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dsRNA-induced expression of thymic stromal lymphopoietin (TSLP) in asthmatic epithelial cells is inhibited by a small airway relaxant

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ABSTRACT

Rationale: Thymic Stromal Lymphopoietin (TSLP) is considered a hub cytokine that activates dendritic cells and T-cells producing asthma-like Th_2-inflammation. Viral stimuli, a major cause of asthma exacerbations, have been shown to induce overexpression of TSLP in asthmatic epithelium. Capsazepine has multiple effects and is of interest because it relaxes human small airways. Here we have explored effects of capsazepine on viral surrogate (dsRNA)-induced TSLP and other cytokines (TNF-alpha, IL-8) in human bronchial epithelial cells (HBEC) from healthy and asthmatic donors.
Methods: HBEC obtained from healthy and asthmatic subjects were grown and stimulated with dsRNA. Cells pre-treated with capsazepine (3–30 μM), dexamethasone (0.1–10 μM) or an IkappaB-kinase inhibitor (PS1145, 30 μM) were also exposed to dsRNA (10 μg/ml). Cells and supernatants were harvested for analyses of gene expression (RT-qPCR) and protein production (ELISA,Western blot).
Results: dsRNA-induced TSLP, TNF-alpha, and IL-8 in asthmatic and non-asthmatic HBEC. Dexamethasone attenuated gene expression and protein release whereas capsazepine dose-dependently, and similar to a non-relaxant NFkB inhibitor (PS1145), completely inhibited dsRNA-induced TSLP and TNF-alpha in both healthy and asthmatic HBEC. Capsazepine reduced dsRNA-induced IL-8 and it prevented dsRNA-induced loss of the NF-κB repressor protein IkBα.
Conclusion: Additional to its human small airway relaxant effects we now demonstrate that capsazepine has potent anti-inflammatory effects on viral stimulus-induced cytokines in HBEC from healthy as well as asthmatic donors. Based on these data we suggest that exploration of structure–activity amongst the multifaceted capsazepinoids is warranted in search for compounds of therapeutic value in viral-induced, steroid-resistant asthma.

1. Introduction

Thymic stromal lymphopoietin (TSLP) is an interleukin 7-like, epithelial cell-derived cytokine that may be a major factor in the pathogenesis of asthma [1,2]. TSLP has several features of a hub cytokine for which redundancy may not apply. Its actions are multifacetted but most authors emphasize TSLP's priming action on dendritic cells to promote Th2 inflammatory responses in the airways [2–4]. Through its potential role in many features of the asthma paradigm TNF-alpha has also been widely implicated in the pathophysiology of this disease including its neutrophilic and refractory states [5,6]. TNF-alpha antibody treatment has been investigated in asthma producing variable results in clinical trials [6]. Bronchial biopsy studies have demonstrated overexpression of TSLP mRNA in the bronchial epithelial lining in severe asthma [7]. Preliminary data suggest also that TNF-alpha mRNA may be overexpressed in bronchial biopsies in severe asthma [8]. Viral infection and exacerbations may contribute to development of severe asthma that often fails to respond well to currently available drugs [9]. Indeed, rhinovirus infection has emerged as the most common cause of severe episodes of asthma in children and adults [10,11]. The potential role of TSLP in viral-induced exacerbations of asthma is additionally supported by the findings that double-stranded RNA (dsRNA), a viral infection surrogate stimulus, can evoke

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overexpression and overproduction of TSLP gene and protein in primary bronchial epithelial cells obtained from asthmatic individuals compared to epithelial cells from non-asthmatic donors [12]. The rapidly increasing attention to asthma as an epithelial disease [1,13,14] and to epithelium-derived TSLP as a disease-inducing cytokine [2,12] has created interest in finding treatments directed against TSLP. Thus, TSLP antagonizing antibodies have been patented for potential future treatment of asthma [15]. Amongst the drugs available today it appears that steroids can attenuate dsRNA-induced generation of TSLP from human bronchial epithelial cells but the efficacy of this mainstay asthma treatment is incomplete and varies [16,17]. There is also an unconfirmed report suggesting that beta agonists may stimulate TSLP generation [18]. Other drugs with known immunosuppressive actions, including cyclosporine and FK506, seem ineffective against dsRNA-induced TSLP [17]. During rhinovirus infection of airway epithelium dsRNA is generated. Through interaction with toll-like receptor 3 (TLR3) it is responsible for the viral-induced biological effects. Hence, dsRNA can be used as a viral infection surrogate in experimental cell studies [12,19,20]. TLR3 and nuclear factor (NF) κB-dependent mechanisms are involved in dsRNA-induced epithelial TSLP [16,21] and likely in the cellular production of TNF-alpha [22]. However, little is as yet known about the possibility of small molecular inhibition of TSLP and TNF-alpha in virally stimulated bronchial epithelial cells from asthmatic patients.

Capsazepine, a widely used transient receptor potential cation channel, subfamily V, member 1 (TRPV1) antagonist, was demonstrated to be a more effective relaxant of human small airways than beta agonists [23] This action was independent of TRPV1 antagonism [23]. Given this recent demonstration and given the fact that several established bronchodilator drug classes have been shown to possess additional anti-inflammatory effects, we considered the possibility that capsazepine could exert anti-inflammatory effects in human airways. In this study we have explored the possibility that capsazepine may reduce viral stimulus-induced generation of TSLP and other pro-inflammatory cytokines such as TNF-alpha and IL-8 in asthmatic bronchial epithelial cells.

2. Material and methods

2.1. Asthmatic subjects

Primary bronchial epithelial cells were obtained from six asthmatic patients as described in Table 1. The asthmatic group had a median age of 40 (range 22–58) and median $FEV_{1.0\%}$ predicted 77.3 (range 63.3–90.1). All asthmatic patients were atopic and were on inhaled corticosteroids. Ethical approval was obtained from regional ethical review board at Lund University, Sweden.

2.2. Epithelial cell culture

Epithelial brushings were obtained by bronchoscopy using a fiberoptic bronchoscope (Olympus, IT160, Tokyo, Japan) and a standard sterile single-sheathed nylon cytology brush was used to sample epithelial cells from the bronchi in accordance with standard published guidelines. On average, 4 consecutive brushings were sampled from the bronchial mucosa of the second and third generation bronchi. Cells were harvested into 5 ml sterile phosphate-buffered saline (PBS) after each brushing. At the completion of the procedure, 5 ml RPMI with 20% fetal bovine serum (FBS) was added and the sample centrifuged at 150×*g* for 5 min to harvest the cells. Epithelial cell purity was assessed by performing differential cell counts of the harvested cell suspension as previously described [12,24].

Primary cultures of human bronchial epithelial cells (HBEC) were established by seeding bronchial brushings from asthmatic subjects into collagen coated tissue culture flasks containing 3 ml of serum-free hormonally supplemented Bronchial Epithelium Growth Medium (BEGM; Clonetics, San Diego, CA) as previously described [12,24]. Additional HBEC from six healthy donors were purchased from Lonza (Walkersville, Maryland, USA) as cryopreserved cells. Cultures were routinely tested for mycoplasma infection. HBEC were seeded into 12-well plates (Nunc; Life technologies) and when 80%–90% confluent the growth medium was replaced with Bronchial Epithelial Basal Medium (BEBM) (Clonetics, San Diego, CA) containing 1% ITS and 0.1% BSA and the cells were rendered quiescent for 24 h before start of the experiment.

2.3. Stimulation of cells with dsRNA and treatment with capsazepine, capsaicin and dexamethasone

For all experiment cells were used at passage 2–4 and stimulated with a dsRNA analogue, polyinosine-polycytidylic acid, (Invitrogen Ltd., Paisley, UK) and tested in the range 0.01–25 μg/ml to establish dose-dependent effects (e-Fig. 1). The optimal concentration of 10 μg/ml dsRNA was chosen to be used in combination with other compounds in this study. Capsazepine, 1–30 μM (Sigma–Aldrich, UK), capsaicin (Sigma–Aldrich, UK), dexamethasone 0.1–10 μg/ml (Sigma–Aldrich, UK), and PS1145, 30 μM (a commonly employed IkK inhibitor [25,26], Sigma–Aldrich, UK) were dissolved in DMSO and added to the cells 1 h prior to stimulation with 10 μg/ml dsRNA. Vehicle control was prepared as having the highest concentration of DMSO present. After the treatment with drugs and control solution, HBEC were stimulated for either 3 h or 24 h by adding dsRNA to the wells. Thus, the compounds remained in contact with HBEC for 1 h + 3 h or 1 h + 24 h. Then cell supernatant was removed for protein analysis and cells lysed and harvested for either mRNA analysis or western blot as described below.

2.4. RNA extraction and quantification of TSLP, TNFα and IL-8 gene expression with RT-qPCR

Total RNA was extracted from primary HBEC using a RNA extraction kit NucleoSpin® RNA II (MACHERY-NAGEL, Düren, Germany), according to manufacturer's instructions. One microgram of mRNA was reverse transcribed to cDNA using a RT-kit (PrimerDesign, Southampton, UK) and quantitative PCR was performed as previously described [27]. Briefly, thermocycling and real-time detection of PCR products were performed on an iCyclerIQ sequence detection system (Stratagene, Mx3000P, La Jolla, CA, USA) with standard cycling parameters. Genes of interest were normalized to the geometric means of Ubiquitin C (UBC) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the delta delta Ct method as previously described [27]. Within group comparisons were normalized to one control sample of a single patient using the delta delta Ct method. Between groups comparison were only conducted where conditions were the same and normalized to the

Table 1
Asthmatic patient characteristics.

Sex	Age	$FEV_{1.0\%}$	PD20	Atopy	Treatment daily dose
M	27	81.7	>2000	Yes	Pulmicort® 800 μg, bricanyl prn[a]
M	22	79.9	251.5	Yes	Symbicort® 320 μg/9 μg
M	58	63.3	68	Yes	Symbicort® 320 μg/9 μg
M	30	90.1	1620	Yes	Symbicort® 320 μg/9 μg, bricanyl prn
F	50	74.7	69.3	Yes	Pulmicort® 800 μg, bricanyl prn
F	50	72.3	138.2	Yes	Pulmicort® 800 μg

[a] prn = prescribed as needed.