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Heli Perttunen a, Eeva Moilanen a, Xianzhi Zhang ab, Peter J. Barnes c, Hannu Kankaanranta ad

a The Immunopharmacology Research Group, Medical School, University of Tampere and Research Unit, Tampere University Hospital, Tampere, Finland
b The Center for Infection and Immunity, Institute of Biophysics, Chinese Academy of Sciences, Beijing, China
c Department of Thoracic Medicine, Imperial College at the National Heart and Lung Institute, London, UK
d Department of Respiratory Diseases, Seinäjoki Central Hospital, Seinäjoki, Finland

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Original Research

β2-Agonists Potentiate Corticosteroid-Induced Neutrophil Survival

Heli Perttunen1(heli.perttunen@uta.fi), Eeva Moilanen1(eeva.moilanen@uta.fi), Xianzhi Zhang1,2(xianzhi.zhang@gmail.com), Peter J. Barnes3(p.j.barnes@imperial.ac.uk), and Hannu Kankaanranta1,4(hannu.kankaanranta@epshp.fi)

1The Immunopharmacology Research Group, Medical School, University of Tampere and Research Unit, Tampere University Hospital, Tampere, Finland
2The Center for Infection and Immunity, Institute of Biophysics, Chinese Academy of Sciences, Beijing, China
3Department of Thoracic Medicine, Imperial College at the National Heart and Lung Institute, London, UK
4Department of Respiratory Diseases, Seinäjoki Central Hospital, Seinäjoki, Finland

ABSTRACT

Neutrophils are considered to play a role in the pathogenesis of chronic obstructive pulmonary disease (COPD) and severe asthma. Recent guidelines recommend the use of a combination of inhaled corticosteroids (ICS) and long-acting β2-agonists (LABA) in the treatment of COPD with exacerbations and asthma not adequately controlled by ICS alone. LABA have been proposed to have a synergistic effect with corticosteroids by activating glucocorticoid receptors. The aim of this study was to investigate the effect of β2-agonists on the inhibitory effects of corticosteroids on human neutrophil apoptosis. In addition, the effects of β2-agonists on spontaneous neutrophil apoptosis and on GM-CSF- and LTB4-afforded survival were also evaluated. Neutrophils were isolated from human blood under sterile conditions and cultured for 16 hours. Apoptosis was assessed by relative DNA fragmentation assay. Morphological analysis was used as a control method to confirm the occurrence of apoptosis. Salbutamol, formoterol and salmeterol prolonged the lifespan of budesonide- and fluticasone propionate-treated neutrophils by inhibiting apoptosis. Formoterol and salbutamol partly reversed the inhibitory effect of GM-CSF on neutrophil apoptosis. In contrast, the effects of β2-agonists on spontaneous neutrophil apoptosis and on LTB4-afforded survival were negligible. β2-agonists potentiate corticosteroid-induced neutrophil survival at clinically relevant drug concentrations. Whether these effects translate into clinically relevant changes in lung neutrophil numbers remains to be demonstrated.

INTRODUCTION

Inhaled corticosteroids (ICS) and β2-agonists are the classes of drugs widely used in the treatment of inflammatory lung diseases such as chronic obstructive pulmonary disease (COPD) and asthma. Regular treatment with LABA is recommended in moderate to very severe COPD (1) and the combination of ICS and LABA has been proposed to be an efficient way to prevent exacerbations in severe COPD (2–5). In asthma, the use of a combination of ICS and long-acting β2-agonists (LABA) is advocated by guidelines when a medium dose of ICS alone fails to achieve control of asthma (6). LABA have been shown to be the most effective of add-on therapy options in asthma not adequately controlled by ICS alone (7).

Neutrophils are polymorphonuclear leucocytes, which are essential in the innate immunity forming the first line defence...
against fungal and bacterial infections. They constitutively undergo apoptosis and thus have the shortest half-life of all the cells in the immune system. The morphological properties of apoptotic neutrophils include the shrinkage of the cells and an appearance of a condensed, rounded nucleus as well as chromatin fragmentation (8). The apoptosis of neutrophils is followed by phagocytosis, which provides a safe mechanism for the removal of neutrophils from inflamed sites. This mechanism is essential for the resolution of inflammation (9). Neutrophils are thought to play a central role in the pathogenesis of COPD (10) and severe asthma (11). Neutrophilia is seen in patients with severe asthma (12–14) using not only high doses of corticosteroids but also β2-agonists. Whether this neutrophilia in severe asthma is iatrogenic remains unknown.

Corticosteroids inhibit neutrophil apoptosis at clinically relevant drug concentrations and the effect seems to be mediated through glucocorticoid receptors (GR) (15). Accordingly, oral (16) and inhaled (17) corticosteroids have been reported to increase neutrophil numbers in the lung tissue. The process of neutrophil apoptosis can also be inhibited by granulocyte-macrophage colony-stimulating factor (GM-CSF), which is reported to be a crucial factor for neutrophil survival in vivo, and by leukotriene B4 (LTB4) (18). In vascular smooth muscle cells and primary human lung fibroblasts, β2-agonists salbutamol and salmeterol induce translocation of GR into the nucleus and enhance the binding of GR to its consensus sequence in the absence of corticosteroids in vitro (19). Combination of a corticosteroid (budesonide) and a LABA (formoterol) resulted in a synergistic effect on cellular signalling at the level of GR activation on airway smooth muscle cells (20). In addition, Spoelstra et al. (21) reported that formoterol exerts an additive effect on the anti-inflammatory effects of budesonide in human lung fibroblasts. Recently this has also been demonstrated after clinical treatment of asthmatic patients in whom a LABA enhances the nuclear translocation of GR in response to corticosteroids (22).

The aim of the present study was to investigate the effects of corticosteroids and β2-agonists on neutrophil apoptosis. Based on the reports that β2-agonists may potentiate corticosteroid-induced GR activation, we hypothesized that β2-agonists could modulate corticosteroid effects on human neutrophils. To test this, the effects of combinations of two commonly used corticosteroids budesonide and fluticasone and β2-agonists salbutamol, formoterol, and salmeterol were studied on human neutrophil apoptosis. Furthermore, to evaluate whether β2-agonists alone would be able to activate GRs i.e., to produce effects similar to corticosteroids, their effects on constitutive apoptosis and GM-CSF- and LTB4-afforded survival of human neutrophils were studied.

METHODS

Neutrophil isolation and culture

Neutrophils were isolated under sterile conditions as previously described (15, 23). Briefly, venous blood (50 ml) from healthy donors was collected into 10 ml of acid citrate dextrose anticoagulant and hydroxyethyl starch solution. White blood cells were obtained after removing supernatant and were overlaid onto Ficoll-Paque (Pharmacia AB, Uppsa, Sweden) and centrifuged at 700g for 30 min at 20°C. Mononuclear cell layer was removed and the remaining pellet containing granulocytes and red blood cells was washed in Hank’s balanced salt solution (HBSS) (BioWhittaker, Verviers, Belgium).

Red blood cells were lysed by hypotonic lysis. The neutrophils were washed by RPMI 1640 (Dutch Modification) (Gibco BRL, Paisley, Scotland, UK) with 10% fetal calf serum (BioWhittaker, Verviers, Belgium) and antibiotics (Gibco BRL, Paisley, Scotland, UK) and counted using microscopic examination in Kimura stain. The purity of neutrophil population was >98%. The cells were then resuspended at 1 × 10⁶ cells/ml and in all experiments cultured for 16 h (37°C, 5% CO₂) in RPMI 1640 (Dutch modification) with fetal calf serum and antibiotics. Blood donors gave written informed consent to a study protocol approved by the ethical committee of Tampere University Hospital (Tampere, Finland).

Relative DNA fragmentation

Apoptosis was determined by propidium iodide (Sigma-Aldrich, St Louis, MO) staining of DNA fragmentation and flow cytometry (FACScan, Becton Dickinson, San Jose, CA) as previously described (15, 23). Briefly, neutrophils were washed in PBS solution, fixed in 70% ethanol and incubated on ice for 1–3 hours. The pellet was then resuspended in propidium iodide solution (25 μg/ml in PBS), and measured by flow cytometry. The cells showing decreased relative DNA content were considered as apoptotic. Unless otherwise stated, the results presented are apoptotic indexes (number of measured apoptotic cells/number of total measures cells) obtained by using relative DNA fragmentation assay.

Morphology

Neutrophils were spun onto cytopsin slides, fixed in methanol and stained with May-Grünwald (Merck, Darmstadt, Germany)-GIemsas (J.T. Baker, Deventer, Holland). The cells showing the typical features of neutrophil apoptosis such as cell shrinkage and nuclear coalescence were considered as apoptotic (8). In a separate set of experiments a Pearson correlation coefficient of 0.94 (n = 35, p < 0.0001) was obtained between morphological examination and propidium iodide staining of neutrophils.

Materials

Salbutamol (Sigma-Aldrich, St Louis, MO), formoterol hemifumarate, and salmeterol (Tocris, Bristol, UK) were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in cells was 0.5%. Budesonide (Sigma-Aldrich, St Louis, MO), and fluticasone propionate (Tocris, Bristol, UK) propionate were dissolved in ethanol. The final concentration of ethanol in cells was 0.2%. DMSO and/or ethanol were added to the respective control samples at the same concentrations. In
all experiments the β₂-agonists (or corresponding solvent) were added on the cells and after 20 minutes incubation at 20°C the steroids, GM-CSF (R&D systems Europe, Abingdon, UK) or LTB₄ (Calbiochem, LaJolla, CA) were added. Thereafter cells were cultured for 16 h, whereafter apoptosis was measured.
Effects of β2-agonists on GM-CSF- and LTB4-induced neutrophil survival

GM-CSF and LTB4 inhibited human neutrophil apoptosis to a similar extent than corticosteroids during culture for 16 h (Figures 1 and 3). As previously reported (15), budesonide and fluticasone potentiated the effects of GM-CSF and LTB4 on neutrophil apoptosis (Figure 3). If β2-agonists alone are able to activate GRs in neutrophils similarly to that reported in other cell types (19, 20), the effect on GM-CSF- and LTB4- afforded survival should be qualitatively, albeit not necessarily quantitatively, similar to that of corticosteroids. Salbutamol and formoterol partially reversed the survival-prolonging effect of GM-CSF on neutrophil apoptosis (Figure 4). The maximal inhibitory effect of formoterol was 23% and was obtained at the concentration of 1 μM.

Similarly, the maximal effect of salbutamol was 31% and was obtained at a drug concentration of 100 μM. With salmeterol, a similar tendency was seen but the results were not statistically significant. Morphological analysis of formoterol-treated (1 μM) neutrophils confirmed a partial reversal of GM-CSF-induced survival (apoptotic indexes 0.35 ± 0.05 and 0.40 ± 0.06, in the absence and presence of formoterol; n = 6, p < 0.05).

The effects of β2-agonists on GM-CSF-afforded survival prompted us to investigate whether the effect found was more commonly associated with receptor-mediated agents inhibiting neutrophil apoptosis. For comparison, a lipid mediator, leukotriene B4, previously shown to inhibit neutrophil apoptosis was chosen. However, salbutamol, formoterol and salmeterol did not affect LTB4-induced neutrophil survival (n = 6–7, data not shown). Budesonide and fluticasone potentiated LTB4-induced neutrophil survival (Figure 3).

Effects of salbutamol, formoterol and salmeterol on constitutive neutrophil apoptosis

Corticosteroids have been reported to inhibit spontaneous neutrophil apoptosis (15). Budesonide and fluticasone inhibited neutrophil apoptosis (Figure 1). Formoterol, but not salbutamol or salmeterol, slightly inhibited constitutive neutrophil apoptosis. By using morphological analysis, the inhibitory effect of formoterol (1 μM) on spontaneous neutrophil apoptosis was confirmed (0.60 ± 0.06 and 0.53 ± 0.06, in the absence and presence of formoterol; n = 6, p < 0.05).

DISCUSSION

In the present study, we demonstrate for the first time that β2-agonists salbutamol, formoterol, and salmeterol potentiate the delayed neutrophil apoptosis induced by corticosteroids budesonide and fluticasone. However, the effects of β2-agonists on spontaneous apoptosis and on GM-CSF- and LTB4- afforded neutrophil survival differ from those of corticosteroids. This suggests that β2-agonists do not alone activate GRs on human neutrophils.

Roth et al. (20) have reported that the combination of formoterol and budesonide activated GR and a transcription factor CCAAT-enhancer binding protein (C/EBP-α) in human bronchial airway smooth muscle cells. Both drugs also activated GR and C/EBP-α when administered alone. Interestingly, when
administered together, the drugs were effective at concentrations that were ineffective when either drug was used alone. Our results provide support to the findings of Eickelberg et al. (19) and Roth et al. (20) and suggest that β2-agonists may potentiate GR activation and that occurs also in human neutrophils. In contrast to that found in bronchial smooth muscle cells β2-agonists alone do not seem to activate GRs in human neutrophils as β2-agonists did not modify human neutrophil apoptosis in a manner similar to corticosteroids. Another possibility is that the interaction between the two drug classes does not involve gene transcription i.e. it occurs at the non-genomic level. This might explain the finding that β2-agonists did not modify human neutrophil apoptosis in a manner similar to corticosteroids.

A statistically significant effect on corticosteroid-induced neutrophil survival was obtained by salbutamol at concentrations from 10 nM to 100 μM, by formoterol from 0.1 nM to 10 μM, and by salmeterol from 1 nM to 10 μM. After inhalation of metered-dose inhaler or nebulizer the peak plasma concentrations of salbutamol have been reported to be 7.5 to 23 nM (24). Cmax of formoterol in plasma after inhalation has been reported to be 0.27 nM (25) and Cmax of salmeterol 1.7 nM (26). Thus the apoptosis delaying effects of all the three β2-agonists were achieved in vitro at clinically relevant drug concentrations. Furthermore, it is reasonable to expect that even higher drug concentrations can be found locally in the lungs after β2-agonist inhalation. However, it is of note that the pharmacokinetics of inhaled drugs may vary between healthy subjects and patients with severe asthma or COPD.

The present study was performed by using neutrophils from peripheral blood of healthy donors. Whether this effect is seen with neutrophils obtained from patients with severe asthma or COPD needs to be studied. The numbers of neutrophils have been reported to be increased in patients with severe asthma. Wenzel et al. (12) reported a persistent neutrophilic inflammation associated with high dose corticosteroids in patients with severe asthma. The numbers of neutrophils were increased in BAL samples as compared with mild asthmatics or healthy controls. Severe asthmatics (n = 16) were treated with high-dose oral and inhaled corticosteroids and 8 of them were treated using inhaled salmeterol.

The relationship between airways inflammation and the severity of asthma was analyzed by Louis et al. (14), and increased neutrophil counts in induced sputum of subjects with severe persistent asthma were found. The severe asthmatics were using oral and/or inhaled corticosteroids and 82% of them were on LABA. According to the studies mentioned here, neutrophilia is seen in patients suffering from severe persistent asthma using high doses of corticosteroids. However, a noticeable proportion of severe asthmatics were also using LABA. Taken together, these results provide in vivo support to our findings that a combination of corticosteroids and LABA may induce a further delay in neutrophil apoptosis. Thus our results may provide an additional explanation for the neutrophilic inflammation seen in severe asthma suggesting a proinflammatory effect for β2-agonists.

One study (27) reported a tendency towards an increase in sputum neutrophil proportion in asthmatic patients after one-year randomized treatment with 100 μg budesonide plus 12 μg formoterol twice daily as compared with asthmatic patients receiving 400 μg budesonide plus placebo twice daily under the same period of time. In a recent study, a combination of salmeterol with fluticasone showed a tendency to increase in the neutrophil numbers in sputum 24 hours after allergen challenge as compared with fluticasone alone (28). In contrast, a combination of salmeterol with fluticasone was reported not to affect the numbers of neutrophils as compared with fluticasone or salmeterol alone in a small group of patients with asthma (29). However, exact cell numbers were not reported.

In summary, there are few clinical data about neutrophil counts in lungs after treatment with a combination of ICS and LABA and the groups of patients in these studies are small. Our present results suggest that the use of a combination of ICS and LABA may induce a delay in neutrophil apoptosis in the airways of patients with asthma. A possibility also exists that neutrophils might contribute to the resolution of lung injury by removing damaged cells and by increasing proliferation of new epithelium as has been described in ozone-induced lung injury (30). Whether this contributes to the good clinical efficacy of combination of ICS with LABA in the treatment of asthma remains to be studied.

A recent study in patients with COPD (31) demonstrates a slight decrease in total sputum neutrophil numbers after 13 weeks of treatment with combined salmeterol/fluticasone propionate. However, the effect is fairly modest and not statistically significant. Another recent study (32) compared the effects of placebo, fluticasone propionate and combination of salmeterol/fluticasone propionate on inflammatory cells in bronchial biopsies. Combination of salmeterol/fluticasone propionate did not significantly change the numbers of neutrophils as compared with placebo (32). To the best of our knowledge, there are no other published studies reporting the neutrophil proportions or numbers in lung samples of COPD patients during treatment with LABAs or ICS/LABA combinations. The accumulation and activation of neutrophils in the airways in COPD has been reported (10). Thus, the delayed apoptosis could result in increased numbers of neutrophils capable to activate in the airways. This may be reflected in the large clinical studies (33) reporting that combinations of LABA and ICS reduce COPD exacerbations (in which eosinophils are involved) but do not affect the decline in lung function (which is possibly neutrophil driven).

In the present study, we demonstrate that β2-agonists salbutamol and formoterol partly reverse the apoptosis delaying effect of GM-CSF on human neutrophils, GM-CSF has been reported to be the main survival-prolonging cytokine for neutrophils in inflammatory lung conditions (34). Hence these findings might...
imply that salbutamol and formoterol could possess some anti-inflammatory effects against cytokine-induced neutrophil survival. In fact, a recent study (35) showed a significant reduction in the absolute sputum neutrophil number caused by formoterol administration. Our result with formoterol could offer one possible explanation for this finding. In contrast, salmeterol did not significantly affect spontaneous apoptosis or GM-CSF-oxidized neutrophil survival. Clinical studies with salmeterol in steroid-naive asthmatics have reported variable results, one (36) reporting a decrease and four others (37–40) reporting no significant changes in neutrophil numbers either in tissues, sputum, or BAL.

It appears that the reversal of GM-CSF-oxidized neutrophil survival by formoterol and salbutamol is not generally associated with agents inhibiting neutrophil apoptosis. β2-agonists did not affect LTβ4-oxidized neutrophil survival. Furthermore, the effects of β2-agonists on spontaneous neutrophil apoptosis were negligible. The findings suggest that formoterol and salbutamol specifically interact with GM-CSF-driven neutrophil inflammation.

Taken together, we have demonstrated that β2-agonists potentiate the delayed neutrophil apoptosis induced by corticosteroids. Thus, the combined use of β2-agonists and corticosteroids may result in enhanced neutrophil accumulation at the sites of inflammation. However, in the absence of corticosteroids, formoterol and salbutamol may have some anti-inflammatory effect by reversing GM-CSF-oxidized neutrophil survival. Whether these findings translate into clinical effects remains to be studied.

REFERENCES


