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Fluticasone and budesonide inhibit cytokine release in human lung epithelial cells and alveolar macrophages

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Glucocorticoids are potent anti-inflammatory agents capable of influencing cytokine release in a number of cell types. The aim of the present study was to investigate whether glucocorticoids, frequently used in the treatment of asthma, interfere with cytokine secretion by lung epithelial cells and alveolar macrophages *in vitro*. Inhalation of swine dust induces airway inflammation with influx of inflammatory cells and release of proinflammatory cytokines in the lungs. Therefore, human lung epithelial cells (A549) and human alveolar macrophages were stimulated with swine dust or lipopolysaccharide (LPS), and the inhibitory effect of budesonide and fluticasone propionate on cytokine release was studied in a dose-response (10^{-13} – 10^{-8} M) manner. The time course for the steroid effect was also investigated. Both steroids caused a dose-dependent, almost total, inhibition of swine dust-induced IL-6 and IL-8 release from epithelial cells and LPS-induced IL-6 and TNF- α from alveolar macrophages. The steroids only partially inhibited IL-8 release from alveolar macrophages. Budesonide was approximately 10 times less potent than fluticasone propionate. Preincubation with the steroids did not inhibit cytokine release more than simultaneous incubation with stimulus and steroid. In conclusion, budesonide and fluticasone propionate, in concentrations that probably occur in the airway lining fluid during inhalational therapy, inhibited cytokine release from human lung epithelial cells (IL-6, IL-8) and alveolar macrophages (TNF- α , IL-6, IL-8). *In vitro*, the onset of this effect was rapid.

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Introduction

Glucocorticoids are potent anti-inflammatory drugs and frequently used in the treatment of various inflammatory

diseases. The lungs are continuously exposed to harmful environmental agents with a potential to cause airway inflammation. Alveolar macrophages and epithelial cells act as the first-line defense, before the migration of other types of inflammatory cells into the airways, against inhaled agents, such as organic dust. Alveolar macrophages and epithelial cells have the capacity to recruit inflammatory cells to the airways via the release of chemoattractants, and the epithelium is also capable of directing inflammatory cell migration via expression and induction of cell-surface molecules (1, 2). Thus, it seems probable that glucocorticoids interact with cytokine release of alveolar macrophages and epithelial cells in the airways after stimulation by inhaled dust.

Interleukin-6 (IL-6), IL-8, and tumor necrosis factor- α (TNF- α) mediate a variety of proinflammatory effects which are of importance in conditions characterized by airway inflammation. One major source of TNF- α in the lung is alveolar macrophages (3), and IL-6 and IL-8 are produced and released from both epithelial cells and alveolar macrophages (4, 5). TNF- α is capable of activating neutrophils, T cells, and macrophages and, like IL-6, it induces production of acute-phase proteins (3, 6). IL-6 stimulates T-cell activation and differentiation and induces fever and production of acute-phase proteins (7–9). IL-8 is recognized as a chemotactic agent for neutrophils (10), T cells (11), and primed eosinophils (12).

Inhalation of organic dust from a swine confinement building induces airway inflammation in healthy human volunteers. A 3-h exposure causes a 75-fold increase in the number of neutrophils and a 2–3-fold increase in alveolar macrophages and lymphocytes in bronchoalveolar lavage (BAL) fluid (13). The exposure may also induce systemic effects such as increased body temperature and increased levels of the acute-phase proteins in peripheral blood (13). In addition, the concentrations of the proinflammatory cytokines IL-6 and TNF- α and the chemokine IL-8 are elevated in nasal and BAL fluids, and TNF- α and IL-6 concentrations increase in peripheral blood after exposure to swine dust (14–16). These findings indicate that IL-6, IL-8, and TNF- α actively participate in the initiation and maintenance of airway inflammation after inhalation of organic dust from a swine house.

One aspect of the glucocorticoid anti-inflammatory effect is related to the ability to inhibit inflammatory leukocyte activation and migration into the airways (17, 18), effects which are partly regulated by different cytokines. Through the effect of glucocorticoids on the transcription of specific target genes, inflammatory mediators, particularly cyto-

kines, can be inhibited (17). In recent years, it has been demonstrated that glucocorticoids are capable of regulating inflammatory genes, not only by direct binding of the glucocorticoid receptor to glucocorticoid responsive elements, but also through interactions with transcription factors (19, 20).

The precise target cells of the glucocorticoid action in the airways still remain to be determined. To elucidate the role of presumptive target cells, we have, in the present study, exposed a human lung epithelial carcinoma cell line (A549) and human alveolar macrophages obtained by BAL to swine dust or *Escherichia coli*-derived lipopolysaccharide (LPS) in the presence of two glucocorticoids, budesonide (BUD) and fluticasone propionate (FP) in a dose-response manner. The release of proinflammatory cytokines (TNF- α and IL-6) and the chemoattractant (IL-8) and the time kinetics for the steroid-induced inhibition of cytokine release were studied.

Material and methods

Stimuli and drugs

Swine dust was collected in a swine confinement building, containing 700–900 pigs, approximately 1.2 m above the floor. Before the experiments, the dust was dissolved in Ham's F-12 medium (Seromed[®] Biochrom KG, Berlin, Germany) supplemented with 1% penicillin/streptomycin (Seromed Biochrom KG, Berlin, Germany) to a final concentration of 1 mg/ml, and put in an ultrasound bath for 10 min. Lipopolysaccharide (LPS; lipopolysaccharide B *E. coli* O111:B4, Difco, Detroit, MI, USA) was dissolved in RPMI 1640 media (Seromed Biochrom KG, Berlin, Germany). BUD was a kind gift from Astra Draco AB, Lund, Sweden, and FP was generously provided by Glaxo Wellcome, Stevenage, UK. A stock solution of 2×10^{-2} M BUD in 99.5% ethanol (Kemetyl, Stockholm, Sweden) was prepared. FP was dissolved in 10^{-2} M dimethylacetamide (J.T. Baker, Deventer, The Netherlands) and diluted with saline/1% Tween 80 (Merck-Schuchardt, München, Germany) to a 10^{-5} M working stock solution. The stock solutions were further diluted with medium to achieve the concentrations used (10^{-6} – 10^{-12} M).

Epithelial cells

The human lung carcinoma epithelial cell line (A549) was purchased from the American Type Culture Collection (Rockville, USA). A549 cells were cultured in Ham's

F12-medium containing 10% heat-inactivated fetal calf serum (Seromed Biochrom KG, Berlin, Germany), supplemented with 1% penicillin/streptomycin, and kept at 37°C, 5% CO₂. For the experiments, A549 cells were grown in six-well plates (Nunc, Roskilde, Denmark) to subconfluency, washed once with F-12 media, and treated as described below in serum-free media.

Alveolar macrophages

Alveolar macrophages were obtained from 15 healthy, nonsmoking, nonatopic subjects (12 men, mean age 30, range 23–49 years) by BAL. All participants denied symptoms of respiratory tract infection during a 2-week period before the BAL. The procedure was approved by the ethics committee of Karolinska Institute. Briefly, bronchoscopy was performed through the nose with a flexible fiberoptic bronchoscope (Olympus Type 4B2) under local anesthesia with 2% lidocaine (Xylocaine®, Astra, Södertälje, Sweden) after premedication with morphine-scopolamine. The bronchoscope was wedged into a subsegment middle lobe bronchus, and sterile saline solution, at 37°C, was instilled in 5×50 ml aliquots. The fluid was gently aspirated and collected in a siliconized plastic bottle kept on ice. The BAL fluid was put through a Dacron prefilter (Type AP 32 Millipore) and centrifuged (200 g, 10 min, 4°C). The pellet was resuspended in RPMI 1640 culture medium. Cell viability was determined by the exclusion of Trypan blue solution (0.5% in normal saline; AMIMED, Allschwil, Switzerland). The total number of cells was 12–40×10⁶ cells per lavage, counted with a hemacytometer. Differential cell counts were performed by flow cytometry (Epics Profile II®, Coulter Electronics, Inc.), and the number of alveolar macrophages in a lavage was 72% (56–85%) of the total cell count. One million cells or 300 000 cells were then added to sterile 6- or 24-well culture plates, respectively; (Nunc, Roskilde, Denmark) and allowed to adhere for 2 h at 37°C, with 5% CO₂ in RPMI medium containing 5% heat-inactivated fetal calf serum, 1% penicillin/streptomycin, and 0.5% gentamicin (Seromed Biochrom KG, Berlin, Germany). The nonadherent cells were then gently removed by washing twice with serum-free medium and incubated overnight with serum-free medium (1% penicillin/streptomycin, 0.5% gentamicin), and then treated in serum-free RPMI-medium, as described below. The actual cell number was calculated by multiplying the number of seeded cells by the proportion of macrophages obtained by flow cytometry.

Experimental design

Epithelial cells were exposed to swine dust (100 µg/ml) for 24 h and alveolar macrophages to LPS (100 µg/ml) for 8 h. For the dose-response curves, BUD or FP (10⁻¹³–10⁻⁸ M) was added 90 min before swine dust or LPS to be present during the 24 h and 8 h of swine-dust and LPS exposure, respectively.

For the time-course experiments, BUD or FP was added, at a fixed concentration, to the cells at different time points before and after the start of the exposure to swine dust or LPS. Thus, at a concentration of 10⁻⁹ M, the glucocorticoids were added to the epithelial cells 24, 6, and 1.5 h before the swine dust, at the same time as the dust and 4, 8, and 12 h after the start of the 24-h swine-dust exposure. The glucocorticoids (BUD 10⁻⁶ M and FP 10⁻⁹ M) were added to the alveolar macrophages 1.5 h before the LPS exposure, at the same time as LPS and 2, 4, and 6 h after the start of the 8-h LPS exposure.

Since the concentration of the diluent (ethanol and dimethylacetamid/Tween 80) of the drugs increases with increasing steroid concentration, control experiments with the diluents (without steroids) corresponding to each steroid concentration were performed. With epithelial cells, different preincubation time periods (24 and 1.5 h) and different swine-dust exposure time periods (6 and 24 h) were used. With alveolar macrophages, this was performed during the same conditions as in the dose-response studies (90-min preincubation and 8-h LPS exposure).

After each experiment, the supernatants were centrifuged (1000 g, 10 min, 20°C) and stored at -70°C until assayed. The

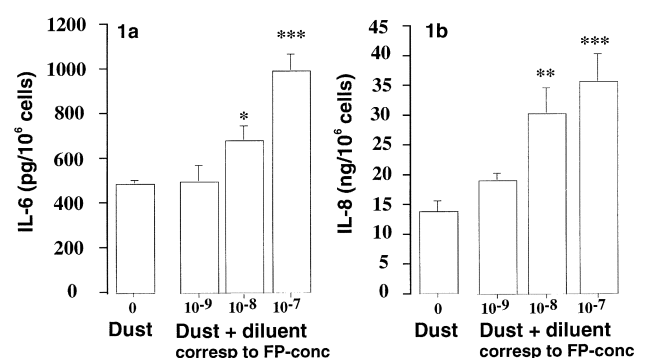


Figure 1. Effect of swine dust alone and swine dust in combination with diluent (dimethylacetamide/Tween 80) corresponding to FP concentrations of 10⁻⁹–10⁻⁷ M on A549 epithelial cells on IL-6 and IL-8 secretion. A549 cells were preincubated with diluent 24 h and then coincubated with swine dust for another 6 h. Swine dust: *n*=6; diluent plus swine dust: *n*=3. Results are presented as IL-6 and IL-8 amount (pg and ng, respectively) per 10⁶ cells. Mean values and SEM. ****P*<0.001; ***P*<0.01; **P*=0.05 compared to swine dust (0).

epithelial cells and the macrophages were detached by exposure to trypsin/EDTA solution (0.05/0.02% in calcium- and magnesium-free phosphate-buffered saline; Seromed Biochrom KG, Berlin, Germany), and cell viability was determined by exclusion of Trypan blue (0.4% in saline, Sera-Lab, Sussex, UK). The epithelial cells were counted in a hemocytometer.

Each experiment was performed in triplicates or quadruplicates.

Cytokine assays

The amount of IL-6, IL-8, and TNF- α secreted into the medium from the epithelial cells was determined by a commercial enzyme-linked immunosorbent assay (ELISA) test kit (QuantikineTM, R&D Systems Europe, Abingdon, UK). The detection ranges for the IL-6, IL-8, and TNF- α assays were 3.13–300 ng/l, 31.2–2000 ng/l, and 15.6–1000 ng/l, respectively. The cytokines were measured in duplicates, and an intra- and interassay coefficient of variation of <10% was accepted.

IL-6, IL-8, and TNF- α in the macrophage cell supernatants were measured with ELISA methods developed in our laboratory; the procedure is described in detail by Larsson et al. (21). Commercially available antibody pairs were used. The monoclonal antihuman capture antibodies were IL-6: MAB206, IL-8: MAB208, and TNF- α : MAB610 (R&D Systems Europe, Abingdon, UK). The detection biotinylated antihuman polyclonal antibodies were IL-6: BAF206, IL-8: BAF208, and TNF- α : BAF210. The recombinant human standards were IL-6: 206-IL-010, IL-8: 208-IL-010, and TNF- α : 210-TA-010 (R&D Systems Europe, Abingdon, UK). Absorbance was read at 450 and 650 nm with a Thermomax 250 reader (Molecular Devices, Sunnyvale, CA, USA). QuantikineTM serum controls at three different concentrations were used as calibrators in all three methods. The cytokines were measured, and production was expressed as pg or ng/10⁶ cells. An intra-assay coefficient of variation of <10% and an interassay coefficient of variation of <20% was accepted. The detection ranges in the IL-6, IL-8, and TNF- α assay were 3–375 pg/ml, 50–3200 pg/ml, and 15.6–1000 pg/ml, respectively.

Statistical analysis

The results are presented as mean (SEM) cytokine production per 10⁶ cells and are expressed as percentage change from the positive control (swine dust or LPS). Comparisons were assessed by analysis of variance (ANOVA) with

Fisher's PLSD test when appropriate and Student's *t*-test. A *P* value of <0.05 was considered significant. Statistical analysis was performed with the StatView[®] program, version 4.5, for Macintosh (Abacus Concepts, Inc., Berkeley, CA, USA).

Results

A549 epithelial cells

In A549 epithelial cells, exposure to swine dust (100 μ g/ml) for 24 h induced a more than 25-fold increase in IL-6 and IL-8 production, while TNF- α did not increase above the detection limit (TNF- α values not shown). The viability of the cells after each experiment was >90%, unless otherwise stated.

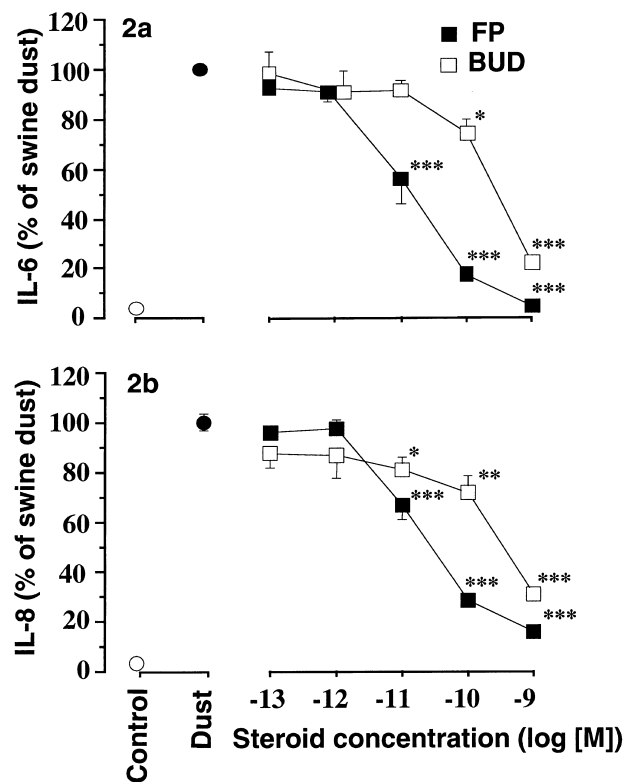


Figure 2. Dose-response curves for IL-6 (a) and IL-8 (b) production from A549 epithelial cells. Cells were preincubated 1.5 h with BUD or FP at each concentration and then exposed to swine dust (100 μ g/ml) for 24 h with drugs present. Results are presented as mean and SEM in percentage of cytokine production induced by swine dust. For IL-6, 100% corresponds to 556 pg/10⁶ cells, and corresponding value for IL-8 is 16.9 ng/10⁶ cells. Curves for FP are based on three independent tests (*n*=12). Curves for BUD are based on two independent tests (*n*=8). At concentration of 10⁻⁹ M, FP data are based on five tests (*n*=19), and BUD data are based on four tests (*n*=15). **P*<0.05; ****P*<0.001 compared to swine dust (positive control).

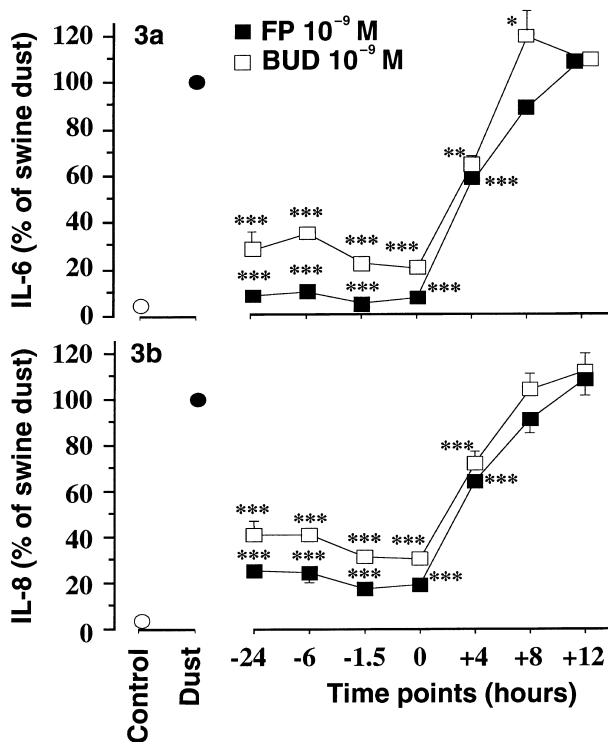


Figure 3. Time-course curves for IL-6 (a) and IL-8 (b) production from A549 epithelial cells. Cells were incubated with BUD or FP 10^{-9} M at different time points before (-24, -6, and -1.5 h), at same time (0), and after (+4, +8, and +12 h) start of swine-dust exposure. Drugs were then present during swine-dust exposure, which lasted for 24 h. Results are presented as mean and SEM in percentage of cytokine production induced by swine dust. For IL-6, 100% corresponds to $515 \text{ pg}/10^6$ cells, and corresponding value for IL-8 is $13.8 \text{ ng}/10^6$ cells. For -24 to 0 h, data are based on two independent tests ($n=7$). For +4 to +12 h, results are based on quadruplicates from one test. *** $P<0.001$; ** $P<0.01$; * $P<0.05$ compared to swine dust (positive control).

Effect of the diluent

The highest concentration of the diluent used in the experiments with epithelial cells, corresponding to the diluent content in BUD or FP at 10^{-9} M, incubated under the same conditions as in the dose-response experiments, did not significantly alter the steroid effect on swine dust-induced cytokine production ($n=8$). However, the diluent corresponding to BUD 10^{-5} M (0.05 % ethanol) enhanced the IL-8 response to swine dust by 28% ($P<0.001$) and IL-6 response by 47% ($P<0.05$, 24 h before incubation plus coincubation 6 h with swine dust; $n=3$). The diluent, corresponding to BUD 10^{-7} M, did not significantly alter the swine dust-induced IL-6 and IL-8 response (24 h preincubation and 6 h coincubation with swine dust, $n=3$).

The FP diluent (preincubation 24 h with dimethylacetamid/saline with Tween 80 and coincubated with swine dust for 6 h) corresponding to a FP concentration of 10^{-8} M enhanced the swine dust-induced IL-6 and IL-8 production

significantly by 35% ($P<0.05$) and 74% ($P<0.01$), respectively. The FP diluent corresponding to a FP concentration of 10^{-7} M more than doubled the IL-6 production and increased IL-8 production more than threefold (cell viability=82%). At the diluent concentration corresponding to FP 10^{-6} M, cell blebbing and a change of pH medium were observed ($n=3$). The viability was not measurable because of cell fragmentation after trypsination. When FP (10^{-6} M) was present during the same conditions, with or without swine dust, the cells also had signs of blebbing ($n=3$). The effect of the FP diluent is shown in Fig. 1. Because of these diluent-induced effects on cytokine release, the highest concentration of BUD and FP was 10^{-9} M in the epithelial cell experiments.

Dose response

BUD and FP (10^{-13} – 10^{-9} M) caused a dose-dependent inhibition of the IL-6 and IL-8 production induced by swine dust. BUD inhibited IL-6 and IL-8 significantly at 10^{-11} – 10^{-10} M and FP at 10^{-11} M (Fig. 2). At 10^{-9} M, BUD inhibited IL-6 release by 80% and IL-8 release by 72%. FP inhibited IL-6 release by 96% and IL-8 release by 85% at 10^{-9} M (Fig. 2).

Time course

BUD and FP (10^{-9} M) inhibited the swine dust-induced IL-6 and IL-8 release in the same order of magnitude regardless of the length (24, 6, and 1.5 h) of preincubation or whether the cells were incubated with the steroid and dust at the same time (Fig. 3). BUD or FP (10^{-9} M) added 4 h after swine dust inhibited the IL-6 production by 34% and 41%, respectively. Corresponding values for IL-8 production were 27% (BUD) and 36% (FP), which is about half the inhibition accomplished with the steroids added simultaneously with the dust. Addition of the steroids 8 and 12 h after the dust did not significantly influence the swine dust-induced cytokine release.

Alveolar macrophages

LPS ($100 \text{ } \mu\text{g}/\text{ml}$ for 8 h) induced an almost 200-fold increase of IL-6 ($n=18$) and TNF- α ($n=21$) production and a 12-fold increase of IL-8 production ($n=21$) compared to control in human alveolar macrophages. The cell viability before the seeding onto plates was >90% in all cases, and the viability of the adherent cells after each experiment was >86% in all cases.

Effect of the diluent

The diluent corresponding to a BUD concentration of 10^{-6} M did not enhance the IL-6, IL-8, or TNF- α response to LPS (data not shown). At an equivalent amount of diluent

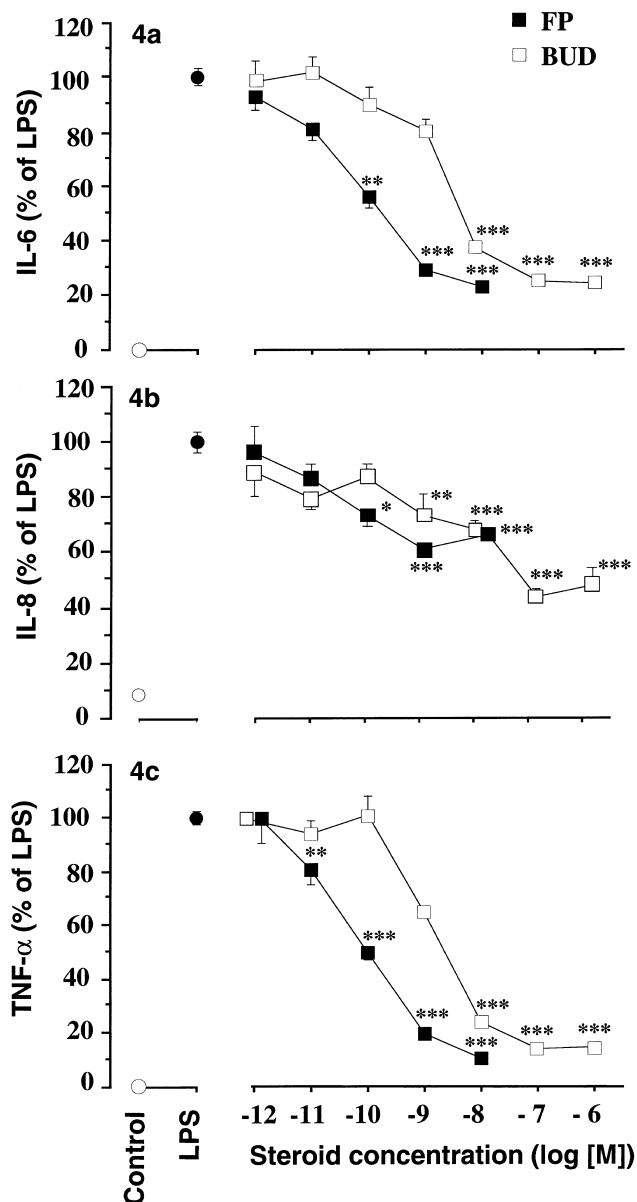


Figure 4. Dose-response curves for IL-6 (a), IL-8 (b), and TNF- α (c) production from human alveolar macrophages. Cells were preincubated with BUD or FP at each concentration for 1.5 h and then exposed to LPS (100 μ g/ml) for 8 h, during which drugs were present. Results are presented as mean and SEM in percentage of cytokine production induced by LPS. For IL-6, 100% corresponds to 11.8 ng/ 10^6 cells, and corresponding values for IL-8 and TNF- α are 46.9 and 14.2 ng/ 10^6 cells, respectively. Each data point is based on 1–6 experiments performed in triplicate or quadruplicate. *** $P<0.001$; ** $P<0.01$; * $P<0.05$ compared to LPS (positive control).

corresponding to FP 10^{-9} M ($n=4$), the IL-6, IL-8, and TNF- α response to LPS was not affected (ratio [diluent plus LPS]/LPS=0.85, 1.08, and 0.93, respectively). The diluent corresponding to a FP concentration of 10^{-8} M did not significantly enhance the IL-8 production (ratio [diluent plus LPS]/LPS=1.34; $n=7$). Because of these diluent-induced

effects on cytokine release, the highest concentration of BUD was 10^{-6} M and of FP 10^{-8} M in the alveolar macrophage experiments.

Dose-response experiments

BUD and FP (10^{-12} – 10^{-6} M) caused a dose-dependent inhibition of the IL-6, IL-8, and TNF- α production (Fig. 4). BUD inhibited IL-8 significantly from 10^{-9} M ($P<0.01$) and IL-6 and TNF- α secretion significantly from 10^{-8} M ($P<0.001$). FP inhibited TNF- α secretion significantly from 10^{-11} M ($P<0.01$), whereas IL-6 and IL-8 secretion was inhibited significantly from 10^{-10} M ($P<0.01$ and $P<0.05$, respectively). Inhibitions of IL-6, IL-8, and TNF- α were about 75%, 33%, and 85% respectively in alveolar macrophages at the highest FP concentration (10^{-8} M).

Time course

BUD (10^{-6} M) added 2, 4, or 6 h after LPS still inhibited the IL-6, IL-8, and TNF- α production significantly ($P<0.001$, Fig. 5). Adding FP (10^{-9} M) 2 or 4 h after LPS still inhibited the IL-6 production significantly ($P<0.001$). Similarly, TNF- α production was inhibited significantly by the addition of FP (10^{-9} M) 2 h ($P<0.001$) and 4 h ($P<0.05$) after the addition of LPS.

Discussion

In the present study, we have shown that BUD and FP inhibit swine dust-induced IL-6 and IL-8 production from a human lung epithelial cell line (A549) and LPS-induced IL-6, IL-8, and TNF- α production from human alveolar macrophages in a dose-dependent manner. Furthermore, we demonstrated that the onset of this steroid effect is rapid; i.e., there is no need for preincubation with the steroid to achieve a maximal inhibitory effect.

The glucocorticoids were diluted in 99.5% ethanol (BUD) or dimethylacetamid in saline/1% Tween 80 (FP). Since it could not be excluded that these diluents may influence the cytokine release, we studied the cytokine response to the diluents in concentrations corresponding to the concentrations of the steroids. When incubating epithelial cells with 0.05% ethanol (corresponding to BUD 10^{-5} M) in combination with swine dust, the IL-6 and IL-8 production was elevated by 47% and 28%, respectively, compared to the cytokine production induced by swine dust alone. Similarly, enhanced cytokine release from the epithelial cells and alveolar macrophages was observed at a concentration of dimethylacetamide/Tween 80 corresponding to a FP

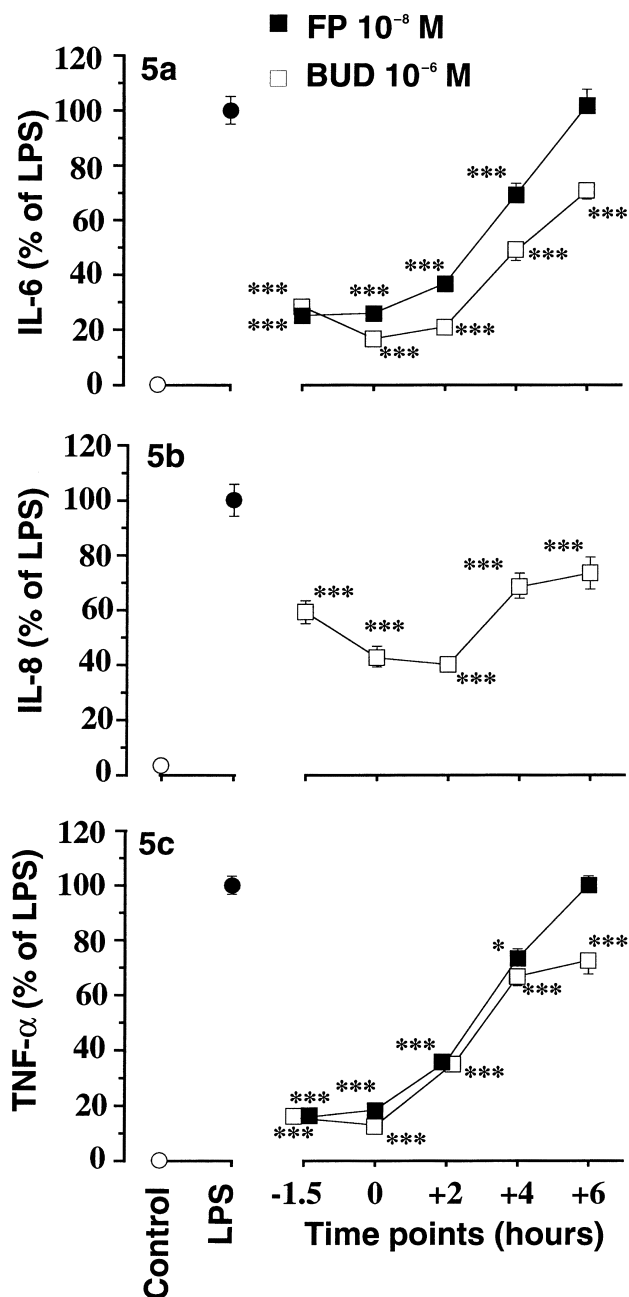


Figure 5. Time-course curves for IL-6 (a), IL-8 (b), and TNF- α (c) production from human alveolar macrophages. Cells were exposed to LPS (100 μ g/ml) for 8 h. BUD (10⁻⁶ M) or FP (10⁻⁹ M) was added 1.5 h before addition of LPS (-1.5 h), simultaneously with LPS (0), or 2, 4, or 6 h after (+2, +4, and +6 h) addition of LPS. Results are presented as mean and SEM in percentage of cytokine production induced by LPS. For IL-6, 100% corresponds to 7.15 ng/10⁶ cells, and corresponding values for IL-8 and TNF- α are 44.6 and 7.62 ng/10⁶ cells, respectively. Results with FP are based on triplicates from two separate tests, and results with BUD are based on quadruplicates from one test. *** P <0.001; * P <0.05 compared to LPS (positive control).

concentration of 10⁻⁸ M. Therefore, we did not use higher concentrations than 10⁻⁶ M for BUD and 10⁻⁸ M for FP in our experiments.

Ryrfeldt et al. showed that inhalation of 500 μ g BUD resulted in a blood plasma concentration of 10⁻⁹ M for

several h (22). The steroid concentration in lung tissue was approximately 10 times higher than in blood plasma (23), and in central lung tissue approximately four times higher than in peripheral lung tissue (24). Kerrebijn has calculated that the concentration of a glucocorticoid in the large airways will be approximately 10⁻⁴–10⁻³ M after inhalation of 0.5 mg (25). Based on these calculations, the highest concentrations used in our experiments (10⁻⁹–10⁻⁸ M) seem to be concentrations which could be anticipated to be achieved in the airways during treatment of asthma with inhaled BUD and FP. From the present data, it is thus very likely that inhalation of BUD and FP, in clinically relevant doses leads to steroid concentrations in the airways that could suppress cytokine release from epithelial cells and alveolar macrophages. It is, however, not possible to draw any firm clinical conclusions regarding these *in vitro* effects and it is not clear whether these effects really occur *in vivo* and constitute a favorable effect in the treatment of asthma with inhaled steroids. Moreover, the cells we have studied do not originate from patients with asthma, and it is not clear whether cells from asthmatics behave differently. In previous studies, we have shown that LPS and swine dust, on a weight basis, stimulate cytokine release from alveolar macrophages in a similarly dose-dependent manner, and since LPS is a more standardized stimulus, we have in the present study chosen to stimulate the macrophages with LPS (26). We have also shown that swine dust stimulates cytokine release from lung carcinoma epithelial cells (A549) and normal human bronchial epithelial cells (NHBE) in a similarly dose-dependent fashion (26), indicating that in this respect the A549 cells are representative of normal epithelial cells.

BUD and FP almost totally inhibited IL-6 and IL-8 release from the epithelial cells and TNF- α release from the macrophages, while IL-6 and IL-8 release from the macrophages was only partially inhibited. FP inhibited IL-8 production only by 33% at 10⁻⁸ M in the alveolar macrophages, and higher concentrations of this drug were infeasible due to the influence of the diluent on the cytokine release (as already discussed). Therefore, we did not test the time course for inhibition of IL-8 by FP. Our results agree with previous studies. Thus, Linden & Brattsand found a partial inhibition of IL-6 release from alveolar macrophages by BUD (27), and Standiford et al. found that dexamethasone (10⁻⁶ M) inhibited LPS-induced IL-8 secretion by 61% in alveolar macrophages (28). The TNF- α response to interferon- γ was found to be inhibited by dexamethasone in alveolar macrophages from lung-transplant recipients (29). Others have found a glucocorticoid-induced inhibition of

IL-8 (30, 31) and IL-6 (30) synthesis in epithelial cells stimulated by IL-1 β or endotoxin from *Haemophilus influenzae*.

It has been claimed that once the glucocorticoid has reached the biophase of the target cell, time is needed from the application of the drug to the appearance of biological effects (32). The present study has clearly demonstrated that the glucocorticoid-induced inhibition of IL-6, IL-8, and TNF- α was not enhanced by preincubating the cells with the steroid for different times before the stimulus (dust or LPS) compared to incubating the cells with the steroid and the stimulus at the same time. Moreover, adding the steroid to the already dust- or LPS-incubated cells showed an inhibition of the cytokine release. For example, a significant inhibition of TNF- α release was still observed when the glucocorticoid was added to the alveolar macrophages 6 h after the stimulus. Since the glucocorticoids probably do not influence the cytokines already produced, these findings suggest that the glucocorticoid-induced inhibition of cytokine release starts very rapidly. The molecular basis for the effect of glucocorticoids is by influencing transcription, by sequence-specific DNA-binding, and by transcriptional repression of protein synthesis mediated through interaction with transcription factors such as NF- κ B and AP-1 (28, 33–35). The very rapid onset of action found in the present study may suggest that the major site of the steroid action on cytokine (IL-6, TNF- α , IL-8) release is probably within the cytoplasm, interacting with transcription factors such as NF- κ B and AP-1, and not in the nucleus (36).

In previous studies, FP has, in many aspects, been found to be more potent than BUD. Thus, FP was more potent than

was BUD with regard to inhibition of PAF-induced eosinophil accumulation in the guinea-pig lung (37). FP was three times more potent than BUD in the ability to produce vasoconstriction in human subjects (38), an effect which is believed to reflect the glucocorticoid clinical anti-inflammatory effect (39). It is generally thought that from a clinical point of view FP has a higher potency than BUD (or is at least as potent) in the treatment of asthma (40). This difference in potency has been attributed to the higher lipophilicity of FP (300-fold more lipophilic than BUD) and the higher affinity of the human glucocorticoid receptor to FP (41). Our results agree with previous data, and we found that FP was approximately 10 times more potent than BUD in inhibition of the release of all three cytokines.

In conclusion, our results suggest that one of the anti-inflammatory effects of glucocorticoids is related to inhibition of proinflammatory cytokine release, such as IL-6 and IL-8 from lung epithelial cells and IL-6, IL-8, and TNF- α from alveolar macrophages, which are produced early in the inflammatory process. The onset of the effect is rapid and our *in vitro* results suggest that the BUD and FP concentration in the airways after regular steroid inhalation might be high enough to lower significantly the IL-6, IL-8, and TNF- α production *in vivo*. The clinical relevance of this remains to be shown.

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