Toxicology Letters 258 (2016) 216–226

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Effects of ceftaroline on the innate immune and on the inflammatory responses of bronchial epithelial cells exposed to cigarette smoke

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HIGHLIGHTS

- Ceftaroline is a prodrug used to treat Community Acquired Pneumonia.
- Ceftaroline counteracts the effects of cigarette smoke in bronchial epithelial cells.
- Ceftaroline exerts immune-regulatory activities.
- Ceftaroline exerts anti-inflammatory activities.
- Ceftaroline improves host anti-microbial activities.

ARTICLE INFO

Article history:
Received 11 March 2016
Received in revised form 24 June 2016
Accepted 29 June 2016
Available online 7 July 2016

Keywords:
Cephalosporins
Cigarette smoke
Inflammation
Bronchial epithelial cells

ABSTRACT

The tobacco smoking habit interferes with the innate host defence system against infections. Recurrent infections accelerated the functional respiratory decline. The present study assessed the effects of ceftaroline on TLR2 and TLR4 and on pro-inflammatory responses in airway epithelial cells (16HBE cell line and primary bronchial epithelial cells) with or without cigarette smoke extracts (CSE 10%).

TLR2, TLR4, LPS binding and human beta defensin 2 (HBD2) were assessed by flow cytometry, NFkB nuclear translocation by western blot analysis, IL-8 and HBD2 mRNA by Real Time PCR; the localization of NFkB on the HBD2 and IL-8 promoters by ChiP Assay.

CSE increased TLR4, TLR2 expression, LPS binding and IL-8 mRNA; CSE decreased HBD2 (protein and mRNA), activated NFkB and promoted the localization of NFkB on IL-8 promoter and not on HBD2 promoter. Ceftaroline counteracted the CSE effect on TLR2 expression, on LPS binding, on IL-8 mRNA, HBD2 and NFkB in 16HBE. The effects of ceftaroline on HBD2 protein and on IL-8 mRNA were confirmed in primary bronchial epithelial cells.

In conclusion, ceftaroline is able to counteract the effects of CSE on the innate immunity and pro-inflammatory responses modulating TLR2, LPS binding, NFkB activation and activity, HBD2 and IL-8 expression in bronchial epithelial cells.

1. Introduction

A key component of the innate immune response against infections is represented by the family of toll like receptors (TLRs), pattern-recognition receptors (Imler and Hoffmann, 2001). TLR2 and TLR4, predominantly expressed by monocytes/macrophages and neutrophils, are also expressed by airway epithelial cells (Imler and Hoffmann, 2001). The activation of TLRs, finalised to the release of antimicrobial peptides including Human Beta-Defensin2 (HBD2), leads also to the activation of pro-inflammatory pathways including NF-kappaB activation and to the release of neutrophil chemotactic molecules such as IL-8 (Kawai and Akira, 2011).

Cigarette smoke is the major risk factor for Chronic Obstructive Pulmonary Disease (COPD) (Brusselle et al., 2011). Recurrent infections in COPD patients concur to disease progression and accelerate functional respiratory decline (Curtis et al., 2007; Simpson et al., 2009). The tobacco smoking habit interferes with

Abbreviations: TLR, toll like receptors; LPS, lipopolysaccharide; HBD2, human beta defensin; COPD, chronic obstructive pulmonary disease; CSE, cigarette smoke extracts; NF-kappaB, nuclear factor kB; IL-8, interleukin 8.

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http://dx.doi.org/10.1016/j.toxlet.2016.06.2105
0378-4274/

the innate host defence system by reducing mucociliary clearance, by disrupting the epithelial barrier and stimulating the migration of inflammatory immune cells, including neutrophils, into the damaged site (Herr et al., 2009). It has been shown in vitro that in the presence of cigarette smoke extracts (CSE) the activation of innate immunity receptors leads to a prevalent pro-inflammatory response and to a reduced protective anti-microbial response (Pace et al., 2008). Accordingly, HBD2 is reduced in the epithelium of central airways in smoker COPD patients and correlates with airway obstruction (Pace et al., 2012).

The macrolide antibiotic azithromycin, which in addition to its antibacterial effects has anti-inflammatory properties, has been shown to be effective in the prevention of COPD exacerbations and in the restoration of the defective phagocytic function of alveolar macrophages in COPD (Hodge et al., 2008). Macrolides can reduce the neutrophil number, the levels of CXCL8, and neutrophil proteases within the airways (Simpson et al., 2009).

The new antibiotic cephalosporin, ceftaroline fosamil, is a prodrug active against typical bacterial pathogens most often associated with Community Acquired Pneumonia (CAP), including resistant Gram-positive pathogens such as multidrug-resistant Streptococcus pneumoniae and methicillin resistant *Staphylococcus aureus* (MRSA) (Lodise et al., 2015; Stryjewski et al., 2015).

Although it is known that antibiotics belonging to the macrolide family are effective because of their anti-inflammatory activities (Hodge et al., 2008), it is unknown whether ceftaroline (the active metabolite of ceftaroline fosamil) exerts immune-modulatory activities in airway epithelial cells. The general aims of the present study were to assess the ability of ceftaroline to counteract the effects of CSE exerting immunoregulatory, anti-inflammatory, and host anti-microbial activities via the modulation of TLR2, LPS binding, IL-8 expression, and HBD2 expression and production in bronchial epithelial cells.

2. Materials and methods

2.1. Cell cultures

16HBE, an immortalized human normal bronchial epithelial cell line (Cozens et al., 1992), a primary human normal bronchial epithelial cell line (NHBE) (American Type Culture Collection (ATCC), Manassas V.A.; PCS-300-010), human foetal lung fibroblasts (HFL-1) (ATCC) were used. Cell cultures were maintained in a humidified atmosphere of 5% CO_2 in air at 37 °C. 16HBE were cultured as adherent monolayers in Eagle's minimum essential medium (MEM) (Gibco, BRL, Germany), supplemented with 10% heat-inactivated (56 °C, 30 min) fetal bovine serum (FBS) (Gibco), 1% MEM (non-essential aminoacids) (Euroclone), 2 mM L-glutamine and 0.5% gentamicin (Gibco). NHBE were grown in complete airway epithelial cell basal medium according to ATCC Primary Cell Solutions™ system. HFL-1 were maintained in Dulbecco's modified Eagle's medium supplemented with glutamine, antibiotics (penicillin and streptomycin) and 10% foetal bovine serum (Gibco).

2.2. Stimulation and treatments of the cells

Cells were grown in Plate 6 well (BD Falcon, Franklin Lakes, New Jersey) for 3 days until 80–90% confluency. 16-HBE cells were then treated in medium 1% FBS with CSE 10%, and ceftaroline (AstraZeneca). For ceftaroline, dose-response (0.6 ng/ml; 6 ng/ml; 60 ng/ml; 600 ng/ml;6 mg/ml) experiments were initially performed. Ceftaroline was added 1 h before CSE cell stimulation, the time of incubation of CSE is 3 h to assess the nuclear translocation of NF-kappaB, and 24 h to assess protein and cytokine expression. In some experiments, the effects of ceftaroline were evaluated in cells stimulated with IL-1 beta alone and combined with CSE. The concentration of CSE and time of incubation were selected on the basis of previous findings (Cozens et al., 1992). NHBE were treated in cell basal medium according to ATCC Primary Cell Solutions™ system. At the end of stimulation, cells, culture supernatants or RNA were collected for further evaluations. Three replicates were performed for each experiment.

2.3. Preparation of cigarette smoke extracts (CSE)

Commercial cigarettes (Marlboro) were used in this study. Cigarette smoke solution was prepared as described previously (Pace et al., 2013a). Each cigarette was smoked for 5 min and one cigarette was used per 10 ml of PBS to generate a CSE-PBS solution. The CSE solution were filtered through a 0.22 micrometer-pore filter to remove bacteria and large particles. The smoke solution was then adjusted to pH 7.4 and used within 30 min of preparation. These solutions were considered to be 100% CSE and diluted to obtain the desired concentration in each experiment. The concentration of CSE was calculated spectrophotometrically measuring the OD as previously described at the wavelength of 320 nm. As previously reported (Pace et al., 2008), the presence of contaminating LPS on undiluted CSE was assessed by a commercially available kit (Cambrex Corporation, East Rutherfort, New Jersey, USA) and was below the detection limit of 0.1 EU/ml.

2.4. Cell viability assay

Cell viability was evaluated by CellTiter 96 Aqueous One Solution Cell Proliferation Assay, (PROMEGA, Madison WI USA) according to the manufacturer's instructions. One Solution reagent contains MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfopheyl)2H-tetrazolium]. Cells were plated in 96-well plate and were treated for 24 h with ceftaroline (0.6 ng/ml; 6 ng/ml; 60 ng/ml; 600 ng/ml;6 mg/ml) and, after drug treatment, 20μL of One Solution reagent was added to each well, and incubated for 20 min for the 16HBE at 37 °C, 5% CO_2. The absorbance was read at 490 nm on the Microplate reader ^{wallac}Victor^2 1420 Multilabel Counter (Perkin Elmer). Results are expressed as absorbance values.

2.5. Annexin V expression (cell apoptosis) by flow cytometry

Cell apoptosis was evaluated by staining with annexin V-fluorescein isothiocyanate and propidium iodide using a commercial kit (Bender MedSystem, Vienna, Austria) following the manufacturer's directions. Cells were analyzed by flow cytometry using a FACSCalibur (Becton Dickinson, Mountain View, CA) analyzer equipped with an Argon ion Laser (Innova 70 Coherent) and Consort 32 computer support. The propidium negative and annexin V negative cells (i.e. viable cells) were present in the lower left quadrant; the propidium positive cells (i.e. necrotic cells) were present in the upper left quadrant; the propidium and annexin V double positive cells (i.e. late apoptotic cells) were present in the upper right quadrant and the single annexin V positive cells (i.e. early apoptotic cells) were present in the lower right quadrant.

2.6. TLR2, TLR4, and HBD2 expression by flow cytometry

The expression of TLR2, TLR4, and HBD2 was evaluated by flow-cytometry. Flow cytometry analyses were performed on a Becton Dickinson FACSCalibur System. The cells (16HBE) were incubated with a mouse PE conjugated anti-human TLR4 or a mouse FITC conjugated anti-human TLR2 (eBioscience, San Diego, CA), or with a rabbit polyclonal antibody anti-HBD2 (Santa Cruz Biotechnology) followed by a FITC conjugated anti-rabbit IgG (Dako). For the detection of intracellular HBD2, cells were cultured overnight with