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# Beclomethasone, budesonide and fluticasone propionate inhibit human neutrophil apoptosis

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**Abstract**

Inhaled glucocorticoids are widely used to treat chronic obstructive pulmonary disease without much evidence of efficiency in this disease where neutrophils may contribute to the pathophysiology. This prompted us to test the effects of several currently used inhaled and systemic glucocorticoids on human neutrophil apoptosis. Beclomethasone, budesonide, dexamethasone, fluticasone propionate, hydrocortisone and prednisolone inhibited apoptosis in a concentration-dependent manner as assessed by flow cytometric analysis, annexin-V binding and morphological analysis. The maximal inhibition of apoptosis was 50–60%. The order of potency was fluticasone propionate ($EC_{50}$ 0.6 ± 0.2 nM) ≈ budesonide ($EC_{50}$ 0.8 ± 0.2 nM) > dexamethasone ≈ prednisolone ≈ beclomethasone ≈ hydrocortisone. The inhibitory effects of glucocorticoids were reversed by mifepristone. Moreover, glucocorticoids slightly enhanced the inhibitory effect of granulocyte-macrophage colony-stimulating factor (GM-CSF) on neutrophil apoptosis. The present data suggests that budesonide and fluticasone propionate prolong human neutrophil survival by inhibiting apoptosis at clinically relevant drug concentrations via an effect on glucocorticoid receptor. 

*Keywords:* Apoptosis; Neutrophil; Fluticasone propionate; Budesonide; Beclomethasone

## 1. Introduction

Neutrophils are crucial to host defense mainly through phagocytosing and destroying infectious agents (Smith, 1994). However, they are also thought to be an important cellular mediator of tissue damage in many acute and chronic inflammatory conditions (Haslett, 1999). As other human phagocytes, neutrophils die through apoptosis during the resolution of inflammation (Haslett, 1999). This process determines the rapid clearance of intact senescent neutrophils by macrophages and thus provides an injury-limiting granulocyte clearance mechanism (Cox et al., 1995b; Haslett, 1999). Distinct from necrosis that loses cell membrane integrity and releases the dying cell's contents in an uncontrolled and possibly harmful manner, apoptotic cell is phagocytosed intact without releasing its contents (Haslett, 1997). Human neutrophils undergo spontaneous apoptosis at a rapid rate in vitro and in vivo (Cox et al., 1995; Haslett, 1999). This process can be inhibited by granulocyte-macrophage colony-stimulating factor (GM-CSF) (Brach et al., 1992; Dibbert et al., 1999) and leukotriene $B_4$ (Haslett, 1999). The lifespan of the mature circulating neutrophil is estimated to be between 8 and 20 h but can be increased to a few days when the cell is recruited into the tissue (Moulding et al., 1998).

It is reasonable to suppose that failure to remove neutrophils and their toxic products from tissues could result in chronic persistent inflammation. Neutrophils are thought to be involved in a variety of inflammatory lung disorders. Chronic obstructive pulmonary disease is believed to be the consequence of chronic airway inflammation (Barnes, 1998; Jeffery, 1999). Increased numbers of inflammatory cells and high levels of inflammatory mediators have been reported in bronchoalveolar lavage fluid and lung tissue in the patients with chronic obstructive pulmonary disease (Barnes, 1998; Jeffery, 1999). Increased numbers/levels

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of neutrophils and neutrophil activating inflammatory mediators such as interleukin-8 and leukotriene $B_4$ as well as neutrophil-derived oxidants and proteases are found in the airway lumen in chronic obstructive pulmonary disease (Jeffery, 1999). Neutrophils thus seem to be involved in the development of chronic obstructive pulmonary disease (Barnes, 1998; O'Byrne and Postma, 1999).

Glucocorticoids are effective in controlling the inflammation in asthma and several other chronic inflammatory diseases. However, the effect of inhaled glucocorticoids in the treatment of chronic obstructive pulmonary disease is controversial. Though there are some studies to suggest that inhaled glucocorticoids may be of benefit in chronic obstructive pulmonary disease, many reports suggest that the inflammatory process in chronic obstructive pulmonary disease is resistant to the anti-inflammatory effect of glucocorticoids, especially in stable chronic obstructive pulmonary disease (Postma and Kerstjens, 1999; Van Grunsven et al., 1999). In some studies in patients with asthma and chronic obstructive pulmonary disease, glucocorticoids have been reported not to reduce, but even to increase, neutrophil numbers in induced sputum (Hoshino and Nakamura, 1996; Inoue et al., 1999; Keatings et al., 1997). Therefore, we hypothesized that inhaled glucocorticoids at low concentrations could delay neutrophil apoptosis.

The aim of the present study was to find out whether the currently used inhaled glucocorticoids beclomethasone, budesonide and fluticasone propionate affect constitutive apoptosis or GM-CSF-afforded human neutrophil survival. A preliminary insight on the mechanism of action of inhaled glucocorticoids was made by investigating whether their effects are mediated via glucocorticoid receptor.

## 2. Materials and methods

### 2.1. Neutrophil isolation and culture

Neutrophils were isolated under sterile conditions. Briefly, a buffy-coat preparation (50 ml) from normal individuals was collected into 10 ml of acid citrate dextrose anticoagulant and hydroxyethyl starch solution. White blood cell pellet was laid onto Ficoll and centrifuged at $700 \times g$ for 30 min at 20 °C. The mononuclear cell layer was discarded and the phagocyte pellet was resuspended and washed in Hank's balanced salt solution (HBSS). Contaminating red blood cells were lysed by hypotonic lysis. The remaining granulocytes were washed twice with RPMI 1640. The neutrophils were counted using microscopic examination in Kimura stain, and the purity of neutrophil population was $> 98\%$. The cells were resuspended at $2 \times 10^6$ cells/ml, cultured for the indicated time (37 °C; 5% $CO_2$) in PRMI 1640 (Dutch modification) with 10% fetal calf serum plus antibiotics.

### 2.2. Flow cytometry

#### 2.2.1. Propidium iodide staining

Neutrophil apoptosis was determined by propidium iodide staining of DNA fragmentation and flow cytometry (FACScan, Becton Dickinson, San Jose, CA). Briefly, the cells were washed in phosphate-buffered saline (PBS) solution, fixed by 70% ethanol and incubated in ice for 30 min. The cell pellet was resuspended in propidium iodide solution (25 μg/ml in PBS), incubated for 60 min and measured by flow cytometry. The cells showing decreased relative DNA content were considered as apoptotic.

#### 2.2.2. Annexin-V

Annexin-V binding and analysis by flow cytometry were performed according to the instructions of the manufacturer. Briefly, the cells ($5 \times 10^5$) were washed in PBS solution and suspended in 195 μl of binding solution (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Five microliters of annexin-V fluorescein isothiocyanate (annexin-V-FITC) (solution containing 50 mM Tris, 100 mM NaCl, 1% bovine serum albumin, 0.02% Sodium Azide, pH 7.4) was added and the cell suspension was incubated at room temperature for 10 min. The cells were then washed and resuspended in binding buffer containing 10 μg/ml propidium iodide and analyzed by flow cytometry.

### 2.3. Morphology

Neutrophils were spun onto cytospin slides (1000 rpm, 7 min), fixed in methanol and stained with May–Grünwald–Giemsa. The cells showing the typical features of neutrophil apoptosis such as cell shrinkage, nuclear coalescence and nuclear chromatin condensation were considered as apoptotic (Haslett, 1997). When the percentages of apoptotic cells obtained by morphological analysis and flow cytometric analysis of relative DNA content were compared, a significant correlation between the two methods was observed ($r = 0.922$, $P < 0.00001$, $n = 75$).

### 2.4. Materials

Beclomethasone, budesonide, dexamethasone, hydrocortisone, mifepristone, prednisone, prednisolone, and propidium iodide were purchased from Sigma (St. Louis, MO, USA). Other reagents were obtained as follows: fluticasone propionate (GlaxoWellcome, County Durham, UK), antibiotics, fetal calf serum and RPMI 1640 (Gibco BRL, Paisley, Scotland, UK), human recombinant GM-CSF (R&D system Europe, Abringdon, UK), Annexin V-FITC kit (Bender medSystems, Vienna, Austria). Dexamethasone was dissolved in HBSS. Stock solutions of all other steroids (50 mM) were prepared in ethanol. The final concentration of ethanol in the culture was 0.2% and was found not to

affect constitutive apoptosis or cytokine-afforded neutrophil survival ($n = 36$, data not shown).

### 2.5. Statistics

Results are expressed as mean ± S.E.M. Apoptosis is expressed as apoptotic index (number of apoptotic cells/total number of cells). The $EC_{50}$ was defined as the concentration of drug producing 50% of its own maximal effect. Statistical significance was calculated by analysis of variance for repeated measures supported by Student–Newman–Keuls test. Differences were considered significant if $P < 0.05$.

## 3. Results

### 3.1. The effects of glucocorticoids on spontaneous neutrophil apoptosis

When neutrophils were cultured in cytokine-deprived conditions for 16 h, the apoptotic index was 0.58 ± 0.02 ($n = 42$) as assessed by flow cytometry measuring the relative DNA content. Fluticasone propionate and budesonide inhibited neutrophil apoptosis in a concentration-dependent manner at the concentration ranges of 0.1–1000 and 1–1000 nM, respectively (Fig. 1). Beclomethasone, prednisolone and dexamethasone significantly delayed constitutive neutrophil apoptosis at the concentration range of 10–1000 nM (Fig. 1A,B), whereas hydrocortisone was effective only at concentrations of 100 nM and above (Fig. 1B). Prednisone, an inactive pro-drug of prednisolone, slightly delayed neutrophil apoptosis at 1 μM drug concentration (Fig. 1B).

Table 1
The $EC_{50}$ values of glucocorticoids in inhibiting neutrophil apoptosis

| Glucocorticoid | $EC_{50}$ (nM) | Relative receptor affinity |
|---|---|---|
| Dexamethasone | 8 ± 4 | 100 |
| Fluticasone | 0.6 ± 0.2 | 1800 |
| Budesonide | 0.8 ± 0.2 | 935 |
| Beclomethasone | 20 ± 3 | 76 |
| Prednisolone | 13 ± 3 | |
| Hydrocortisone | 38 ± 15 | 7.8 |

Neutrophils were cultured in the presence of glucocorticoids for 16 h. Apoptosis was assessed by flow cytometry measuring the relative DNA content of propidium iodide-stained neutrophils. Values are the mean ± S.E.M. of five or six duplicate experiments with cells isolated from different donors. For comparisons, the relative receptor affinities by Högger and Rohdewald (1998) are listed.

The $EC_{50}$ values for inhibition of neutrophil apoptosis by glucocorticoids are shown in Table 1. The order of potency of inhaled glucocorticoids for inhibition of neutrophil apoptosis was fluticasone propionate ≈ budesonide > beclomethasone. Fluticasone propionate and budesonide were significantly more potent than beclomethasone ($P < 0.001$). The $EC_{50}$ values of dexamethasone, beclomethasone, prednisolone and hydrocortisone did not significantly differ from each other. At 1 μM drug concentration, the degree of inhibition of neutrophil apoptosis was about 50–60% and it was not significantly different among the tested glucocorticoids except prednisone. Similar results were obtained when the effects of glucocorticoids (all at 1 μM) were analyzed by using the morphological criteria

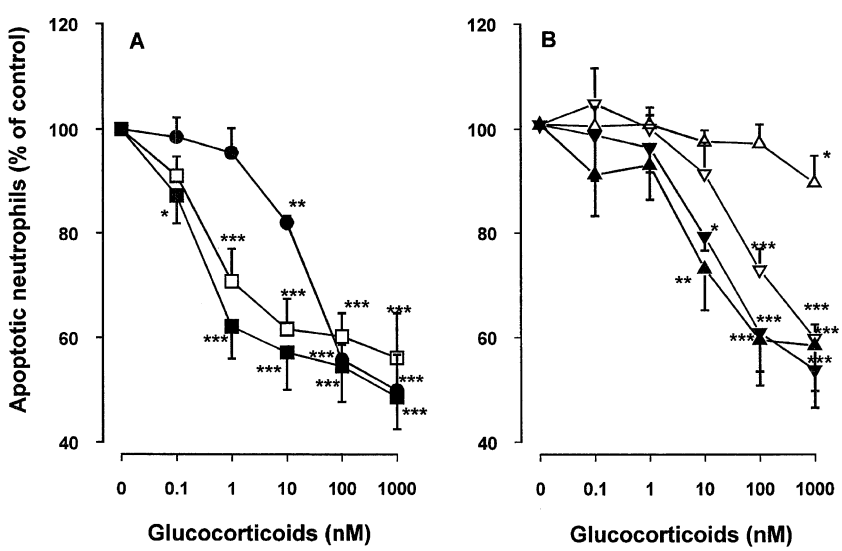


Fig. 1. The effects of (A) beclomethasone (●), budesonide (□), fluticasone (■) and (B) dexamethasone (▲) hydrocortisone (▽), prednisolone (▼) and prednisone (△) on constitutive apoptosis of cytokine-deprived human neutrophils. Neutrophils were cultured in the absence or presence of glucocorticoids for 16 h and apoptosis was assessed by flow cytometry measuring the relative DNA content of propidium iodide-stained neutrophils. Each data point represents the mean ± S.E.M. of five or six independent determinations using neutrophils from different donors. Results are expressed as percentage of control. Solvent control in the absence of glucocorticoids is set as 100%. * indicates $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ as compared with the respective solvent control.

Table 2
The effects of glucocorticoids (all at 1 μM) on human neutrophil apoptosis in the absence and in the presence of mifepristone (10 μM)

| | Apoptotic index | |
|---|---|---|
| | −mifepristone | +mifepristone |
| Solvent (HBSS) | 0.51 ± 0.07 | 0.55 ± 0.06 |
| Dexamethasone | 0.28 ± 0.04[a] | 0.54 ± 0.05 |
| Solvent (EtOH) | 0.47 ± 0.08 | 0.54 ± 0.05 |
| Fluticasone | 0.28 ± 0.06[b] | 0.49 ± 0.05 |
| Budesonide | 0.31 ± 0.05[b] | 0.52 ± 0.04 |
| Beclomethasone | 0.30 ± 0.03[b] | 0.57 ± 0.05 |
| Prednisone | 0.44 ± 0.06 | 0.58 ± 0.05 |
| Prednisolone | 0.29 ± 0.04[b] | 0.55 ± 0.06 |
| Hydrocortisone | 0.26 ± 0.05[b] | 0.59 ± 0.05 |

Shown is the apoptotic index after 16-h incubation as analysed by morphological criteria. Values are the mean ± S.E.M. of six independent determinations using neutrophils from different donors.

[a] Indicates $P < 0.01$ as compared with the respective solvent control in the absence of mifepristone and glucocorticoids.

[b] Indicates $P < 0.05$ as compared with the respective solvent control in the absence of mifepristone and glucocorticoids.

(Table 2) and analysis of annexin-V binding (Table 3). Both flow-cytometric analysis of neutrophils double-stained with annexin-V and propidium iodide and the morphological analysis revealed the absence of significant induction of primary necrotic cell death in glucocorticoid (all at 1 μM)-treated neutrophils ($n = 42$ and $n = 28$, respectively, data not shown). None of the glucocorticoids affected neutrophil apoptosis at drug concentrations of 0.1–10 pM ($n = 5$–6, data not shown).

### 3.2. The effect of mifepristone on glucocorticoid-induced inhibition of neutrophil apoptosis

To investigate whether the effects of glucocorticoids on neutrophil apoptosis are mediated via glucocorticoid receptor, we studied the effect of mifepristone, an antagonist of the glucocorticoid receptor (Agarwai, 1996). Mifepristone (10 μM) itself had no significant effect on neutrophil apoptosis as assessed by flow cytometry measuring the relative DNA content ($n = 6$, data not shown). The apoptosis-delaying effects of beclomethasone, budesonide, dexamethasone, fluticasone propionate, hydrocortisone and prednisolone (all at 1 μM) on neutrophils were reversed by mifepristone (10 μM) as assessed by flow cytometry measuring the relative DNA content of propidium iodide-stained cells (Fig. 2). Similar results were also obtained when apoptosis was analyzed using morphological criteria (Table 2).

Table 3
The effects of glucocorticoids (all at 1 μM) and GM-CSF (0.7 nM) on human neutrophil apoptosis

| | Apoptotic index |
|---|---|
| Solvent (HBSS) | 0.66 ± 0.07 |
| GM-CSF | 0.52 ± 0.09[a] |
| Dexamethasone | 0.39 ± 0.08[b] |
| Solvent (EtOH) | 0.64 ± 0.09 |
| Fluticasone | 0.41 ± 0.08[b] |
| Budesonide | 0.46 ± 0.09[b] |
| Beclomethasone | 0.44 ± 0.06[b] |
| Prednisone | 0.67 ± 0.05 |
| Prednisolone | 0.53 ± 0.10[a] |
| Hydrocortisone | 0.57 ± 0.08 |

Shown is the apoptotic index after 12-h incubation as determined by flow cytometric analysis of double-stained cells with annexin-V and propidium iodide. Values are the mean ± S.E.M. of four duplicate experiments with cells isolated from different donors.

[a] Indicates $P < 0.05$ as compared with the respective solvent control.

[b] Indicates $P < 0.001$ as compared with the respective solvent control.

### 3.3. The effects of glucocorticoids on GM-CSF-afforded neutrophil survival

The maximal inhibitory effect of GM-CSF on neutrophil apoptosis was obtained at 0.7 nM concentration ($n = 4$, data not shown) during 16-h incubation as assessed by flow cytometry measuring the relative DNA content of propidium iodide-stained cells. In the presence of GM-CSF (0.7 nM), dexamethasone and fluticasone propionate

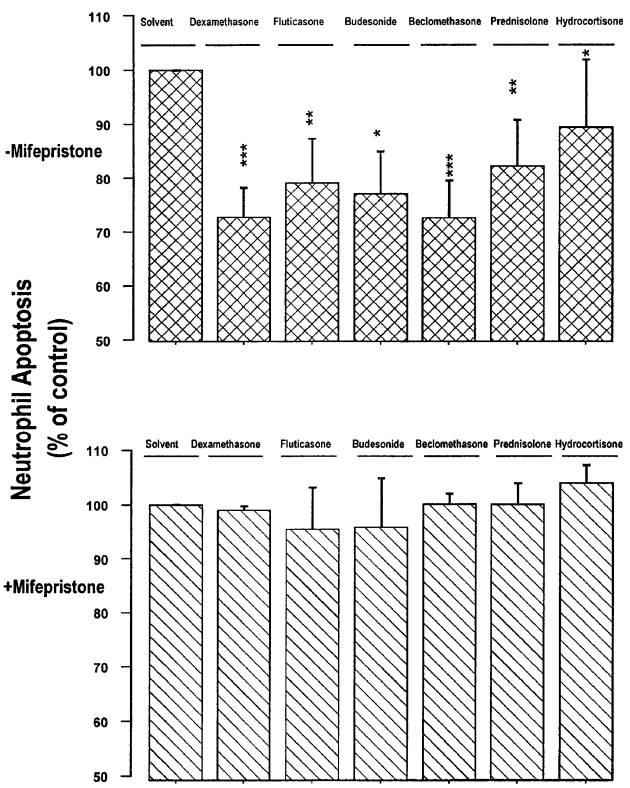


Fig. 2. The effect of mifepristone (10 μM) on the induction of neutrophil apoptosis by beclomethasone, budesonide, and fluticasone, dexamethasone, hydrocortisone, prednisone and prednisolone (all at 1 μM) during 16-h culture. Apoptosis was assessed by flow cytometry measuring the relative DNA content of PI-stained neutrophils. Each data point represents the mean ± S.E.M. of six independent determinations using neutrophils from different donors. Results are expressed as percentage of control. Solvent control in the absence of glucocorticoids is set as 100%. * indicates $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared with the respective control in the absence of mifepristone and glucocorticoids.

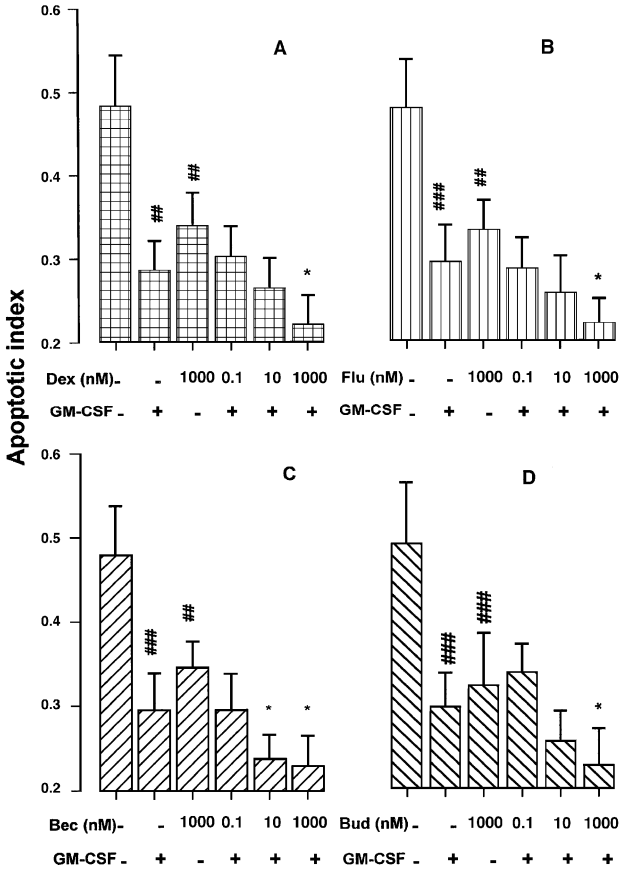


Fig. 3. The effects of dexamethasone, fluticasone, beclomethasone and budesonide on GM-CSF (0.7 nM)-induced neutrophil survival during 16-h incubation. Apoptosis was assessed by flow cytometry measuring the relative DNA content of propidium iodide-stained neutrophils. Each data point represents the mean ± S.E.M. of six or seven independent determinations using neutrophils from different donors. * indicates $P < 0.05$ as compared with the respective control in the presence of GM-CSF. ## indicates $P < 0.01$ and ### $P < 0.001$ as compared with the respective control in the absence of GM-CSF and glucocorticoids.

(1 μM) slightly enhanced the inhibitory effect of GM-CSF on neutrophil apoptosis (Fig. 3A,B). However, at lower drug concentrations tested (0.1 and 10 nM) dexamethasone and fluticasone propionate did not significantly affect the inhibitory effect of GM-CSF on neutrophil apoptosis (Fig. 3A,B). Similar results were obtained when the effects of beclomethasone and budesonide on GM-CSF-afforded survival were studied (Fig. 3C,D).

## 4. Discussion

The present results show that the commonly used inhaled glucocorticoids beclomethasone, budesonide and fluticasone propionate inhibit constitutive apoptosis of human neutrophils in vitro. Our study extends the previous reports (Kato et al., 1995; Liles et al., 1995; Meagher et al., 1996) which showed that dexamethasone and/or methylprednisolone delay neutrophil apoptosis.

The $EC_{50}$ values of budesonide and fluticasone propionate for the inhibition of neutrophil apoptosis were 0.8 and 0.6 nM, respectively. The maximal concentration in plasma ($C_{max}$) has been reported to be in a range of 2.2–5.6 nM for budesonide and 0.18–2 nM for fluticasone after administration by inhalation (Meibohm et al., 1998; Thorsson et al., 1994, 1997). The tissue concentrations of budesonide and fluticasone in the lung have been reported to be substantially higher than those in plasma (Barnes, 1998; Barnes et al., 1998; Högger and Rohdewald, 1998; Thorsson et al., 1997). Thus, the inhibitory effects of budesonide and fluticasone propionate on neutrophil apoptosis are obtained at drug concentrations that are found in the lung during inhalation therapy. The $EC_{50}$ of beclomethasone in inhibiting neutrophil apoptosis in the present study was 20 nM, whereas $C_{max}$ has been reported to be 2.3 nM after inhalation (Harrison et al., 1999). However, it may be possible that beclomethasone concentrations locally in the lung would be high enough to inhibit neutrophil apoptosis. This idea is supported by the finding of Hoshino and Nakamura (1996) that treatment with inhaled beclomethasone increased the numbers of neutrophils in bronchial biopsies. The $EC_{50}$ values of prednisolone and hydrocortisone in inhibiting neutrophil apoptosis were 13 and 38 nM, respectively. The $C_{max}$ of prednisolone was reported to be 500 nM after oral administration (Barnes et al., 1998). Intravenous hydrocortisone produced $C_{max}$ of 1–1.6 μM (Aalto-Korte and Turpeinen, 1995). Hence, prednisolone and hydrocortisone inhibit neutrophil apoptosis at highly clinically relevant drug concentrations.

There is evidence that neutrophil numbers are increased in the airways of subjects with status asthmaticus and during exacerbations of asthma. Analysis of bronchoalveolar lavage fluid and bronchial biopsies (Wenzel et al., 1997) and induced sputum (Jatakanon et al., 1999; Louis et al., 2000) revealed significantly increased neutrophil numbers in severe asthma as compared with mild asthma or normal control subjects. At present, it is not known whether neutrophilic inflammation is involved in the pathogenesis of severe asthma or whether it is an iatrogenic phenomenon. In the study of Jatakanon et al. (1999), all patients with severe asthma associated with neutrophilia were treated with oral prednisolone combined with high-dose inhaled steroids. In the study of Wenzel et al. (1997), severe asthma was classified based on the use of oral steroids at doses equivalent or greater than 20 mg/day prednisolone. Furthermore, Louis et al. (2000) reported that the numbers of neutrophils in induced sputum in those asthmatic patients who were treated with high doses of inhaled glucocorticoids were increased, but not in those treated with low doses. Since oral and inhaled glucocorticoids increase neutrophil survival by reducing apoptosis at clinically relevant drug concentrations, it is possible that treatment with high dose glucocorticoids contributes to the neutrophilic inflammation in severe asthma.

Glucocorticoids are effective to control the inflammation in asthma and several other chronic inflammatory diseases. In contrast, the role of glucocorticoids in the treatment of chronic obstructive pulmonary disease is not clear. There are more than 100 studies that have investigated the effects of glucocorticoids on the inflammation in chronic obstructive pulmonary disease (Postma and Kerstjens, 1999) but the results are conflicting. Neutrophils have been shown to be present in increased numbers in induced sputum in chronic obstructive pulmonary disease (Jeffery, 1999; O'Byrne and Postma, 1999). Since it is now well recognized that apoptosis provides a granulocyte clearance mechanism that would tend to limit tissue injury and promote resolution of inflammation (Haslett, 1999), a delay in constitutive apoptosis induced by glucocorticoids may increase the numbers of neutrophils in the airways of patients with chronic obstructive pulmonary disease. Furthermore, during apoptosis neutrophils lose their capacity to degranulate (Haslett, 1999). Thus, modulation of apoptosis in neutrophils by glucocorticoids regulates their "functional longevity" (Cox, 1995). Thus, the inhibition of constitutive neutrophil apoptosis by currently used inhaled or oral glucocorticoids may inhibit the course of resolution of the neutrophilic inflammation. Therefore, there exists a possibility that long-term treatment with oral or inhaled glucocorticoids may not be of benefit to the patients with chronic obstructive pulmonary disease.

The basic mechanism of glucocorticoid actions is that glucocorticoids penetrate into the cell and bind to glucocorticoid receptor molecules in the cytoplasm (Barnes et al., 1998). Glucocorticoids differ in their receptor affinity. The agents that have high glucocorticoid-receptor affinity, e.g. fluticasone propionate and budesonide (Table 1), were more potent in inhibiting neutrophil apoptosis than beclomethasone and dexamethasone. An antagonist of the glucocorticoid receptor, mifepristone (Agarwai, 1996), completely reversed the effects of budesonide and fluticasone propionate on neutrophil apoptosis. This suggests that budesonide and fluticasone propionate modulate neutrophil apoptosis through glucocorticoid receptor. We (Zhang et al., 2000) and others (Adachi et al., 1996; Meagher et al., 1996) have previously shown that inhaled glucocorticoid enhance human eosinophil apoptosis at clinically relevant drug concentrations via glucocorticoid receptor. The reason for the opposing effects of glucocorticoids on eosinophil and neutrophil apoptosis remains unknown at the present.

Neutrophil apoptosis can be delayed by inflammatory mediators such as GM-CSF (Dibbert et al., 1999). Glucocorticoids are effective in inhibiting GM-CSF synthesis in several human cell types (Barnes et al., 1998). Recently, Dibbert et al. (1999) reported that peripheral blood and bronchoalveolar lavage neutrophil apoptosis is delayed in inflammatory lung diseases such as cystic fibrosis, pneumonia and idiopathic fibrosis. Furthermore, GM-CSF and granulocyte-colony stimulating factor were shown to be the main survival-prolonging factors for neutrophils in these diseases (Dibbert et al., 1999). Thus, it is possible that the slight enhancement of the anti-apoptotic effect of GM-CSF by inhaled glucocorticoids has clinical relevance in some situations. It is, however, difficult to estimate the clinical net effect by glucocorticoids on neutrophilic inflammation as they (a) inhibit GM-CSF production, (b) inhibit neutrophil apoptosis and (c) enhance GM-CSF-afforded survival.

In conclusion, we have shown that the currently used inhaled glucocorticoids (budesonide and fluticasone propionate) inhibit human neutrophil apoptosis at clinically relevant drug concentrations via an effect on glucocorticoid receptor.

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