Fluticasone propionate reduces bacterial airway epithelial invasion

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ABSTRACT: Fluticasone propionate reduces the frequency and severity of the episodes of exacerbation of chronic obstructive pulmonary disease (COPD). *Streptococcus pneumoniae* and *Haemophilus influenzae* are frequently isolated in these episodes. Both express phosphorylcholine, an epitope that mediates their interaction with airway epithelial cells via the platelet-activating factor receptor (PAFR).

The present work studies the effects of fluticasone propionate on the expression of PAFR on human airway epithelial cells, the invasion of these cells by *S. pneumoniae* and *H. influenzae*, and the course of pneumococcal infection *in vivo*. The following were used in the experiments: *S. pneumoniae* and *H. influenzae* isolated from patients with COPD, cell cultures of type II pneumocytes and bronchoepithelial cells, and a mouse model of lung infection.

Fluticasone propionate was found to reduce PAFR expression on the surface of the two cells types studied. All *S. pneumoniae* and *H. influenzae* isolates expressed phosphorylcholine. Treatment of both cells lines with fluticasone propionate reduced invasion of both microorganisms and reduced the bacterial load of mice infected with *S. pneumoniae*.

Fluticasone propionate reduces the invasion of airway epithelial cells by *Streptococcus pneumoniae* and *Haemophilus influenzae* through its effect on platelet-activating factor receptor. These results may help explain the beneficial effects of fluticasone propionate on chronic obstructive pulmonary disease exacerbations.

KEYWORDS: Airway epithelial cells, bacterial infections, chronic obstructive pulmonary disease, fluticasone

Patients with chronic obstructive pulmonary disease (COPD) often suffer episodes of exacerbations of COPD during the course of the disease [1]. These episodes impact negatively on health status, worsen prognosis and are extremely costly. Treatment with inhaled steroids (alone or in combination with long-acting β_2 -agonists) reduces the number of exacerbations of COPD [2], particularly in the subgroup of patients with moderate-to-severe disease [2, 3] in whom pulmonary inflammation is enhanced [4].

Streptococcus pneumoniae and *Haemophilus influenzae* are frequently isolated in airway secretions of patients with COPD, particularly during exacerbations [5]. Phosphorylcholine is a surface epitope of *S. pneumoniae* and *H. influenzae* [6, 7] that binds specifically to the high-affinity platelet-activating factor receptor (PAFR) expressed on the surface of, among other cells types, airway epithelial cells [8]. The interaction between phosphorylcholine and PAFR is a key pathogenic element of *S. pneumoniae* and *H. influenzae* infections [9, 10]. This is demonstrated by the fact that PAFR-deficient mice are more resistant

to pneumococcal pneumonia than wild-type mice [11] and that intratracheal administration of *S. pneumoniae* and a PAFR antagonist to rabbits reduces bacterial loads in lung lavages [9]. Likewise, phosphorylcholine favours the persistence of *H. influenzae* in the airway epithelia and promotes the formation of biofilms [12, 13].

Given that the expression of PAFR is elevated in chronically inflamed airways [9, 10], such as those found in COPD patients, the present authors hypothesised that steroids may reduce exacerbations by downregulating PAFR expression. To test this hypothesis, the *in vitro* effect of fluticasone propionate on the following was investigated: 1) the expression of PAFR on airway epithelial cells; 2) the invasion of airway epithelial cells by *S. pneumoniae* or *H. influenzae*; and 3) the course of pneumococcal infection *in vivo*.

MATERIALS AND METHODS

Bacterial isolates and reagents

S. pneumoniae and *H. influenzae* strains were isolated from spontaneous sputum samples from patients with stable and exacerbated COPD.

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STATEMENT OF INTEREST

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Bacterial isolates were identified and maintained following standard techniques. PAFR antagonist (1-O-Hexadecyl-2-acetyl-*sn*-glycerol-3-phospho-(N,N,N-trimethyl)-hexanolamine) was purchased from Calbiochem (San Diego, CA, USA). The fluticasone propionate was kindly provided by Glaxo-SmithKline (London, UK).

Cell culture and treatments

The human bronchoepithelial immortalised cells 16HBE14o- and the human lung carcinoma cells A549 (ATCC CCL185) derived from type-II pneumocytes were used in the present study. The cells were propagated in Earl's minimal essential medium plus 1% L-glutamine culture medium and in RPMI 1640 plus 1% HEPES (hydroxyethyl piperazine ethane sulphonic acid), respectively, supplemented with 10% foetal calf serum and 1% penicillin-streptomycin (Sigma Aldrich, St Louis, MO, USA). Cells were incubated in 24-well tissue culture plates at 37°C and 5% CO₂ until confluence was reached ($\sim 5 \times 10^5$ cells per well) and then used in the experiments. Monolayers of each cell line were treated at 37°C with fluticasone propionate (0.1 µM, 4 h) or PAFR antagonist (0.05 µM, 30 min), which were both dissolved in the cell culture medium. The invasion assays were performed as previously described [14]. Briefly, after the incubation of the epithelial cells with the bacterial suspension, the wells were washed with PBS and incubated with fresh medium containing gentamicin (100 µg·mL⁻¹) to kill extracellular bacteria. After 1 h, an aliquot of the medium was plated to confirm killing of extracellular bacteria, and the gentamicin-containing medium was rewashed. The epithelial cells were lysed and intracellular bacteria were quantified by plating appropriate dilutions on blood agar plates.

Flow cytometry

Monolayers of treated cells were detached with 0.25% trypsin-EDTA (Sigma Aldrich), washed with PBS and fixed for 18 h at 4°C with 1% paraformaldehyde in PBS. Cells were then washed, resuspended at a concentration of 5×10^4 cells·mL⁻¹ in PBS-1% bovine serum albumin (BSA), and incubated for 3 h at 25°C with rabbit anti-PAFR diluted at 1:500 (Alexis Biochemicals, San Diego, CA, USA). After washing, cells were incubated for 1 h at 25°C with goat anti-rabbit immunoglobulin (Ig)G conjugated fluorescein isothiocyanate diluted at 1:500. Finally, cells were resuspended in isoton buffer for analysis. Analysis was carried out in an Epics XL flow cytometer using the Expo32 software (both Beckman Coulter, Madrid, Spain). Nonspecific binding was corrected with the corresponding isotype-matched antibodies.

Immunoblot assay

Phosphorylcholine expression by the bacterial isolates was assessed by immunoblotting, as described previously [7]. Bacterial suspensions were spotted on to nitrocellulose membranes. After blocking in PBS-1% BSA, membranes were sequentially incubated with specific monoclonal antibody (mAb) against phosphorylcholine (TEPC-15; Sigma Aldrich) and a goat anti-mouse IgA/alkaline phosphatase conjugate (Caltag Laboratories, Burlingame, CA, USA). Washing with PBS was included after incubations. Alkaline phosphatase was visualised on the blots using a BCIP/NBT fast kit (Sigma Aldrich). Finally, the filters were analysed by densitometry

using a BioImage densitometer and Whole band 3.1 software (both Millipore, Barcelona, Spain). *Klebsiella pneumoniae*, a microorganism that does not present phosphorylcholine on its surface [15], was used as a negative control.

Murine model of lung infection

Male (20–25 g) ICR-CD1 mice (Harlan Ibérica, Barcelona, Spain) were anaesthetised and inoculated intratracheally with 10⁶ colony-forming units of *S. pneumoniae* using a blunt-end feeding needle. Pneumococci were inoculated with saline or saline solutions of PAFR antagonist (0.25 µg) or fluticasone propionate (10 µg; n=16 per group). At 24 and 48 h, eight animals of each group were sacrificed. The lungs were aseptically removed and homogenised for quantitative bacterial cultures by plating appropriate dilutions on blood agar plates [14]. All animal experiments were carried out according to institutional and national guidelines and approved by the Experimental Animal Committee of the Universitat Illes Balears (Palma de Mallorca, Spain).

Statistical analysis

All values are presented as mean ± SEM. A two-tailed unpaired t-test was used to compare the effect of each treatment with the control. A p-value of ≤0.05 was considered significant.

RESULTS

Fluticasone propionate reduces PAFR expression on airway epithelial cells.

Pneumocytes type-II derived cells A549 and bronchoepithelial cells 16HBE14o- constitutively expressed PAFR (fig. 1). Treatment with the PAFR antagonist reduced the expression of the receptor in both cell types compared with untreated cells (p<0.05 for each cell type *versus* control). Interestingly, fluticasone propionate also reduced the expression of PAFR in A549 and 16HBE14o- (p<0.05 for each cell type *versus* control).

Effect of fluticasone propionate on the invasion of airway epithelial cells by *S. pneumoniae* and *H. influenzae*

The presence of phosphorylcholine on the surface of the various *S. pneumoniae* and *H. influenzae* isolates from COPD patients was investigated first, as the expression of phosphorylcholine undergoes phase variation in both microorganisms [12, 16]. This was examined using immunoblot analysis with the mAb TEPC-15, which is highly specific for the phosphorylcholine epitope [17]. All *S. pneumoniae* and *H. influenzae* isolates reacted with TEPC-15 but a wide variability in the expression of phosphorylcholine was observed among different isolates of either *S. pneumoniae* or *H. influenzae* (fig. 2).

The effect of fluticasone propionate on the invasion of the epithelial cells by both microorganisms was studied by selecting one strain of each species in which the phosphorylcholine expression was intermediate (fig. 2). In preliminary experiments, it was observed that neither PAFR antagonist nor fluticasone propionate affected bacteria or cell viability. The effect of different fluticasone propionate doses (0.05, 0.1 and 0.5 µM) on the invasion of either *H. influenzae* and *S. pneumoniae* to A549 cells was also tested. All concentrations tested reduced the invasion of both microorganisms in a dose-dependent manner (data not shown). The intermediate concentration (0.1 µM) was selected for further experiments because it was the lowest concentration that produced the best

FIGURE 1. The effect of fluticasone propionate on the expression of platelet-activating factor receptor (PAFR) on the airway epithelial cells surface. a) A549 and b) 16HBE14o- cells were treated with PAFR antagonist (0.05 μ M for 30 min) or fluticasone propionate (0.1 μ M for 4 h). The cell surface-associated PAFR was determined by flow cytometry analysis using a monoclonal antibody, anti-PAFR. The results are from five independent experiments. *: $p < 0.05$ versus untreated cells.

results for treatment comparison. As shown in figure 3, treatment of monolayers of both types of airway epithelial cells with 0.1 μ M of fluticasone propionate significantly reduced the invasion of *H. influenzae* and *S. pneumoniae* with respect to the untreated control ($p < 0.05$ for each pathogen and cell type *versus* control). Invasion was reduced between 60 to 28% depending on the microorganism and the epithelial cell type. PAFR antagonist was used as a positive control and, as expected, reduced the capacity to invade the epithelial cells of both microorganisms.

Effect of fluticasone propionate on the lung colonisation of *S. pneumoniae* in vivo

Fluticasone propionate administered at 10 μ g per mouse reduced pneumococcal lung colonisation by almost 50% at 24 h and 48 h after infection ($p < 0.05$ for each group *versus* control; fig. 4). As previously described [9], the inoculation of PAFR antagonist also significantly reduced the pneumococcal load of the infected animals.

DISCUSSION

The main findings of the present study were that fluticasone propionate reduces: 1) the expression of PAFR in human lung

FIGURE 2. Analysis of the phosphorylcholine expression on *Streptococcus pneumoniae* or *Haemophilus influenzae* isolates from chronic obstructive pulmonary disease (COPD) patients. Bacterial cells from COPD patients' isolates were spotted on membranes and phosphorylcholine was detected with the specific monoclonal antibody TEPC-15 (see Materials and Methods section). Quantification was carried out by densitometric analysis of the spots using a Bioluminescence densitometer and Whole band 3.1 software (both Millipore, Barcelona, Spain). Data are presented as mean $\pm$ SD of three independent experiments. Control values, such as phosphorylcholine expression by *Klebsiella pneumoniae*, were removed from experimental results to give the data shown. #: *S. pneumoniae* and *H. influenzae* isolates selected for further experiments.

epithelial cells; 2) the invasion of these cells by *H. influenzae* and *S. pneumoniae*; and 3) pneumococcal lung colonisation *in vivo*. These experimental findings can be relevant for the observed effects of fluticasone propionate in COPD.

It is generally accepted that bacterial infection/colonisation is a key factor in the pathogenesis of COPD exacerbations. Interestingly, *S. pneumoniae* and *H. influenzae*, which are frequently isolated in patients with COPD exacerbations [15, 18], share a common epitope, phosphorylcholine. This bacterial surface epitope mediates the interaction of these pathogens with PAFR, which is present on airway epithelial cells. The phosphorylcholine-PAFR interaction is critical for the pathogenesis of infections by both pathogens [9, 10]. The present results show that fluticasone propionate reduces the expression of PAFR in two predominant cell types of the airway epithelia. As a consequence, the capacity of both pathogens to invade the epithelial cells is impaired. DOWLING *et al.* [19] reported that fluticasone propionate also has the ability to reduce the total number of *Pseudomonas aeruginosa* that adhere to the respiratory mucosa. DOWLING *et al.* [19] suggested that this reduction was due to a decrease in the amount of mucosal damage caused by the microorganism, to which the bacteria preferentially adhered. According to the present results, it can be hypothesised that downregulation of PAFR expression might also have contributed to the effects of fluticasone propionate on the adherence of *P. aeruginosa* to respiratory mucosa, as this respiratory pathogen also expresses phosphorylcholine [20]. The current results also support previous studies that described the key role of the bacterial epitope, phosphorylcholine, in the interaction of the respiratory pathogens with the host cells [9, 10]. However, phosphorylcholine expression undergoes phase variation in *S. pneumoniae* and *H. influenzae* [12, 16]. The fact that all isolates

FIGURE 3. The effect of fluticasone propionate on the invasion of the airway epithelial cells by *Streptococcus pneumoniae* and *Haemophilus influenzae*. a, c) Monolayers of A549 cells and b, d) 16HBE14o- cells were treated with platelet-activating factor receptor (PAFR) antagonist or fluticasone propionate and incubated with 10^7 colony-forming units (cfu) of *S. pneumoniae* (a and b) or *H. influenzae* (c and d). Intracellular bacteria was determined as described in the Materials and Methods section. Data are presented as mean $\pm$ SEM of five independent experiments. *: $p < 0.05$ versus controls; **: $p < 0.01$ versus controls.

FIGURE 4. The effect of fluticasone propionate on the *Streptococcus pneumoniae* lung infection in vivo. Mice were intratracheally inoculated with *S. pneumoniae* resuspended with saline (control) or with saline solutions containing platelet-activating factor receptor (PAFR) antagonist or fluticasone propionate. The number of bacterial cells $\cdot g^{-1}$ of lung homogenate was determined at 24 h (■) and 48 h (□). cfu: colony-forming unit. *: $p < 0.05$ versus controls.

tested in the present study expressed phosphorylcholine on their outer surfaces suggests that phosphorylcholine may represent an important adaptation to the abnormal lung environment encountered by *S. pneumoniae* and *H. influenzae* in the airways of COPD patients.

The present study found that airway epithelial cells express PAFR constitutively. CUNDELL *et al.* [9] showed that activation of airway epithelial cells with interleukin-1 upregulates the surface expression of PAFR which, in turn, facilitates the invasion by *S. pneumoniae* via the interaction between PAFR and the phosphorylcholine component of the bacterial surface. To the current authors' knowledge, there are no data in the literature reporting the level of expression of PAFR on airway epithelial cells of patients with COPD. However, it is conceivable that the chronic inflammatory response that characterises COPD [4] can upregulate the expression. In fact, this is the case for other receptors involved in the interaction with bacterial pathogens, such as the protein G-coupled receptor intercellular adhesion molecule-I [21] or Toll-like receptor-2 [22].

The present study did not investigate the molecular mechanism that mediates the reduction of PAFR due to fluticasone propionate. However, several nonexclusive mechanisms can

be predicted. First, fluticasone propionate can reduce PAFR expression indirectly [9] through the reduction of lung inflammation [1]. Secondly, fluticasone propionate can increase the synthesis of lipocortin, which in turn inhibits the production of lipid phospholipase A and, hence, the production of lipid mediators such as leukotrienes, prostaglandins and platelet-activating factor [23]. Finally, fluticasone propionate can prevent airway mucosal damage [19]. These mechanisms alone or in combination can explain the current *in vivo* observations. However, further studies are required to establish the molecular and cellular basis of the effect of fluticasone propionate on the PAFR expression *in vitro* and *in vivo*.

The present study demonstrates that fluticasone propionate reduces PAFR expression on airway epithelial cells, and that it prevents the invasion of *S. pneumoniae* and *H. influenzae* *in vitro* and *in vivo*. These novel molecular effects can explain the reduction in the frequency of exacerbations observed in patients treated with fluticasone propionate. They seem to differ to the recently reported increase in pneumonia in the same patients [24]. Several nonexclusive factors might explain this apparent discrepancy. First, in the previous study the diagnosis of pneumonia in patients treated with fluticasone propionate was often not confirmed by chest radiography [24]. Thus, the accuracy of a clinical diagnosis of "pneumonia" is questionable. Secondly, no attempt was made to obtain a specific microbiological diagnosis in these patients in the previous study [24], and several bacteria and viruses may have accounted for such pneumonia. In contrast, in the present study, the effect of fluticasone propionate was tested using two well-characterised bacterial pathogens. Finally, the current results indicate that fluticasone propionate reduces bacterial load during the first steps of pulmonary infection, but the results do not exclude the possibility that fluticasone propionate may also inhibit the recruitment of phagocytes to the lung, thus increasing the susceptibility to pneumonia. In this context, it is worth mentioning that the reduced bacterial invasion of the epithelial cells induced by fluticasone propionate may also reduce the expression of certain inflammatory cytokines, which might be critical for the recruitment of phagocytes to the infection site.

In conclusion, the current results demonstrate that fluticasone propionate reduces the invasion of the airway epithelial cells by *Streptococcus pneumoniae* and *Haemophilus influenzae*. However, further studies are required to understand the biological significance of this phenomenon, particularly in the context of the airway infection/colonisation that often occurs in patients with chronic obstructive pulmonary disease.

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