TITLE: Fluticasone propionate reduces bacterial airway epithelial invasion

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RUNNING TITLE: Steroids and bacterial infection
ABSTRACT
Fluticasone propionate (FP) 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, a epitope that mediates their interaction with airway epithelial cells via the platelet-activating factor receptor (PAFR).

We studied the effects of FP 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.

For experiments, we used *S. pneumoniae* and *H. influenzae* isolated from patients with COPD, cell cultures of type II pneumocytes and bronchoepithelial cells, and a mice model of lung infection.

We found that: FP reduced the expression of PAFR on the surface of two types of cells studied; All *S. pneumoniae* and *H. influenzae* isolates expressed phosphorylcholine; Treatment of both cells lines with FP reduced invasion of both microorganisms; and, FP reduced the bacterial load of mice infected with *S. pneumoniae*.

FP reduces the invasion of airway epithelial cells by *S. pneumoniae* and *H. influenzae* through its effect on PAFR. These results may contribute to explain the beneficial effects of FP on COPD exacerbations.

Key words: Airway epithelial cells, Bacterial infections, Chronic Obstructive Pulmonary Disease, Fluticasone.
INTRODUCTION

Patients with chronic obstructive pulmonary disease (COPD) often suffer episodes of exacerbation (ECOPD) during the course of their disease [1]. These episodes impact negatively on their health status, worsen their prognosis and associate a very significant cost. Treatment with inhaled steroids (alone or in combination with long-acting $\beta_2$ agonists) reduce the number of ECOPD [2] particularly in the subgroup of patients with moderate to severe disease [2, 3] in whom pulmonary inflammation is enhanced [4].

*S. pneumoniae* and *H. 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], as shown by the fact that PAFR-deficient mice are more resistant to pneumococcal pneumonia than the wild-type mice [11] and that intratracheal administration of *S. pneumoniae* and a PAFR antagonist to rabbits reduced bacterial loads in lung lavages [9]. Likewise, phosphorylcholine favours the persistence *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 those found in COPD patients, we hypothesized that steroids may reduce exacerbations by down-regulating PAFR expression. To test this hypothesis, we investigated the *in vitro* effect of FP on: (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*.
METHODS 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. 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. FP was kindly provided by GlaxoSmithKline.

Cell culture and treatments

The human bronchoepithelial immortalized cells 16HBE14o- and the human lung carcinoma cells A549 (ATCC CCL185) derived from type II pneumocytes were used in this study. These cells were propagated in Earls’MEM + 1% L-glutamine culture medium and in RPMI 1640 + 1% HEPES, respectively, supplemented with 10 % fetal calf serum and 1% penicillin-streptomycin (Sigma). Cells were incubated in 24-well tissue culture plates at 37°C and 5% CO₂ until reaching confluence (≈ 5 x 10⁵ cells per well) and then used for the studies. Monolayers of each cell line were treated at 37°C with FP (0.1 µM, 4 h) or PAFR antagonist (0.05 µM, 30 min), both dissolved in the cell culture medium. The invasion assays were performed as described [14]. Briefly, after the incubation of the epithelial cells with the bacterial suspension, the wells were washed with PBS and then incubated with fresh medium containing gentamicin (100 µg/ml) to kill extracellular bacteria. After one hour, an aliquot of the medium was plated to confirm killing of extracellular bacteria, and the gentamicin-containing medium was washed again. 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), 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 $5 \times 10^4$ cell/ml in PBS-1% bovine serum albumin (BSA), and incubated for 3 h at 25°C with rabbit anti-PAFR (Alexis) diluted 1:500. After a washing step, cells were incubated 1 h at 25°C with a goat anti-rabbit IgG FITC conjugated diluted 1:500, and finally, resuspended in isoton buffer for their analysis. Analysis was carried out in an Epics XL flow cytometer using the Expo32 software. Non-specific binding was corrected with the corresponding isotype matched antibodies.

**Immunoblot assay**

Phosphorylcholine expression by the bacterial isolates included in this study was assessed by immunoblotting, essentially as described previously [7]. Bacterial suspensions were spotted onto nitrocellulose membranes. After blocking in PBS-1% BSA, membranes were sequentially incubated with specific monoclonal antibody against PC (mAb TEPC-15 (Sigma)) and with a goat anti-mouse IgA/alkaline phosphatase conjugate (Caltag). Washing steps with PBS were included after incubations. Alkaline phosphatase was visualized on the blots using BCIP/NBT fast kit (Sigma). Finally, the filters were analyzed by densitometry using a Bio Image densitometer and Whole band 3.1 software (Millipore). *Klebsiella pneumoniae*, a microorganism that does not present PC on its surface (17), was used as negative control.
**Murine model of lung infection**

Male (20-25 g) ICR-CD1 mice (Harlan Ibérica, S.L.) were anesthetized and inoculated intratracheally with $10^6$ colony-forming units (CFU) of *S. pneumoniae* using a blunt end feeding needle. Pneumococci were inoculated with saline or with saline solutions of PAFR antagonist (0.25 µg), or FP (10 µg) (n = 16 per group). At 24 and 48 h, 8 animals of each group were sacrificed and lungs were aseptically removed and homogenized for quantitative bacterial cultures by plating appropriate dilutions on blood agar plates [14]. All animal experiments were done according to institutional and national guidelines and approved by the Experimental Animal Committee of the institution.

**Statistical analysis**

All values are present as the mean ±SEM. A two tailed t test was used to compare the effect of each treatment with the control. A p-value of less than or equal to 0.05 was considered significant.
RESULTS

*Fluticasone propionate reduces PAFR expression on airway epithelial cells.*

Both, pneumocytes type II derived cells A549 and bronchoepithelial cells 16HBE14o-constitutively expressed PAFR (figure 1). Treatment with the PAFR antagonist reduced the expression of the receptor in both cell types as compared to untreated cells \((p<0.05\) for each cell type versus control). Interestingly, FP also reduced the expression of PAFR in A549 and 16HBE14o- \((p<0.05\) for each cell type versus control).

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

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

For the studies of the effect of the FP on the invasion of the epithelial cells by both microorganisms, we selected one strain of each species in which the phosphorylcholine expression was intermediate (indicated with an asterisk in Figure 2). In preliminary experiments, we observed that neither PAFR antagonist nor FP affected bacteria or cell viability. We also tested the effect of different FP doses \((0.05\)
µm, 0.1 µm, and 0.5 µm) on the invasion of either *H. influenzae* and *S. pneumoniae* to A549 cells. All concentrations tested reduced the invasion of both microorganisms in a dose-dependent manner (data not shown). We selected the intermediate concentration (0.1 µm) for further experiments because was the lowest concentration that gave the best results to compare treatments. As shown in figure 3, treatment of monolayers of both types of airway epithelial cells with 0.1 µm of FP significantly reduced the invasion of both *H. influenzae* and *S. pneumoniae* respect to the untreated control (p< 0.05 for each pathogen and cell type versus control). Invasion was reduced between 60-28% depending on the microorganism and the epithelial cell type. As positive control we used PAFR antagonist which, as expected, reduced the capacity to invade the epithelial cells of both.

*Effect of FP on the lung colonization of S. pneumoniae in vivo*

FP administered at 10 µg/mouse reduced pneumococcal lung colonization by almost 50% at 24 h and 48 h after infection (p< 0.05 for each group versus control). (Figure 4). As previously described [9], the inoculation of PAFR antagonist also reduced significantly the pneumococcal load of the infected animals.
DISCUSSION

The main and novel findings of this study are that FP reduces: (1) the expression of PAFR in human lung epithelial cells; (2) the invasion of these cells by *H. influenzae* and *S. pneumoniae*; and, (3) pneumococcal lung colonization *in vivo*. These experimental findings can be relevant for the observed effects of FP in COPD.

It is generally accepted that bacterial infection/colonization is a key factor in the pathogenesis of COPD exacerbations. Interestingly, *S. pneumoniae* and *H. influenzae*, that are frequently isolated in patients with COPD exacerbations [17, 18], share a common epitope, phosphorylcholine. This bacterial surface epitope mediates the interaction of these pathogens with PAFR present on the airway epithelial cells. The phosphorylcholine-PAFR interaction is critical for the pathogenesis of infections by both pathogens [9, 10]. Our results show that FP 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. reported that FP has also the ability to reduce the total number of *Pseudomonas aeruginosa* adhering to the respiratory mucosa [19]. They 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 our results, however, it can be hypothesized that downregulation of PAFR expression might have contributed also to the effects of FP upon the adherence of *P. aeruginosa* to respiratory mucosa since this respiratory pathogen also expresses phosphorylcholine [20]. Our results also support previous studies that described the key role of bacterial phosphorylcholine epitope 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, 15]. The fact that all isolates tested in our study expressed PC 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.

We found that airway epithelial cells express PAFR constitutively. Cundell et al showed that activation of airway epithelial cells with IL-1 up-regulates the surface expression of PAFR which, in turn, facilitates the invasion by S. pneumoniae via the interaction between PAFR and the PC component of the bacterial surface [9]. To our knowledge, there is 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 characterizes COPD [4] can upregulate it. In fact, this is the case for other receptors involved in the interaction with bacterial pathogens, such as the protein G-coupled receptor ICAM-I [21] or TLR-2 [22].

We did not investigate the molecular mechanism that mediates the reduction of PAFR due to FP. Yet, several, non exclusive mechanisms can be envisaged. First, FP can reduce PAFR expression indirectly [9] through the reduction of lung inflammation [1]. Second, FP 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 PAF [23]. Finally, FP can prevent airway mucosal damage [19]. These mechanisms alone or in combination can contribute to explain our in vivo observations. However, further studies are required to establish the molecular and cellular basis of the effect of FP on the PAFR expression in vitro and in vivo.
Our study demonstrates that FP 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 contribute to explain the reduction in the frequency of exacerbations observed in patients treated with FP. By contrast, they seem at variance with the recently reported increase in pneumonia in these same patients [24]. Several, non exclusive, factors might explain this apparent discrepancy. First, the diagnosis of pneumonia in patients treated with FP was often not confirmed by chest x-ray [24]. Thus, the accuracy of a clinical diagnosis of “pneumonia” is questionable. Second, no attempt was made to obtain a specific microbiologic diagnosis in these patients [24], and several bacteria and viruses may have accounted for such a “pneumonia”; by contrast, in our study we tested the effect of FP using two well characterized bacterial pathogens. Finally, our results indicate that FP reduces bacterial load during the first steps of a pulmonary infection, but they do not exclude the possibility that FP 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 FP may also reduce the expression of certain inflammatory cytokines that may be critical for the recruitment of phagocytes to the infection site.

In conclusion, our results demonstrate that FP reduces the invasion of the airway epithelial cells by *S. pneumoniae* and *H. influenzae*. However, further studies are required to understand the biological significance of this phenomenon, particularly in the context of the airway infection/colonization that often occurs in patients with COPD.
ACKNOWLEDGMENTS

We thank Teresa de Francisco (Serveis Cientificotècnis, Universitat de les Illes Balears; Palma de Mallorca, Spain) for her assistance in the animal experiments. This study has been supported by an unrestricted educational grant from GlaxoSmithKline (GSK) and by the Spanish Network for the Research in Infectious Diseases (REIPI RD06/0008). Ciberes is an initiative of Instituto de Salud Carlos III.
REFERENCES


Figure 1

Figure 2

Figure 3
Figure 4