefore assessed in primary human nasal epithelial cells the expression of DPP-IV and BDNF under basal and inflammatory conditions.*

Methods: *Human epithelial cells were isolated from nasal polyps and middle turbinates, and grown on collagen-coated polycarbonate filters with an air liquid-interface. After three weeks of culture, constitutive cellular expression of DPP-IV and BDNF was assessed by measuring its activity and by ELISA, respectively. To mimick in vivo inflammatory conditions, cells were exposed apically and basolaterally to 50 ng/ml of TNFα, IL-1β, and IFN-γ for two days. DPP-IV activity and BDNF protein expression were measured in cell lysates and in the apical and basolateral media.*

Results: *Constitutive DPP-IV activity was similar in polyps and turbinates cells. In contrast, polyps epithelial cells expressed higher amounts of BDNF compared to turbinates derived cells. On the other hand, TNFα, IL-1β, and IFN-γ did not affect DPP-IV activity but significantly increased the cellular expression and the basolateral secretion of BDNF.*

Conclusions: *Our data show for the first time that primary human airway epithelial cells produced DPP-IV and BDNF under basal conditions. Furthermore, the production and secretion of BDNF were markedly increased in response to pro-inflammatory cytokines, confirming the involvement of BDNF in airway inflammation.*

Key words: allergic airway diseases, dipeptidyl-peptidase IV, neuropeptides, neurotrophins

INTRODUCTION

The concept of neurogenic inflammation events in the airways and their potential importance for airway diseases such as allergic rhinitis and asthma have been proposed [1,2]. Inflammatory mediators may modulate cholinergic and sensory nerves in the airways through the activation of receptors on nerve terminals, and sensory nerves in turn may amplify inflammation in the airways through the release of neuropeptides such as substance P (SP) and neurokinin A (NKA). SP and NKA are potent vasodilators and bronchoconstrictors, and stimulate mucin secretion from human submucosal glands in vitro. These neuropeptides are degraded and inactivated by peptidases namely neutral endopeptidase (NEP) and dipeptidyl peptidase (DPP-IV) located at the surface of airway epithelial cells, airway smooth muscle cells, submucosal gland cells as well as fibroblasts [3]. Thus reduced activity of these peptidases leads to exaggeration of the inflammatory response evoked by peptides released from peripheral endings of sensory nerves [4].

A further family of biologically active peptides which may interact with sensory neurons and thereby propagating airway neurogenic inflammation are the neurotrophins [5]. The most prominent members of the neurotrophin family are nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin (NT)-3 and NT-4/5. To date, most studies concerned NGF. Subjects with allergic rhinitis have decreased NGF mRNA in nasal scrapings and increased NGF protein in nasal lavage fluids [6]. Asthmatic patients exhibit significantly enhanced NGF levels in the bronchoalveolar lavage fluid and intense NGF-immunoreactivity in bronchial epitheli-

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um, smooth muscle cells and infiltrating inflammatory cells in the submucosa [7]. The biological effects of neurotrophins are mediated through signalling by different neurotrophin receptors, which are expressed not only on nerve cells but also on immune cells including monocytes, mast cells, B cells and T cells. Thus, NGF increases expression of SP in sensory neurons, induces mast cell degranulation, cytokine synthesis and regulates antibody production. Additionally, NGF is a chemoattractant and activation factor for eosinophils [8].

Recent studies have reported an inverse relationship between DPP-IV enzymatic activity and inflammation in the nasal mucosa [4] and a positive correlation between SP content in the plasma and sputum and airway inflammation [2]. These observations led us to evaluate in this study the expression of DPP-IV, a peptidase known to be involved in SP degradation, and BDNF, a neurotrophin involved in SP synthesis, in primary human nasal epithelial cells derived from middle turbinates and polyps. Specifically, we asked whether: 1) nasal epithelial cells constitutively express DPP-IV and BDNF, if so 2) the expression of DPP-IV and BDNF is different between cells derived from turbinates and polyps, and 3) DPP-IV and BDNF expression is affected by proinflammatory cytokines.

MATERIALS AND METHODS

Cell culture

Human airway epithelial cells were obtained from different patients after surgical polypectomies and partial middle turbinectomies. Patients gave informed consent and the protocol was approved by the institution's ethical committee. Cells were isolated by pronase (Roche, Mannheim, Germany) digestion as described [9]. Freshly isolated cells were seeded at a density of 5x105 cells/cm^2 onto 0.6-cm^2 collagen-coated Millicell polycarbonate filters (Millipore, Molsheim, France). Twenty hours after plating, the mucosal media was removed and the cells are allowed to grow at the air-liquid interface, which will allow the cells to develop a morphological and functional phenotype that closely resembles in vivo airway epithelium. The culture media consists of a 1:1 mix of DMEM:F12 (Invitrogen, Life Technologies, Basel, Switzerland), 2% Ultroser G (Pall-Biosepra, Cergy-Saint-Christophe, France), 100 U/ml penicillin and 100 μ-g/ml streptomycin. After 15-20 days, the epithelial monolayers developed a transepithelial resistance of >500 ohm.cm^2.

Experimental conditions

Constitutive expression: The basal expression of DPP-IV and BDNF was measured in cells after three weeks of culture, at which time the cells were fully differentiated to polarized respiratory epithelia that displayed histologic and biochemical characteristics similar to those observed *in vivo*.

Cytokine treatment: Polarized cells were treated apically and basolaterally with 50 ng/ml of TNFα·, IL-1β, and IFN-γ (Sigma, Sigma-Aldrich Chemie, Buchs, Switzerland) for two days. After the treatment, the apical and basolateral media were removed, the cells were washed with PBS and scraped in 100 mM Tris pH 8,0 containing 2% Triton-X100 and sonicated. An aliquot was kept for protein assay and the rest was used for measurement of DPP-IV activity and BDNF protein.

Measurement of DPP-IV activity

DPP-IV activity was measured as previously described [4]. The fluorogenic substrate used was the Gly-Pro-AMC (Bachem, Budendorf, Switzerland). Gly-Pro-AMC (5 mM) were incubated for 3h at 37°C with varying volumes of cell extract in Tris–HCl buffer, pH 8,0. AMC production was monitored by fluorescence measurement (λ_{ex}=370 nm, λ_{em}=460 nm). Fluorescence intensity was related to an AMC (Bachem, Budendorf, Switzerland) standard curve, and results were normalized to protein content.

Quantification of BDNF by enzyme-linked immunoabsorbent assay (ELISA)

To quantify BDNF protein levels in cell lysates and media, commercially available ELISA kit was used according to the manufacturer's instructions (Promega, Madison WI, USA). Plates were read in a microplate reader at 405 nm. Prior to analysis, the media were concentrated using the Amicon Centricon and Microcon centrifugal filter devices (Millipore, Billerica, MA, USA) with a molecular weight cut off of 3000 Da, according to the protocol of the manufacturer.

Statistical analysis

The significance of the difference between groups ($p < 0.05$) was determined with the nonparametric Kruskal-Wallis test and the Wilcoxon signed rank test for paired samples using the Statview program for Windows (Version 5.0.1, SAS Institute Inc.). Results are given as means ± SEM.

RESULTS

DPP-IV activity and BDNF protein expression in polyps and turbinates epithelial cells

DPP-IV expression was assessed by measuring its activity and BDNF expression was measured by ELISA in cell lysates. Epithelial cells obtained from either turbinates or polyps expressed significant DPP-IV activity and BDNF protein amount. A trend toward a higher level of DPP-IV activity was observed in polyps epithelial cells compared to turbinates cells, however this difference did not reach statistical significance (Figure 1A). Polyps epithelial cells also expressed a larger amount of BDNF protein than turbinates epithelial cells, and this difference was statistically significant (Figure 1B).

Effects of pro-inflammatory cytokines on DPP-IV and BDNF

Next we tested the effects of the pro-inflammatory cytokines TNFα, IL-1β, and IFN-γ (cytomix) on DPP-IV activity and BDNF expression in nasal epithelial cells. DPP-IV activity and BDNF protein amounts were measured in both cell lysates and in the apical and basolateral media. The results are shown in Figures 2 and 3. In untreated cells, DPP-IV and BDNF were essentially released in the apical and not in the basolateral

compartment. Cytomix affected neither cellular DPP-IV activity (Figure 2A) nor the apical release of DPP-IV (Figure 2B). In contrast, cytomix significantly increased BDNF expression in cells (Figure 3A) and the secretion of BDNF into the basolateral compartment (Figure 3B).

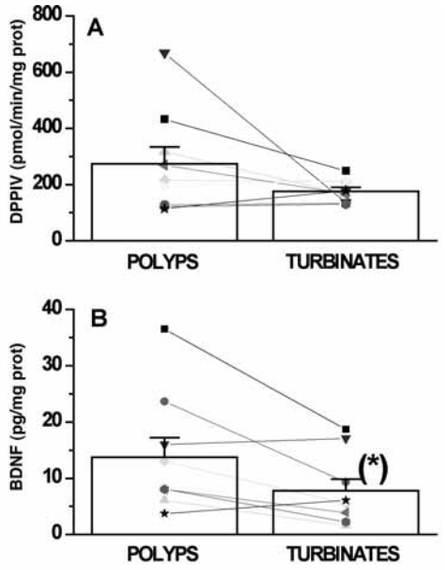

Figure 1. Basal expression of DPP-IV (A) and BDNF (B) in polyps and turbinates derived epithelial cells. Each symbol represents a patient, of which a polyp and turbinate biopsy were used to generate epithelial cell cultures. Means ± SEM (n=8) are also given, (*) $p<0.05$.

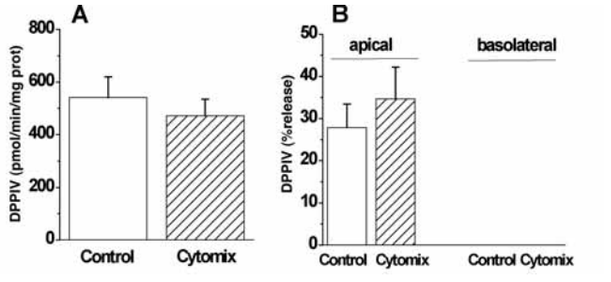

Figure 2. Effects of TNFα+IL-1β+IFNγ (cytomix) on DPP-IV activity in cell lysates (A) and in the apical and basolateral compartments (B). Results are shown as means ± SEM (n=6). % release = (DPP-IV activity in medium / total DPP-IV activity in cells + media) *100.

DISCUSSION

The results presented in this study demonstrate for the first time that 1) primary human nasal epithelial cells in culture produced DPP-IV and BDNF, 2) the production of BDNF and not of DPP-IV was higher in polyps cells than in turbinates cells, and 3) the production and basolateral secretion of BDNF and not of DPP-IV increased in response to the pro-inflammatory cytokines TNFα·, IL-1β, and INFγ. These results clearly suggest that BDNF may be involved in airway inflammation and hyper-reactivity, either through increasing production of

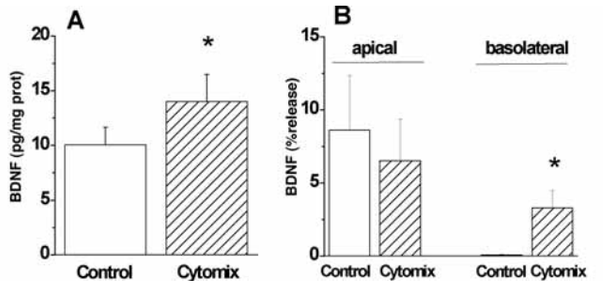

Figure 3. Effects of TNFα+IL-1β+IFNγ (cytomix) on BDNF protein levels in cell lysates (A) and in the apical and basolateral compartments (B). (*) $p<0.05$. Results are shown as means ± SEM (n=4-9). % release = (BDNF in medium / total BDNF in cells + media) *100.

neuropeptides by sensory neurons or by affecting immune cell function or both.

Neurogenic inflammation through the release of neuropeptides such as substance P, neurokinins A/B and C from nerve terminals has been suggested to have an important role in the development and progression of chronic inflammatory airway diseases. Neuropeptides are potent bronchoconstrictors and vasoactive agents. They have also potent effects on airway secretions and on inflammatory and immune cells. The bioactivity and thus the physiological effects of neuropeptides is terminated by their degradation by specific peptidases namely NEP and DPP-IV located on the membrane of a number of cell types within the lung [3]. Here we show for the first time that primary human nasal epithelial cells produced DPP-IV constitutively. This is in line with the immunostaining of human nasal mucosa where DPP-IV immunoreactivity was found in some epithelial cells [4]. Surprisingly, the proinflammatory cytokines TNFα, IL-1β, and IFNγ had no effect on DPP-IV production and secretion in our cells. Consistent with previous findings on MDCK and Caco-2 cells [10], DPP-IV was apically secreted in nasal epithelial cells. The presence of glycans on the DPP-IV ectodomain has been shown to be responsible for this apical targeting. Further studies are needed to assess the physiological significance of the apical localization of DPP-IV in nasal epithelial cells.

In situ expression of the different neurotrophins in the normal human lung was recently reported. Neurotrophins are constitutively and differentially expressed by the resident cells of human lung including bronchial mucosal epithelial cells, bronchial smooth muscle cells, bronchial gland cells, pulmonary macrophages as well as intrapulmonary arteries [11]. To date, almost all invitro studies focused on NGF, which production was demonstrated in human lung interstitial cells namely fibroblasts [12] and bronchial smooth muscle cells [13]. So far, data concerning neurotrophin expression and secretion by primary polarized human airway epithelial cells are missing. At present, the expression of NGF was reported only in the human lung epithelial A549 cell line [14,15]. Whereas A549 cells are representative of airway epithelial cells in some properties,

they are nevertheless tumour cells and it is well known that tumours have a modified expression pattern of growth and survival factors. To our knowledge, our present study demonstrates for the first time that primary polarized human nasal epithelial cells constitutively produced BDNF. Interestingly, a higher level of BDNF was found in polyps cells compared with turbinates cells. Furthermore, proinflammatory cytokines increased the intracellular protein level of BDNF and the basolateral but not the apical release of BDNF. This is of special interest since only neurotrophin secretion towards the basement membrane is of biological importance for signalling within the tissue. We can reasonably assume that BDNF might function like NGF which has been demonstrated to contribute to the inflammation and hyper-responsiveness associated with allergic diseases via effects on inflammatory cells. NGF promotes the infiltration and survival of eosinophils, suppresses apoptosis while inducing proliferation of mast cells [8], and increases tachykinin expression in airway sensory neurons [16]. It is noteworthy that, like DPP-IV, constitutive BDNF is apically secreted in primary nasal epithelial cells. In this respect, contradictory data has been reported. Thus, BDNF has been shown to be secreted predominantly in the basolateral compartment in LA-4 murine bronchial epithelial cells [17]. In transfected MDCK cells, BDNF was released either apically (18) or basolaterally [19].

In conclusion, this is the first study demonstrating DPP-IV and BDNF production by primary polarized human nasal epithelial cells. Furthermore, our study shows that the proinflammatory cytokines TNFα, IL-1β, and INFγ enhance the production and the basolateral secretion of BDNF. This finding suggests that, in vivo, the respiratory epithelia may amplify the ongoing local inflammatory process, by contributing to neurogenic inflammation through increased secretion of neurotrophins. It is now being recognized that local overproduction of neurotrophins during allergic inflammation modulate the activity of sensory neurons resulting in increased synthesis and release of neuropeptides such as substance P [20,21]. The activities of substance P include a broad range of functional responses of immune cells including lymphocytes, eosinophils, mast cells and macrophages leading to activation and differentiation of these cells [22,23]. Thus high concentrations of neurotrophins found in the serum [24] and the bronchoalveolar lavage fluid of asthmatic patients [7], as well as in the nasal fluid of patients with allergic rhinitis [25], may act as potent mediators of inflammation causing plasma extravasation and attracting inflammatory cells to the site of release. Therefore, neurotrophins may be considered as mediators of a vicious cycle of neuroimmune interactions that amplify airway inflammation and airway hyperresponsiveness in allergic diseases.

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