Glucocorticoids Enhance or Spare Innate Immunity: Effects in Airway Epithelium Are Mediated by CCAAT/Enhancer Binding Proteins

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Glucocorticoids Enhance or Spare Innate Immunity: Effects in Airway Epithelium Are Mediated by CCAAT/Enhancer Binding Proteins

Ning Zhang, Quynh Ai Truong-Tran, Brian Tancowny, Kathleen E. Harris, and Robert P. Schleimer

Although it is widely accepted that glucocorticoids (GC) are a mainstay of the treatment of diseases characterized by airway inflammation, little is known about the effects of GC on local innate immunity. In this article, we report that respiratory epithelial cells manifested a local "acute phase response" after stimulation with TLR activation and TNF-α and that GC spared or enhanced the epithelial expression of molecules that are involved in host defense. Studies using Western blotting, EMSA, and functional analysis indicated that the selective effects of GC are mediated through activation of the transcription factor C/EBP. Knockdown of C/EBP by small interfering RNA blocked the enhancement by GC of host defense molecule expression but had no effect on inflammatory gene expression. These results suggest that GC spare or enhance local innate host defense responses in addition to exerting anti-inflammatory actions. It is possible that the known ability of GC to reduce the exacerbation of diseases in which infectious organisms serve as triggering factors (e.g., asthma, allergic bronchopulmonary aspergillosis, and chronic obstructive pulmonary disease) may result in part from enhanced innate immune responses in airway mucosa. The Journal of Immunology, 2007, 179: 578–589.

In response to infections and injury, a spectrum of molecules constituting the acute phase response (APR) is mobilized with the purpose of combating the infection and preventing tissue damage (1). Acute phase proteins such as C-reactive protein (CRP) and serum amyloid A (SAA) are released by the liver during both adaptive and innate immune responses and have proven to be useful as markers of systemic disease activity or severity (2, 3). The airway epithelium is exposed to a large number of potentially pathogenic airborne particles such as microorganisms, allergens, and pollutants and has been shown to produce APR proteins such as C2, C3, C4, C5, factor B (4, 5), CRP, and SAA (6, 7). Based on these studies and the fact that hepatocytes and the airway epithelium come from the same germinal layer, it appears that airway epithelium can manifest a local version of the acute phase response following stimulation.

Glucocorticoids (GC) are a mainstay of the treatment of diseases characterized by airway inflammation including asthma, rhinitis, and chronic rhinosinusitis. Although it is widely accepted that their success directly reflects their anti-inflammatory effects, such as inhibition of the expression of the cytokines and chemokines that activate endothelial cells, leukocytes, and lymphocytes, the precise mechanisms by which steroids improve lung function remain unclear (8). During preliminary microarray studies, we found that epithelial expression of some of the acute phase proteins involved in innate immunity, including C3 and SAA, was not inhibited by GC and in some cases was actually increased (9). Because GC can enhance the hepatic acute phase response, we reasoned that they may also enhance the manifestations of an APR in airway epithelial cells. In support of this possibility, previous reports indicate that GC enhanced the expression of mRNA for C3 and secretory leukocyte protease inhibitor (SLPI) in lung epithelial cells (10, 11). Evidence thus exists to suggest that GC spare or even enhance several innate immune responses of airway epithelial cells while they inhibit inflammatory responses (12).

The APR in the liver is known to be mediated to a great extent by activation of the transcription factor C/EBP, which belongs to the basic region–leucine zipper transcription factor family (13). Six members (C/EBPα–C/EBPδ) have been characterized in mammals; these proteins have been shown to play pivotal roles in numerous cellular responses, including the APR (14, 15). C/EBPβ and C/EBPδ are rapidly and dramatically induced by LPS or cytokines in the liver and in turn activate the expression of various genes involved in the APR (13, 16, 17). Most of the proteins produced in the liver during the APR contain C/EBP binding sites in their promoters and their induction is regulated by C/EBPβ and C/EBPδ (16, 18). C/EBP proteins have been detected in the lung (19), and recently the role of C/EBP in lung physiology and pathophysiology has become a topic of great interest (20, 21). C/EBPα, C/EBPβ, and C/EBPδ were found to be expressed in lung epithelial...
cells and were involved in the regulation of expression of surfactant protein (Sp) A, SpD, the Clara cell secretory protein, and the P450 enzyme CYP2B1 during development (22).

GC are known to enhance the hepatic APR by a mechanism that involves C/EBP proteins (23). We report that: 1) epithelial cells exhibit a response reminiscent of the hepatic APR; 2) this response is potentiated by GC; and 3) C/EBPβ is an important mediator of the epithelial APR to inflammatory stimuli and GC.

Materials and Methods

Cell culture

Human primary bronchial epithelial cells (PBEC). PBEC were harvested from human lungs that had been rejected for transplantation purposes (obtained from the National Disease Research Interchange, Philadelphia, PA) using a modification of a previously described protocol (24) approved by the Northwestern University Institutional Review Board. Cells were seeded in LHC-9 medium (BioSource International) and changed to LHC-8 medium to eliminate any effects of hydrocortisone 48 h before GC treatment. Cells were used only during their primary passage.

BEAS-2B cells. An adenovirus 12-SV40 hybrid virus-transformed human bronchial epithelial cell line, BEAS-2B (a gift from Dr. C. Harris, National Cancer Institute, Bethesda, MD), was cultured in DMEM/F12 (Invitrogen Life Technologies) supplemented with 5% FBS, 2 mM t-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were used between passages 38 and 45 and cultured for 48 h in DMEM/F12 without FBS before use in experiments with GC.

A549 cells. A549 cells, a tumor cell line with properties of type II alveolar epithelial cells, were cultured in DMEM as described above.

dsRNA stimulation and GC treatment of cultured cells

Cells were used for experiments when they reached 80% confluence. Cells were treated with synthetic dsRNA (polynosinic acid:cytidylic acid (poly(I:C); Amersham Biosciences) at 25 μg/ml or TNF-α (R&D Systems) at 100 ng/ml for 18 h (except in time course experiments). Cells were treated with fluticasone propionate (FP) (Sigma-Aldrich) at 100 ng/ml for 18 h (except in time course studies). Cells were treated with synthetic dsRNA (polyinosinic acid:cytidylic acid) at 100 ng/ml for 18 h (except in time course studies). Cells were treated with synthetic dsRNA (polyinosinic acid:cytidylic acid) at 100 ng/ml for 18 h. Cells were used only during their primary passage.

NFκB was stored as a 0.1 M stock in DMSO. Control cells were treated with fluticasone propionate (FP) (Sigma-Aldrich) at 100 nmol/L.

C3 factor was stored as a 0.1 M stock in DMSO. Control cells were treated with fluticasone propionate (FP) (Sigma-Aldrich) at 100 nmol/L.

GM-CSF was stored as a 0.1 M stock in DMSO. Control cells were treated with fluticasone propionate (FP) (Sigma-Aldrich) at 100 nmol/L.

HBD2 was stored as a 0.1 M stock in DMSO. Control cells were treated with fluticasone propionate (FP) (Sigma-Aldrich) at 100 nmol/L.

IFNβ was stored as a 0.1 M stock in DMSO. Control cells were treated with fluticasone propionate (FP) (Sigma-Aldrich) at 100 nmol/L.

CXCL8 was stored as a 0.1 M stock in DMSO. Control cells were treated with fluticasone propionate (FP) (Sigma-Aldrich) at 100 nmol/L.

Table I. Minor groove-binding (MGB) probes and primers used for quantitative TaqMan real-time polymerase chain reaction analysis of gene expression

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Forward Primer (5’ to 3’)</th>
<th>Reverse Primer (5’ to 3’)</th>
<th>MGB Probe (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3</td>
<td>ATCCACGCCGCTGCCCTCCTCA</td>
<td>GAATGCAGCTTAGAGGATTCA</td>
<td>AAGAATGGAGGAAGACT</td>
</tr>
<tr>
<td>Factor B</td>
<td>GGTTCGATAGAGTTTGATTGTAGTCAC</td>
<td>GACGCCGAGTAGGAGG</td>
<td>AGCTCCCTCCGTCTCA</td>
</tr>
<tr>
<td>Factor H</td>
<td>GGATTTGTTAGAGTTTGATTGTAGTCAC</td>
<td>GACGCCGAGTAGGAGG</td>
<td>AGCTCCCTCCGTCTCA</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>AGGGCCCTTGTGGAGG</td>
<td>GACAGCTCCTTGGAGGATATTT</td>
<td>AAGAATGGAGGAAGACT</td>
</tr>
<tr>
<td>HBD2</td>
<td>GATTTGTTAGAGTTTGATTGTAGTCAC</td>
<td>GACGCCGAGTAGGAGG</td>
<td>AGCTCCCTCCGTCTCA</td>
</tr>
<tr>
<td>IFNβ</td>
<td>TCTTCCACAGACGCTTCTCTCA</td>
<td>GACAGCTCCTTGGAGGATATTT</td>
<td>AAGAATGGAGGAAGACT</td>
</tr>
<tr>
<td>CXCL8 (IL-8)</td>
<td>TGCCTGTGCTCTCTCTTG</td>
<td>TTGGCAAATCTTTAGATGTCTCC</td>
<td>CAGCCCTTGTGCTTCTGCTG</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>CAGACGCCTGCCCTGCAAT</td>
<td>TGCTGCCCTCCAAAATGCA</td>
<td>TGACCCAGATATCAAT</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>AGGGCCCTTGTGGAGG</td>
<td>GACAGCTCCTTGGAGGATATTT</td>
<td>AAGAATGGAGGAAGACT</td>
</tr>
<tr>
<td>MASP1</td>
<td>GACGGCCGAGTAGGAGG</td>
<td>GACAGCTCCTTGGAGGATATTT</td>
<td>AAGAATGGAGGAAGACT</td>
</tr>
<tr>
<td>MASP2</td>
<td>ATCCACGCCGCTGCCCTCCTCA</td>
<td>GAATGCAGCTTAGAGGATTCA</td>
<td>AAGAATGGAGGAAGACT</td>
</tr>
<tr>
<td>MBL</td>
<td>GCCTTGTTAGAGTTTGATTGTAGTCAC</td>
<td>GACGCCGAGTAGGAGG</td>
<td>AGCTCCCTCCGTCTCA</td>
</tr>
<tr>
<td>MIP-3α</td>
<td>TCTTCCACAGACGCTTCTCTCA</td>
<td>GACAGCTCCTTGGAGGATATTT</td>
<td>AAGAATGGAGGAAGACT</td>
</tr>
<tr>
<td>SAA</td>
<td>CATTTCTCTCTCTCTGTCTCTCTCT</td>
<td>TGATTTTTGTTATGATGTCTTCTTCAT</td>
<td>AGCCAGGATGGTTAACAT</td>
</tr>
<tr>
<td>CCL5</td>
<td>TGCTGCTGCTGCCCTTGTGCTA</td>
<td>TCAGCTTGTGCTGCTGCTG</td>
<td>CTGGCACACCCACCTTCTGC</td>
</tr>
</tbody>
</table>

(dRNTES)

Cells were treated with small interfering RNA (siRNA) transfection methods containing a C/EBPβ consensus site (5′-TGCAGATTGCGCAATC-3′) using the Odyssey infrared imaging system (LI-COR). Luciferase activity was measured using the Promega luciferase assay system. The C/EBPβ luciferase activity was normalized to the control luciferase activity and the protein concentration of the lysates. The normalized relative luciferase activity was expressed as the fold induction of luciferase activity above the control condition, which was given a value of 1.

EMSA

EMSA was performed with IRDye-labeled double-stranded oligonucleotides containing a C/EBPβ consensus site (5′-TGCAGATTGCGCAATC-3′) using the Odyssey infrared imaging system (LI-COR). The DNA binding reactions were set up by incubating 10 μg of the nuclear protein and 50 fmol of oligonucleotides for 30 min at room temperature in 10 μl of binding buffer (10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10 mM DTT, and 1% Tween 20). Orange G loading dye (1 μl at 10X) was added, the gels were scanned, and the signals were quantified using the Odyssey infrared imaging system (LI-COR).

RNA interference

After BEAS-2B cells reached 60% confluence in 12-well plates, the medium was replaced with small interfering RNA (siRNA) transfection medium (Santa Cruz Biotechnology) at half volume. C/EBPβ siRNA (3.6 μl...
at 10 μM and siRNA transfection reagent (2.4 μl) were mixed in 80 μl of transfection medium and incubated at room temperature for 20 min according to the manufacturer’s instructions. The cells in each well were then transfected with this mixture. After 6 h, the medium volume was restored according to the manufacturer’s instructions. The cells in each well were then transfected with C/EBP siRNA from Qiagen targeted to (antisense). Some of (sense) and 5'-AA AGACAGCCUCUCUACUAGGGCGAGCUUTT-3' (sense) and 5'-AA GUCCGCGCUCGUAGAAGCCGCGCUGUCTT-3' (antisense). Some of the cells were transfected with C/EBP siRNA from Qiagen targeted to nonoverlapping sequences with those above (HS_C/EBPB_5 HP Validated siRNA (Qiagen); S02777292) (S2) or negative control RNA at 5 nM using HiPerFect transfection reagent (Qiagen) following the manufacturer’s instruction.

ELISA and cytometric bead array

The concentration of C3 in culture supernatants was assayed by a previously described ELISA (25) with 1.67 ng/ml detection Ab. ELISA kits from R&D Systems and Tri-Delta Diagnostics were used to quantitate SLPI and SAA. The detection limit of ELISA for C3, SLPI, and SAA is 1 ng/ml, 25 pg/ml, and 0.3 ng/ml, respectively.

Levels of IL-8 and RANTES in supernatants were detected using the human chemokine cytometric bead array kit (BD Pharmingen) in a BD FACSArray instrument.

Statistical Analysis

Data are expressed as the mean value ± SEM from the number of replicate experiments indicated in the text. Statistical significance of differences was determined with a Student’s t test. Differences were considered statistically significant at p < 0.05.

Results

DsRNA induced a local APR in respiratory epithelial cells

We first investigated whether the respiratory epithelium could manifest a response resembling an APR and a host defense response upon stimulation with dsRNA. Lower values represent higher expression and each unit represents a 2-fold change in expression. Ct > 37 indicates no or minimal detectable expression. *, No expression in either basal or stimulating condition. ±, No expression in basal condition but induced by stimulation; †, Ct ≤ 37 in both conditions. Bold type indicate genes induced by at least 6-fold. *, p < 0.05 for the comparison of stimulated to controls. BPI, bactericidal/permeability-increasing protein; PAI, plasminogen activator inhibitor.

Table II. Expression of host defense and inflammatory molecules in human bronchial and alveolar epithelial cells

<table>
<thead>
<tr>
<th>Host Defense Molecules</th>
<th>BEAS-2B (n = 4–7)</th>
<th>A549 (n = 2)</th>
<th>PBEC (n = 4–7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complement</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3</td>
<td>+ 23/21 (3)*</td>
<td>+ 24/23 (2)</td>
<td>+ 25/20 (9)*</td>
</tr>
<tr>
<td>Factor B</td>
<td>+ 22/18 (6)*</td>
<td>+ 21/19 (7)</td>
<td>+ 25/17 (160)*</td>
</tr>
<tr>
<td>Factor H</td>
<td>+ 33/29 (8)*</td>
<td>+ 24/22 (4)</td>
<td>+ 27/23 (12)*</td>
</tr>
<tr>
<td>Defensins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBD1</td>
<td>—</td>
<td>+ 36/33 (11)</td>
<td>+ 28/26 (4)*</td>
</tr>
<tr>
<td>HBD2</td>
<td>—</td>
<td>+ 35/32 (9)</td>
<td>+ 35/24 (262)*</td>
</tr>
<tr>
<td>HBD4</td>
<td>—</td>
<td>+ 31/31 (1)</td>
<td>+ 35/22 (185)*</td>
</tr>
<tr>
<td>Collectins</td>
<td>± 40/34 (12)*</td>
<td>± 40/33 (226)</td>
<td>+ 36/36 (1)</td>
</tr>
<tr>
<td>MASP1</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>MASP2</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>SpA</td>
<td>—</td>
<td>—</td>
<td>± 40/35 (5)*</td>
</tr>
<tr>
<td>SpD</td>
<td>—</td>
<td>+ 34/34 (1)</td>
<td>+ 33/31 (2)</td>
</tr>
<tr>
<td>Pentraxins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Pentraxin3</td>
<td>+ 25/21 (11)*</td>
<td>+ 31/29 (4)</td>
<td>+ 31/29 (4)*</td>
</tr>
<tr>
<td>Other antimicrobial proteins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysozyme</td>
<td>+ 34/33 (1)</td>
<td>+ 30/28 (4)</td>
<td>+ 30/29 (1)</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>+ 34/34 (1)</td>
<td>—</td>
<td>+ 32/31 (2)</td>
</tr>
<tr>
<td>SLPI</td>
<td>+ 29/25 (8)</td>
<td>+ 23/23 (1)</td>
<td>+ 21/20 (1)</td>
</tr>
<tr>
<td>BPI</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>LBP</td>
<td>—</td>
<td>+ 28/26 (5)</td>
<td>—</td>
</tr>
<tr>
<td>SAA</td>
<td>+ 24/19 (10)*</td>
<td>+ 30/28 (7)</td>
<td>+ 24/17 (6)*</td>
</tr>
<tr>
<td>Coagulation proteins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>—</td>
<td>+ 24/24 (1)</td>
<td>—</td>
</tr>
<tr>
<td>PAI</td>
<td>+ 23/22 (1)</td>
<td>+ 23/23 (1)</td>
<td>+ 23/21 (1)</td>
</tr>
<tr>
<td>Inflammatory molecules</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytokines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFNβ</td>
<td>+ 33/29 (28)*</td>
<td>+ 35/32 (13)</td>
<td>+ 35/26 (55)*</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>+ 30/21 (33)*</td>
<td>+ 33/31 (4)</td>
<td>+ 30/21 (81)*</td>
</tr>
<tr>
<td>Chemokines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RANTES</td>
<td>+ 29/17 (1805)*</td>
<td>+ 27/23 (13)</td>
<td>+ 31/19 (55)*</td>
</tr>
<tr>
<td>IL-8</td>
<td>+ 37/32 (14)*</td>
<td>+ 35/33 (5)</td>
<td>+ 40/28 (106)*</td>
</tr>
<tr>
<td>MIP-3α</td>
<td>+ 36/26 (29)*</td>
<td>+ 26/24 (4)</td>
<td>+ 29/24 (36)*</td>
</tr>
<tr>
<td>Reference</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>+ 19/19 (1)</td>
<td>+ 19/19 (1)</td>
<td>+ 19/19 (1)</td>
</tr>
</tbody>
</table>

* Values on the left are mean basal Ct and values on the right are mean Ct after stimulation with dsRNA. Lower values represent higher expression and each unit represents a 2-fold change in expression. Ct > 37 indicates no or minimal detectable expression. —, No expression in either basal or stimulating condition. ±, No expression in basal condition but induced by stimulation; †, Ct ≤ 37 in both conditions. Bold type indicate genes induced by at least 6-fold. *, p < 0.05 for the comparison of stimulated to controls. BPI, bactericidal/permeability-increasing protein; PAI, plasminogen activator inhibitor.
mean raw Ct from two to seven independent experiments; the value on the left for each gene is the basal level, the value on the right represents the stimulated level, and the value in parentheses is the approximate mean fold stimulation. Higher Ct values represent lower levels of expression and a value of >37 indicates the gene was undetected. Genes induced by dsRNA at least 6-fold are indicated by bold text.

PBEC were found to express a broad spectrum of host defense molecules including human β-defensins (HBD1, 2, and 4), lysozyme, lactoferrin, SLPI, SpD, SAA, C3, factor B, factor H, plasminogen activator inhibitor, and MIP-3α. A549 cells expressed most of these same genes with the exception of lactoferrin. Unlike the PBEC and BEAS-2B, the A549 cells also expressed the LPS binding protein (LBP) and fibrinogen. BEAS-2B cells expressed fewer antimicrobial genes than PBEC. The constitutive expression of some of these genes, such as HBD1, HBD2, HBD4, and SpD, may be lost as a result of transformation of these cells by the adenovirus 12-SV40 hybrid virus. Lysozyme and SLPI were expressed at higher levels in PBEC than in BEAS-2B cells. Bactericidal/permeability-increasing protein, lectin-associated serine protease (MASP) 1, MASP2, and SpA were not expressed by any of the three types of cells. Stimulation with dsRNA increased the expression of the genes studied in the PBEC and to a lesser extent in the immortalized cell lines (see bold values in Table II; ≥6-fold or 2.5 Ct change). The stimulus had no effect on levels of the housekeeping gene GAPDH.

These results, together with our former study on the presence and function of TLRs in airway epithelial cells (7), indicate that a...
broad spectrum of inflammatory and host defense acute phase genes is expressed by respiratory epithelial cells and can be induced by TLR3 activation.

Selective effects of GC on host defense and inflammatory genes

As demonstrated in Table II, dsRNA induced many host defense and inflammatory genes in epithelial cells. We next determined the effects of the potent GC FP on these dsRNA-stimulated responses. FP (100nmol/L) was added 2 h before 18 h of stimulation with dsRNA (25 μg/ml). Data in Fig. 1 show a selective effect of FP on gene expression by TLR3 stimulation in PBEC (Fig. 1A) and BEAS-2B cells (Fig. 1B). FP efficiently inhibited the expression of inflammatory genes (IFNβ, GM-CSF, RANTES, and IL-8) but enhanced the expression of C3, SAA, CRP, and MIP-3α and failed to inhibit the expression of HBD1 and factor B. Most of the genes enhanced or spared by FP are host defense molecules. We use the term “host defense genes” to refer to those proteins that can directly interact with and/or kill microorganisms, recognizing that some of these genes can also provoke the signs of inflammation. Most of the genes inhibited by FP were inflammatory genes and

FIGURE 1. (continued)
not host defense molecules (with two exceptions, HBD2 and HBD4). As shown in Fig. 1B, BEAS-2B cells showed a similar pattern. The only difference was that FP alone directly induced the expression of C3, SAA, CRP, and mannose-binding lectin (MBL) from 3- to 25-fold in BEAS-2B cells. The pattern of GC “sparring” innate immune response genes was also observed in TNF-α-stimulated cells (Fig. 1C), which indicates that the effects of steroids are not stimulus specific.

We next checked the concentration response and time course of FP on the expression of several of these target genes in resting BEAS-2B cells (Fig. 2). These studies confirmed the pattern of suppression of inflammatory genes and the enhancement or sparing of host defense genes. Both the IC50 of FP for the inhibition of expression of inflammatory genes and the EC50 for the induction of host defense molecules were ~0.5 nmol/L. Twenty-four hours of treatment with FP had the maximum effect. To further confirm these selective effects of GC, we performed ELISA and a cytometric bead array assay to detect protein levels of C3, SAA, SLPI, pentraxin 3, RANTES, and IL-8 (Fig. 3). The protein data further confirmed the results from studies of mRNA. FP reduced the expression of the chemokines RANTES and IL-8 that were synthesized and secreted by PBEC upon dsRNA stimulation while it spared or enhanced the release of C3, SAA, and pentraxin 3. Consistent with the mRNA data, levels of SLPI did not significantly change. These data suggest that GC have selective effects on epithelial gene expression, sparing several of those involved in host defense and inhibiting those responsible for inflammation (Figs. 1–3).

GC increase C/EBPβ and C/EBPδ mRNA and protein levels
To investigate the role of C/EBP proteins in the regulation of gene expression by GC, we first determined whether mRNA of C/EBPα−γ were expressed in respiratory epithelial cells. We detected mRNA for all C/EBP with the exception of C/EBPγ in primary cells and the BEAS-2B cell line (Fig. 4). At the base of each graph the basal Ct is shown. According to the basal Ct, C/EBPβ and C/EBPδ were expressed at a relatively high level. dsRNA induced C/EBPβ and C/EBPδ mRNA in both primary cells and the

FIGURE 2. Resting BEAS-2B cells were treated with FP for 18 h at the indicated concentration (A) or 100 nmol/L FP for the indicated times (B). The levels of selected host defense and inflammatory molecules were evaluated by real-time PCR. Gene expression is expressed relative to the respective control groups. *, p < 0.05. The values results shown are the mean of three independent experiments.

FIGURE 3. PBEC were treated with diluent (Con, control), 25 μg/ml dsRNA (dsR; poly(I:C)) (A) or 100 ng/ml TNF-α (B), 100 nmol/L FP, or the combination of dsRNA and FP (RF) (A) or TNF-α and FP (TNFα+FP) (B) for 18 h. In the RF group, cells were treated with FP for 2 h before stimulation with dsRNA. Supernatants were collected and proteins were detected using ELISA (C3, SLPI, and SAA) or cytometric bead array assay (RANTES and IL-8); n = 4–6. *, p < 0.05.
BEAS-2B cell line. FP alone significantly increased the mRNA for C/EBPβ and the C/EBPδ mRNA in BEAS-2B cells, and the combination of dsRNA and FP produced the highest levels of C/EBPβ and C/EBPδ in both cell types. In contrast to C/EBPβ and C/EBPδ, C/EBPα expression was inhibited by dsRNA in primary cells and FP had no effect. C/EBPγ mRNA levels did not change in any of the conditions examined, and the levels of C/EBPγ were undetectable in all conditions (not shown).

We next performed Western blot analysis to determine the effects of dsRNA and FP on levels of C/EBPβ proteins (Fig. 5). Results were complicated as we detected five isoforms of C/EBPβ: bands of 40 kDa (possibly liver activating protein (LAP*)), 35 kDa (LAP), 25 kDa (liver inhibiting protein (LIP)), 50 kDa, and 46 kDa. Using the ratio of LAP/LIP as an indicator of activated (LAP), 25 kDa (LIP)), 50 kDa, and 46 bands of 40 kDa (possibly liver activating protein (LAP*)), 35 kDa effects of dsRNA and FP on levels of C/EBP

**FIGURE 4.** PBEC (A) and BEAS-2B (B) cells expressed mRNA for C/EBPα, C/EBPβ, C/EBPγ, and C/EBPδ but not C/EBPε. Basal Ct is indicated below the x-axis (higher numbers indicates lower basal levels; 40 = undetected). Levels of C/EBPε were 40 (data not shown). dsR, dsRNA; Values are mean ± SEM of expression relative to the diluent control (Con). *, p < 0.05.

**FIGURE 5.** Assessment of the expression of C/EBPβ in BEAS-2B cells treated as indicated for 18 h by using Western blotting. Bands at 50 kDa, 46 kDa, 40 kDa (LAP*), 35 kDa (LAP), and 25 kDa (LIP) were detected. NF-κB p65 and GC receptor (GR) were assessed as controls. A. One representative experiment of three independent experiments is shown. B. The mean ± SEM relative density of LAP/LIP from three independent experiments is shown. *, p < 0.05. Con, control; dsR, dsRNA; RF, dsRNA plus FP.

**Effects of GC on the function of C/EBPβ**

To test whether GC can regulate the function of C/EBPβ in addition to increasing its expression, C/EBPβ binding activity in BEAS-2B cells was studied using an EMSA. GC treatment resulted in an increased binding to a C/EBPβ consensus binding site in nuclear extracts as shown in a representative gel (Fig. 6A) and in a densitometric evaluation of three experiments (Fig. 6B). The effect of GC on C/EBPβ transactivation was studied using transfection of a luciferase reporter gene into the BEAS-2B cells. A small but reproducible effect was observed. After treatment with 10^{-7} M FP for 18 h, reporter gene activity was increased 1.7-fold. FP and dsRNA had an additive or possibly synergistic effect; the reporter gene activity increased 2.3-fold in the presence of the combination of these two stimuli (Fig. 6C). One experiment with primary cells confirmed that FP spared the expression and function of C/EBP in primary cells. These results indicate that GC increase the DNA binding activity and the transactivational function of C/EBPβ in epithelial cells.

**C/EBP siRNA blocked the enhancement by GC of host defense molecule expression but had no effect on inflammatory gene expression**

To determine whether the selective effects of GC on host defense and inflammatory responses are mediated by the transcription factor C/EBP, we transiently transfected subconfluent BEAS-2B cells with C/EBPβ siRNA for 24 h and then repeated the experiments assessing the effects of dsRNA stimulation and FP treatment. After the blockade of C/EBPβ expression, the induced expression of mRNA for CRP, SAA, MBL, and factor H was significantly but not completely reduced. In contrast, the inhibition by the GC of the expression of the inflammatory genes RANTES, GM-CSF, and IL-8 was not affected (Fig. 7). The same trend was observed with C3 as well as other host defense molecules. Interestingly, transfection with siRNA for C/EBPδ instead of C/EBPβ blocked the induction of C3 by GC, indicating that C/EBPδ rather than C/EBPβ may be important for the effects of GC on C3. Western blot analysis confirmed that both of the siRNA that we used (Qiagen and Santa Cruz Biotechnology) reduced (but did not eliminate) C/EBP protein compared with scrambled control siRNA (data not shown). Both of the C/EBP siRNA we used failed to induce IFNγ. Taken together, these data indicate that enhancement of the expression of host defense genes in epithelial cells by GC may be mediated by the transcription factor C/EBP.
Discussion

The innate immune response to an infectious challenge is partly mediated by the production of APR proteins by the liver. For this article, we studied the manifestation of a local APR by airway epithelium, a response that may play a role in host defense in the airways. The key findings that we have made are: 1) TLR stimulation of epithelial cells induces the expression of numerous host defense molecules; 2) GC spare or enhance this response; and 3) the epithelial APR and GC responses are mediated by C/EBP. The immune and inflammatory proteins that we found to be induced by dsRNA included complement proteins, small cationic antimicrobial peptides (β-defensins), larger antimicrobial proteins (lysozyme, lactoferrin, and SLPI), collectins (MBL and SpD), collectins (MBL and SpD), cytokines (IFNβ and GM-CSF), and chemokines (RANTES and IL-8). Many of these epithelial-expressed molecules are classical acute phase proteins and display microbicidal activity or inhibit the growth of microorganisms.

The collectin family proteins SpA and SpD induce aggregation and opsonization and can directly cause bacterial lysis by permeabilizing membranes of a broad spectrum of pathogens (27, 28). Another collectin, MBL, and its two associated proteases, MASP1 and MASP2, recognize and bind to mannose and other carbohydrates on bacteria, yeast, and viruses and initiate complement activation (29). Deficiencies of MBL are associated with susceptibility to bacterial and viral infections (30). We observed a trend toward the induction of SpD by dsRNA, FP, and the combination of dsRNA and FP. MBL mRNA was induced by dsRNA and GC in the immortalized BEAS-2B cells but not in primary epithelial cells. Although the explanation for this difference is not clear, the results with SpD and MBL suggest that epithelial collectin expression is inducible by TLR pathways and that this response is not inhibited by GC.

Complement is a centrally important component of the APR and is essential in immunity. Its main physiologic activities include opsonization, chemotaxis, leukocyte activation, direct lysis of bacteria and infected cells, and augmentation of Ab responses (31). Although hepatocytes are the main source of complement biosynthesis, epithelial cells including keratinocytes (32) and renal tubular epithelial cells (33) have been shown to be capable of synthesizing complement proteins. We observed that the basal level of C3 in airway epithelial cells is relatively high at the mRNA level (the raw Ct was 22–25 whereas that of GAPDH was 18–21) and protein level (20 ng/1 × 10⁶cells). The production of C3 in airways may be important for host defense at a major pathogen entry site. Upon stimulation with dsRNA, the mRNA level of C3 increased 9-fold while protein levels increased almost 3-fold. This magnitude of change is much higher than the pattern in blood after a moderate inflammatory stimulus (34). The other complement factors we studied in airway include factor B and factor H, two important components of the alternative pathway. When factor B binds to C3b it is activated by factor D and forms the C3 convertase C3bBb, which then initiates the cleavage of C3. Factor H is a dominant complement control protein that initiates the inactivation of C3 by factor I. Local production of these regulatory molecules may help regulate C3 function. Our results indicate that expression of these important complement proteins by airway epithelial cells can be induced by TLR activation and enhanced by GC, an effect that may boost innate immunity in patients treated with GC.

SAA and CRP are acute phase proteins whose serum concentrations can increase by as much as 100- to 1000-fold in disease (35). Recently, Raynes and coworkers discovered that SAA binds rapidly and firmly to a surprisingly large number of Gram-negative bacteria through the outer member protein A (36). Stimulation of polymorphonuclear cells with SAA increases the secretion of lactoferrin and enhances polymorphonuclear cell phagocytic activity against Candida albicans (37), suggesting that SAA is an important molecule in innate immunity. We observed an impressive increase in the expression of SAA by dsRNA and FP individually, and the combination of dsRNA and FP induced the greatest increase of SAA expression by epithelial cells. Once again, these results are compatible with the hypothesis that GC enhance local innate immune responses.

CRP is the prototypical member of the pentraxin family. It has been used as a clinical marker of inflammatory processes for many years, although the roles of CRP in host defense and disease are not completely understood. CRP transgenic mice are resistant to experimental pneumococcal sepsis and display reduced bacteremia following an i.p. inoculation of pneumococci due to the high level of CRP expression (38). Gould and Weiser found that CRP was present in secretions of the human respiratory tract (6). When the secretions were pretreated to remove CRP, the complement-dependent bactericidal activity of normal nasal airway surface fluid and sputum against Haemophilus influenzae was abolished (6). The protective effect of CRP is thought to be mediated by binding to phosphorylcholine, a constituent of the membrane of major bacterial pathogens of the human respiratory tract, and initiating the classical complement pathway through interaction with C1q (39). We observed a consistent increase of CRP in epithelial cells exposed to the glucocorticoid FP and found enhanced CRP expression by cells exposed to FP and dsRNA.

As has been published by us and many other groups, GC are excellent inhibitors of the expression of chemokines by airway...
epithelial cells and other airway cell types (12, 40, 41). Indeed, we found that FP inhibited the expression of all of the chemokines studied with the exception of MIP-3α/H9251. MIP-3α/H9251 is the unique ligand for CCR6, a receptor with a restricted distribution. In addition to being a potent chemoattractant for immature dendritic cells and T cells, MIP-3α also possesses antibacterial activity of greater potency against *Escherichia coli* and *Staphylococcus aureus* than HBD1 and HBD2, which have also been found to bind and activate CCR6 (42). Thus, MIP–3α appears to respond to GC in our in vitro system more like the other host defense molecules than the chemokines and other inflammatory genes.

Another exception to the pattern that we have observed (i.e., sparing of host defense genes and suppression of inflammatory genes) is the findings with the defensins. HBD are small (3–5 kDa)
protease-resistant antimicrobial peptides that permeabilize the membranes of bacteria, fungi, and enveloped viruses. β-Defensin-deficient mice had delayed clearance of H. influenzae from the lung (43) and inhibition of the synthesis of HBD1 resulted in decreased antimicrobial activity of airway surface fluid (44). It is of note that GC failed to inhibit the expression of HBD1 but suppressed HBD2 and HBD4. HBD1 is constitutively expressed in enteric epithelial cells unlike HBD2–4, which are inducible (45). These results may indicate that HBD1 plays a more important role in resistance to commensal organisms. Interestingly, we found that HBD1 was inducible by TLR3 activation in our studies. HBD2 has been suggested to have a pathophysiological role as a proinflammatory mediator (46). It is not clear why HBD2 and HBD4 are regulated differently from the other antimicrobial proteins. The HBD1 promoter contains a NF-IL-6 (C/EBP regulated differently from the other antimicrobial proteins. 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whereas others, such as NF-κB and AP-1, are more important for the expression of inflammatory genes and are thus GC sensitive. We hasten to point out that many effects of GC are exerted posttranscriptionally and there may be selective effects of GC on these mechanisms as well (65).

Taken together, the results of the present study demonstrate that TLR activation triggers the respiratory epithelium to manifest a response while sparing or enhancing the local innate host defense response. These selective effects of GC are partially mediated through activation of the transcription factor C/EBP by GC. The results presented here are worthy of consideration during ongoing efforts to develop improved GC for the treatment of airway inflammatory diseases.

Disclosures

The authors have no financial conflict of interest.

References


