



Effect of formoterol and budesonide on chemokine release, chemokine receptor expression and chemotaxis in human neutrophils

Karin Strandberg*, Kristin Blidberg, Karin Sahlander, Lena Palmberg, Kjell Larsson

Lung and Allergy Research, The National Institute of Environmental Medicine, Karolinska Institutet, Box 287, SE-171 77 Stockholm, Sweden

ARTICLE INFO

Article history:

Received 9 April 2009
Received in revised form
9 March 2010
Accepted 13 March 2010

Keywords:

Neutrophils
Chemotaxis
CXCR1
CXCR2
Formoterol
Budesonide

ABSTRACT

Severe persistent asthma and chronic obstructive pulmonary disease (COPD) are associated with neutrophil influx into the airways. It is not clear whether neutrophil chemotaxis is influenced by β_2 -agonists and glucocorticoids, drugs commonly used in treatment of asthma and COPD. The effect of a long-acting β_2 -agonist (formoterol), and a glucocorticosteroid (budesonide) on chemokine/cytokine release (CXCL8, CXCL1, IL-6), regulation of chemokine receptors (CXCR1, CXCR2), and migration were assessed in neutrophils from 10 non-allergic, healthy donors. Formoterol enhanced and budesonide inhibited IL-6, CXCL8 and CXCL1 release from LPS-stimulated neutrophils. Formoterol up-regulated both CXCR1 and CXCR2 expression, whereas budesonide up-regulated the expression of CXCR2 only. Despite the effects on chemokine release and drug-induced up-regulation of CXCR1 and CXCR2, no influence on neutrophil chemotaxis could be demonstrated.

We conclude that a β_2 -agonist and a glucocorticoid, commonly used in the treatment of obstructive lung diseases, influence chemokine release and receptor sensitivity but the functional consequences of these findings remain unclear.

© 2010 Elsevier Ltd. All rights reserved.

1. Introduction

The recruitment of neutrophils to sites of inflammation is to a large extent regulated by chemokines [1,2]. Cysteine-X-cysteine (CXC) chemokines act via two related G protein-coupled receptors, CXCR1 and CXCR2, which are expressed predominantly on neutrophils [3]. Both CXCR1 and CXCR2 are of importance for neutrophil chemotaxis, but only CXCR1 mediates cytotoxic and cross-regulatory signals [4]. Of the CXC chemokines, CXCL8 (interleukin-8, IL-8), CXCL1 (growth-related oncogene- α , GRO- α), and CXCL5 (epithelial-derived neutrophil activator, ENA-78) are of particular interest as chemoattractants and activators of neutrophils. These chemokines mediate their effects by stimulating CXCR2, while CXCL8 also activates CXCR1 [5]. Like other G protein-coupled receptors, CXCR1 and CXCR2 are rapidly desensitized following a burst of agonist-mediated signalling [6,7].

Severe persistent asthma is associated with increased number of neutrophils in the airways and in peripheral blood [8] and asthma exacerbations are associated with increased number of neutrophils in the airways [9,10]. The combination of sputum neutrophilia and normal numbers of eosinophils is a characteristic feature of non-

atopic asthmatic subjects who respond poorly to treatment with inhaled corticosteroids [11]. The number of airway neutrophils is also increased in chronic obstructive pulmonary disease (COPD), and is higher in severe COPD than in mild disease [12].

β_2 -Agonists and glucocorticoids are commonly used in the treatment of asthma and COPD. It has been convincingly shown that, from a clinical perspective, the combination of these two types of drugs is more favourable than is either drug alone [13–18]. A synergy between β_2 -agonists and steroids with regard to inhibition of CXCL8 release from human bronchial epithelial cells and human airway smooth-muscle cells has been described [19,20]. Synergistic effects at the molecular level have also been shown - glucocorticoids enhance β_2 -adrenergic receptor function and β_2 -agonists in turn facilitate glucocorticoid receptor (GR) nucleus localization and enhance glucocorticoid receptor binding to DNA [10,21]. *In vitro* studies have demonstrated that β_2 -agonists enhance the release of pro-inflammatory cytokines (IL-6 and CXCL8) from bronchial epithelial cells [19,22,23] and airway smooth-muscle cells [20], and corticosteroids inhibit IL-6 and CXCL8 release from bronchial and alveolar epithelial cells [23,24]. Although not being a direct chemoattractant for neutrophils, IL-6 contributes to leukocytosis by release of neutrophils from bone marrow and induces the production of cytokines, including CXCL8, CCL5, GM-CSF and TNF [5].

It is not unlikely that the beneficial clinical effects of β_2 -agonists and steroids in the treatment of asthma and COPD to some extent

* Corresponding author. Fax: +46 8 300 619.

E-mail address: karin.strandberg@ki.se (K. Strandberg).

are mediated by influence on cell migration. The aims of this *in vitro* study were to elucidate the effect of a long-acting β_2 -agonist (formoterol), and a glucocorticosteroid (budesonide) on mechanisms involved in neutrophil migration. Therefore, drug effect on chemokine/cytokine release (CXCL8, CXCL1, IL-6), regulation of chemokine receptors (CXCR1, CXCR2), and migration towards CXCL8 and CXCL1 as a functional response was investigated in isolated human blood neutrophils from healthy donors.

2. Material and methods

2.1. Study design

In vitro experiments were performed on isolated peripheral blood neutrophils from 10 non-smoking, non-allergic healthy donors (5 females, age 22–54 years). The blood sample was drawn in the morning. Neutrophils were isolated and used for studies on cytokine release, chemotaxis and receptor expression. For the cytokine release drugs were added to the neutrophils which were seeded into cell growth plates and incubated for 8 h. For chemotaxis neutrophils were incubated with calcein and then incubated with the different drugs and added to the chemotaxis plates. Neutrophils used for receptor expression studies were kept on ice until incubated with the drugs and antibodies before flow-cytometry analysis.

2.2. Stimuli and drugs

Formoterol was a generous gift from AstraZeneca AB, Lund, Sweden. Budesonide and propranolol were purchased from Sigma–Aldrich, Stockholm, Sweden. Formoterol was dissolved in 0.01 M HCl (10^{-4} M), budesonide in 99.5% ethanol (10^{-2} M) and propranolol in water (0.1 M). The stock solutions were kept at -70°C until used. At the time of each experiment, stock solutions were diluted in buffer or cell growth medium to achieve final concentrations.

Lipopolysaccharide (LPS) extracted from *Escherichia coli* serotype O111:B4 and purified by gel filtration (Sigma–Aldrich) was used for stimulation of the neutrophils in the cell cultures. Recombinant human CXCL8 (208-IL-010) and CXCL1 (275-GR) were purchased from R&D Systems Europe Ltd., Abingdon, UK. For the blocking experiments in the neutrophil migration assays, monoclonal anti-human CXCR1 (MAB330) and monoclonal anti-human CXCR2 (MAB331) antibodies were used (R&D). For detection of CXCR1 and CXCR2 by flow cytometry, phycoerythrin (PE)-conjugated anti-CXCR1 (FAB330P) and anti-CXCR2 (FAB331P) antibodies and a PE-conjugated mouse IgG_{2A} (IC003P) were used (R&D).

2.3. Isolation of peripheral blood neutrophils

Peripheral blood was collected in heparin Venoject® tubes (Terumo Sweden AB, Västra Frölunda, Sweden) and mixed with Dulbecco's PBS (D-PBS) without Ca^{2+} and Mg^{2+} (GIBCO, Paisly, Scotland, UK) containing 2% dextran (Pharmacosmos, Holbaek, Denmark) and allowed to sediment for 40 min. The leukocyte containing supernatant was then carefully layered onto Lymphoprep™ (Mediatech AB, Lidingö, Sweden). After centrifugation at 600 g for 25 min the granulocyte containing fraction was collected followed by hypotonic lysis of the remaining erythrocytes. The cells were washed twice with D-PBS before they were re-suspended in RPMI 1640 medium (Sigma–Aldrich) supplemented with L-glutamin (GIBCO), 1% penicillin/streptomycin (GIBCO) and 5% heat-inactivated foetal bovine serum (RPMI-FBS) and kept on ice until used. Neutrophil populations isolated with this technique were >98% viable as assessed by Trypan blue (Sigma–Aldrich) staining.

2.4. Cell culturing

Neutrophils in RPMI 1640 medium (Sigma–Aldrich) supplemented with L-glutamin (GIBCO), 1% penicillin/streptomycin (GIBCO) and 5% heat-inactivated foetal bovine serum (RPMI-FBS) were seeded into 24-well plates (Nunc, Roskilde, Denmark) at a seeding density of 10^6 cells per well. The cells were incubated for 8 h with or without LPS 1 $\mu\text{g}/\text{ml}$ and the different drugs at 37°C in humidified atmosphere containing 5% CO_2 . The supernatants were then collected and centrifuged at 1000 g for 10 min to remove cell debris, and stored at -70°C until analysis.

2.5. Cytokine assays

The IL-6 and CXCL8 concentrations were determined with an in house ELISA method [25]. Commercially available antibody pairs, the monoclonal anti-human capture antibodies MAB206 (IL-6) and MAB208 (CXCL8), and the detection biotinylated anti-human polyclonal antibodies BAF206 (IL-6) and BAF208 (CXCL8) (R&D) were used. The recombinant human standard for IL-6 was 206-IL-010 and for CXCL8 it was 208-IL-010 (R&D). Absorbance was read at 450 and 650 nm with a Thermomax 250 reader (Molecular Devices, Sunnyvale, CA, USA). The CXCL1 concentration was assessed by the same method as for IL-6 and CXCL8, using the DuoSet ELISA kit, DY275, from R&D and absorbance was read at 450 and 540 nm.

For duplicates an intra-assay coefficient of variation (CV) of <10% and an inter-assay CV of <20% was accepted. The detection ranges were 3–375 pg/ml for IL-6, 50–3200 pg/ml for CXCL8, and 31–2000 pg/ml for CXCL1.

2.6. Chemokine receptors

The drugs were mixed with 25 μl cell suspension containing 100 000 neutrophils in minisorp tubes and incubated at 37°C for 30 min. To study the influence of formoterol and budesonide on CXCR1 and CXCR2 regulation, the neutrophils were pre-incubated with the drugs for 30 min at 37°C before CXCL8 or CXCL1 was added and the incubation continued for another 30 min at 37°C . Twenty-five μl of each drug/cell suspension were then added to flow-cytometry tubes containing 10 μl of the different PE-conjugated, titrated antibodies and the incubation continued with the tubes placed on ice in the dark for 30 min. The neutrophils were then washed with PBS and centrifuged at 400 g for 5 min three times after which the pellets were re-suspended in 100 μl fixative (CellFIX™, BD Biosciences, Stockholm, Sweden) and placed on ice. The samples were analysed on a FACSCalibur flow cytometer (BD Biosciences) with CellQuest™ (BD Biosciences).

Results are expressed as relative mean fluorescence intensity (rMFI) calculated as the ratio between mean fluorescence intensity (MFI) for CXCR1 or CXCR2 and MFI for the isotype control.

2.7. Chemotaxis

Chemotaxis was performed as described by Frevert et al. [26] with minor modifications. The neutrophils were incubated with Calcein AM (Invitrogen, Stockholm, Sweden) for 30 min at room temperature, and then washed with D-PBS and re-suspended in RPMI-FBS to a final concentration of 5×10^6 cells/ml. The neutrophils were incubated for 20 min at 37°C with formoterol (10^{-10} , 10^{-8} , 10^{-6} M), budesonide (10^{-10} , 10^{-8} , 10^{-6} M), the combination of budesonide (10^{-6} M) and formoterol (10^{-8} M), anti-CXCR1 (5 $\mu\text{g}/\text{ml}$), anti-CXCR2 (5 $\mu\text{g}/\text{ml}$) or the combination of anti-CXCR1 (5 $\mu\text{g}/\text{ml}$) and anti-CXCR2 (5 $\mu\text{g}/\text{ml}$). Following incubation the neutrophils were directly added to the upper surface of a polycarbonate filter with 5 μm pores (ChemoTx™, Neuro Probe, Gaithersburg, USA). The wells

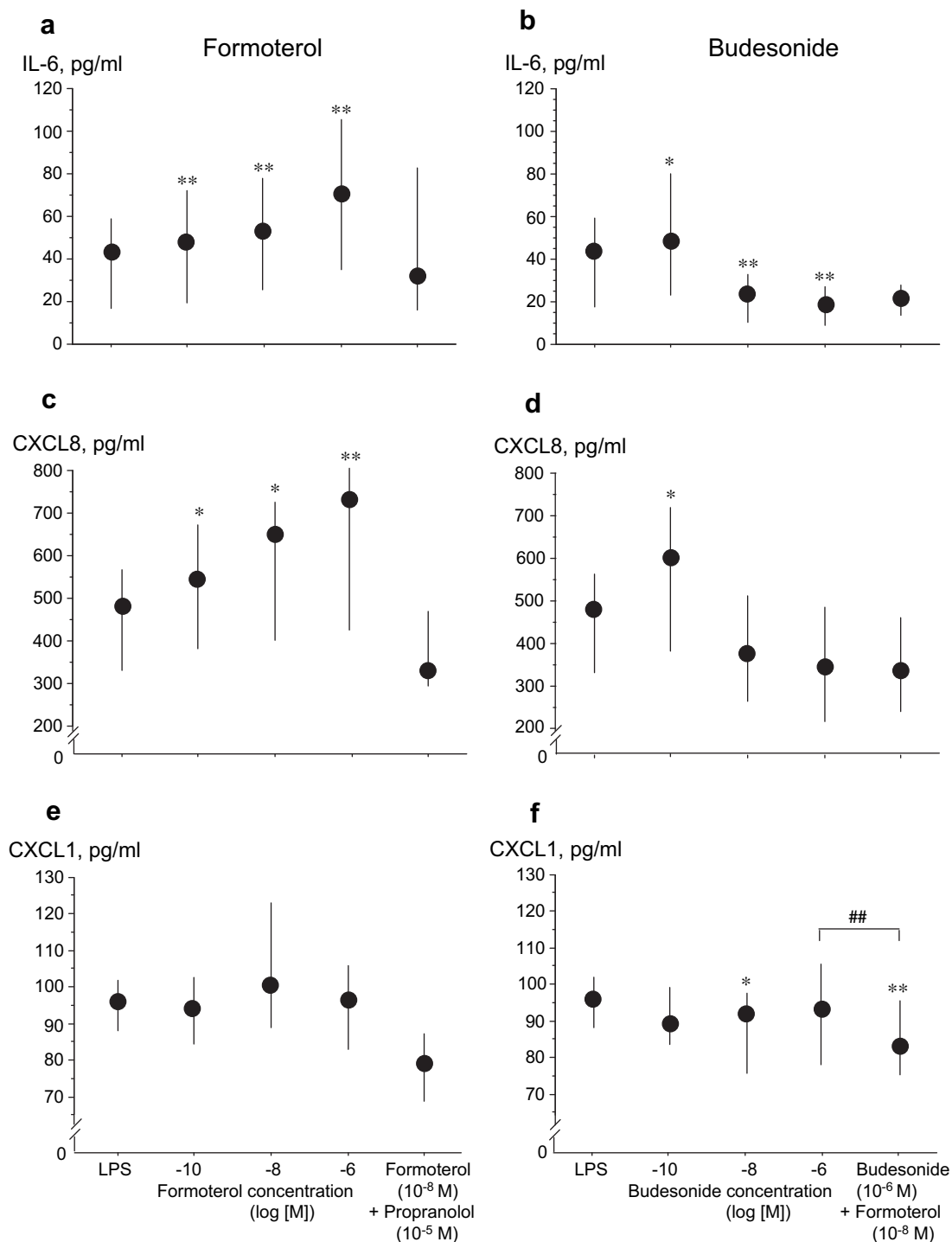


Fig. 1. Effect of formoterol and budesonide on IL-6, CXCL8 and CXCL1 release from LPS-stimulated (8 h) neutrophils from 10 healthy subjects. Propranolol was used for blocking of the formoterol effects. Data are presented as median and 25th–75th percentiles. * $P < 0.05$, ** $P < 0.01$ indicate comparison with LPS-stimulated neutrophils.

below the filter were filled with the applicable doses of chemo-attractant (CXCL8 100 ng/ml or CXCL1 100 ng/ml) with or without drugs (concentrations see above). Following incubation for 1 h at 37 °C the non-migrated cells and the filter were removed, and the fluorescence of the migrated cells was read in a multi-well fluorescent plate reader (SpectraMAX Gemini, Molecular Devices, Downingtown, PA, USA), (excitation, 485 nm; emission, 530 nm).

Cell migration is expressed as percentage of maximum migration (cells added directly to the bottom of the wells) and is

corrected for spontaneous migration. Each sample was run in triplicate and a CV of <15% was accepted.

2.8. Statistics

The results are presented as median values (25th – 75th percentiles) and statistical calculation were made by using non-parametric tests (Friedman test followed by Wilcoxon signed rank test when appropriate). A p value <0.05 was considered statistically

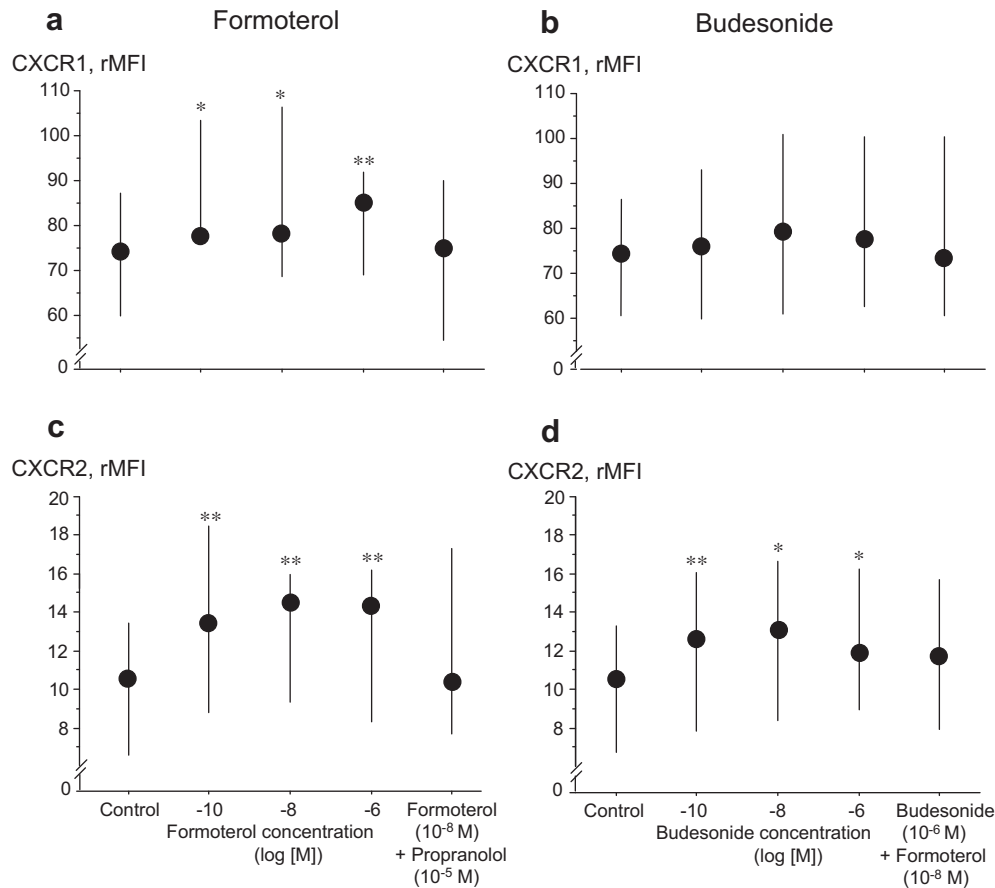


Fig. 2. Effect of 30 min incubation with formoterol and budesonide on CXCR1 and CXCR2 expression on neutrophils from 10 healthy subjects. Propranolol was used for blocking of the formoterol effects. Results are expressed as relative mean fluorescence intensity (rMFI) and presented as median and 25th–75th percentiles. * $P < 0.05$, ** $P < 0.01$ indicate comparison with control values.

significant. Statistical analysis was performed using the StatView® program, version 4.02 (Abacus Concepts Inc., Berkeley, CA, USA).

3. Results

3.1. Cytokine/chemokine release

In the supernatant of non-stimulated neutrophils the IL-6 and CXCL8 concentrations were below detection limit and the concentration of CXCL1 was 48 ± 13 pg/ml. Formoterol and budesonide did not influence basal IL-6, CXCL8 or CXCL1 release (data not shown).

In LPS-stimulated neutrophils, formoterol increased the release of IL-6 ($p < 0.0001$, Fig. 1a) and CXCL8 ($p < 0.0001$, Fig. 1c) in a concentration-response manner but did not affect CXCL1 release (Fig. 1e). Budesonide inhibited IL-6 ($p < 0.0001$, Fig. 1b) and CXCL1 release ($p = 0.026$, Fig. 1f) in a concentration-response manner in LPS-stimulated neutrophils. A similar pattern was observed for budesonide on CXCL8 release ($p = 0.0001$, Fig. 1d). Formoterol enhanced the effect of budesonide on the release of CXCL1 ($p = 0.0069$, Fig. 1f) but not IL-6 and CXCL8 (Fig. 1b, d). Formoterol-induced effects were blocked with propranolol (Fig. 1).

3.2. Chemokine receptor expression

Formoterol induced increased expression of CXCR1 ($p = 0.021$) and CXCR2 ($p = 0.0001$) at all concentrations (10^{-10} – 10^{-6} M). The formoterol effect on both chemokine receptors was blocked by

propranolol (Fig. 2). Budesonide did not influence the expression of CXCR1 (Fig. 2b), but increased CXCR2 expression ($p = 0.0038$) (Fig. 2d) at all concentrations. Maximal drug effect was found already at the lowest budesonide concentration, and no further increase was observed at higher concentrations. Formoterol did not add to the effect of budesonide.

CXCL8 induced decreased expression of CXCR1 by 52% ($p < 0.0003$) and CXCR2 by 76% ($p < 0.0003$, Fig. 3a, c) while CXCL1 decreased CXCR2 expression by 60% ($p < 0.0003$, Fig. 3b, d). CXCL1 tended to increase the CXCR1 expression but only in the absence of formoterol and/or budesonide (Fig. 3b). Agonist-induced down-regulation of CXCR1 and CXCR2 was not counteracted by pre-treatment with formoterol and/or budesonide (Fig. 3a, c, d).

3.3. Chemotaxis

CXCL8 was a more efficient chemoattractant for neutrophils than was CXCL1, the migration rate being 43% and 17% of maximum migration (i.e. cells added directly to the bottom of the wells), respectively (Fig. 4). Neither formoterol nor budesonide, alone or in combination, influenced chemotaxis (Fig. 4).

The migration towards CXCL8 was reduced by 26% when blocking CXCR1 and by 36% in the presence of antibodies against both CXCR1 and CXCR2 ($p = 0.0018$). Blocking CXCR2 only did not significantly influence migration (Fig. 5a). No significant blocking with CXCR1 and CXCR2 antibodies was observed when CXCL1 was used as chemoattractant (Fig. 5b).

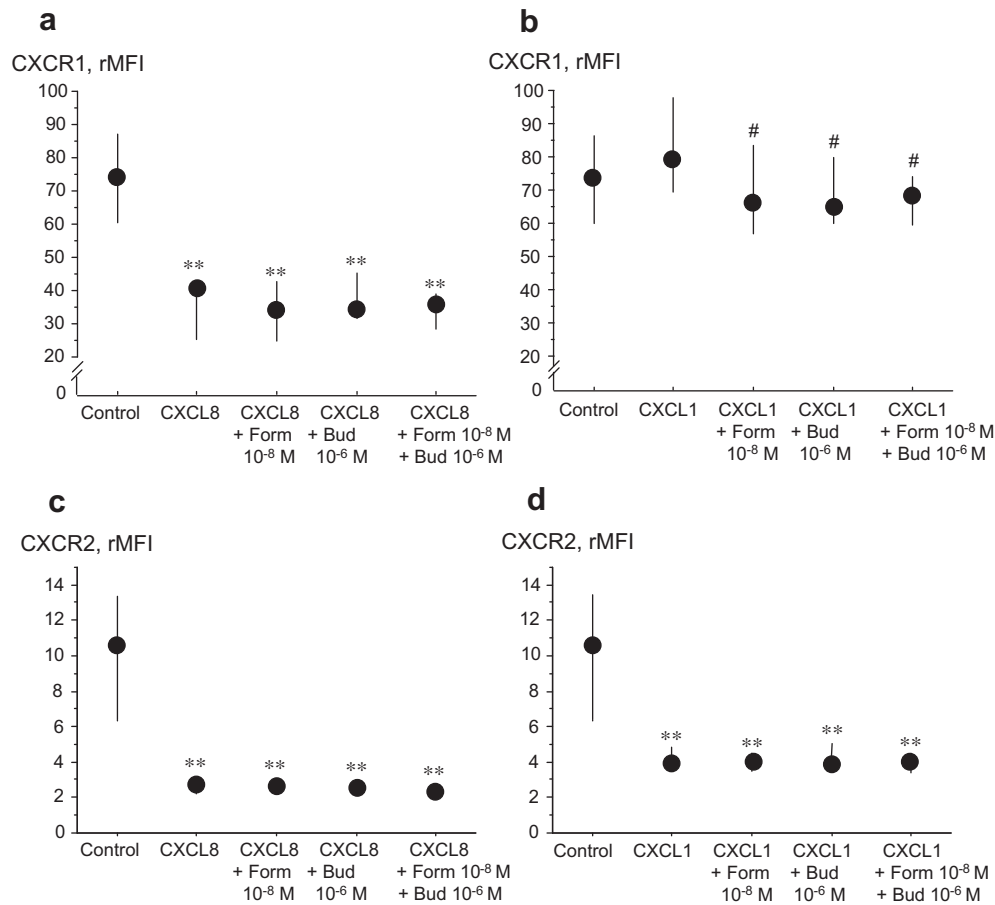


Fig. 3. Effect of 30 min incubation with formoterol (Form) and budesonide (Bud) on CXCR1 and CXCR2 down-regulation induced by CXCL8 and CXCL1 on neutrophils from 10 healthy subjects. Results are expressed as relative mean fluorescence intensity (rMFI) and presented as median and 25th–75th percentiles. ** $P < 0.01$ indicate comparison with control values. # $P < 0.05$, indicate comparison with CXCL1 without drugs.

4. Discussion

In the present study we have demonstrated that IL-6, CXCL8 and CXCL1 release from neutrophils from healthy subjects activated by LPS *in vitro* was stimulated by formoterol and inhibited by budesonide. The formoterol effects were blocked with propranolol indicating β -adrenoceptor mediated effects. Formoterol up-regulated both CXCR1 and CXCR2 expression, whereas budesonide up-regulated the expression of CXCR2 only. It was further shown that CXCR1 and CXCR2 were down-regulated by CXCL8 and CXCL1, and that CXCL1 was the weaker agonist in this respect and active on CXCR2 only. Despite these clear drug-induced effects on chemokine release and sensitivity the β_2 -agonist and the glucocorticosteroid did not influence chemotaxis *in vitro*.

Similar to previous studies in airway epithelial cells [19,22,23] it was demonstrated that a β_2 -agonist increased IL-8 (CXCL8) and IL-6 release. In a study by de Coupade et al. isoprenaline induced release of an undefined chemotactic factor from non-stimulated neutrophils obtained from females. The chemotaxis induced by this chemoattractant was antagonized by CXCR2 receptor blockade [27]. In the present study no detectable IL-6 or CXCL8 release from non-stimulated cells was shown, neither with nor without the β_2 -agonist (formoterol) being present. A low basal release of CXCL1 was found which was not affected by formoterol. We further showed that the β_2 -agonist up-regulated receptors for CXCL8 and other chemokines (CXCR1 and CXCR2) on neutrophils, an effect that, from a functional perspective, most likely should have

influenced the migration rate. We did not, however, find that formoterol altered neutrophil migration. Although the effect of β_2 -agonists on neutrophil chemotaxis has been previously studied it is far from clear how this class of drugs influences cell migration. Straub et al. found a stimulation of monocyte chemotaxis by β -agonists [28], and Koyama et al. showed that eosinophil migration was inhibited by β_2 -adrenoceptor stimulation of bronchial epithelial cells [29]. Formoterol at high concentrations (10^{-6} – 10^{-4} M, $n = 3$) inhibited fMLP- and LL-37-induced neutrophil chemotaxis [30] and fenoterol, another β_2 -agonist, has been shown to reduce neutrophil chemotaxis [31]. In a study by Sadowska et al. salbutamol inhibited fMLP-induced neutrophil migration and, somewhat unexpected, CXCL8 release from neutrophils [32]. Both these effects appeared in an inverse bell-shaped, concentration dependent, manner. The results of that study were, in part, consistent with the findings of Coupade et al. who found isoprenaline-induced down-regulation of chemotaxis in neutrophils obtained from women but not from men [27]. In that study the specificity of the effects were established by control experiments showing a lack of β_1 -adrenoceptors on neutrophils and inhibition of migration by β_2 -adrenoceptor blockade [27].

In previous studies of β_2 -agonist effects on neutrophil migration, chemoattractants such as fMLP, LL-37, C5a, and pro-inflammatory cytokines (TNF, IL-1) have been used. In the study showing inhibition of chemotaxis only in women, recombinant CXCL8 was used as chemoattractant [27]. In the present study of 5 women and 5 men we found no significant β_2 -agonist-induced influence on

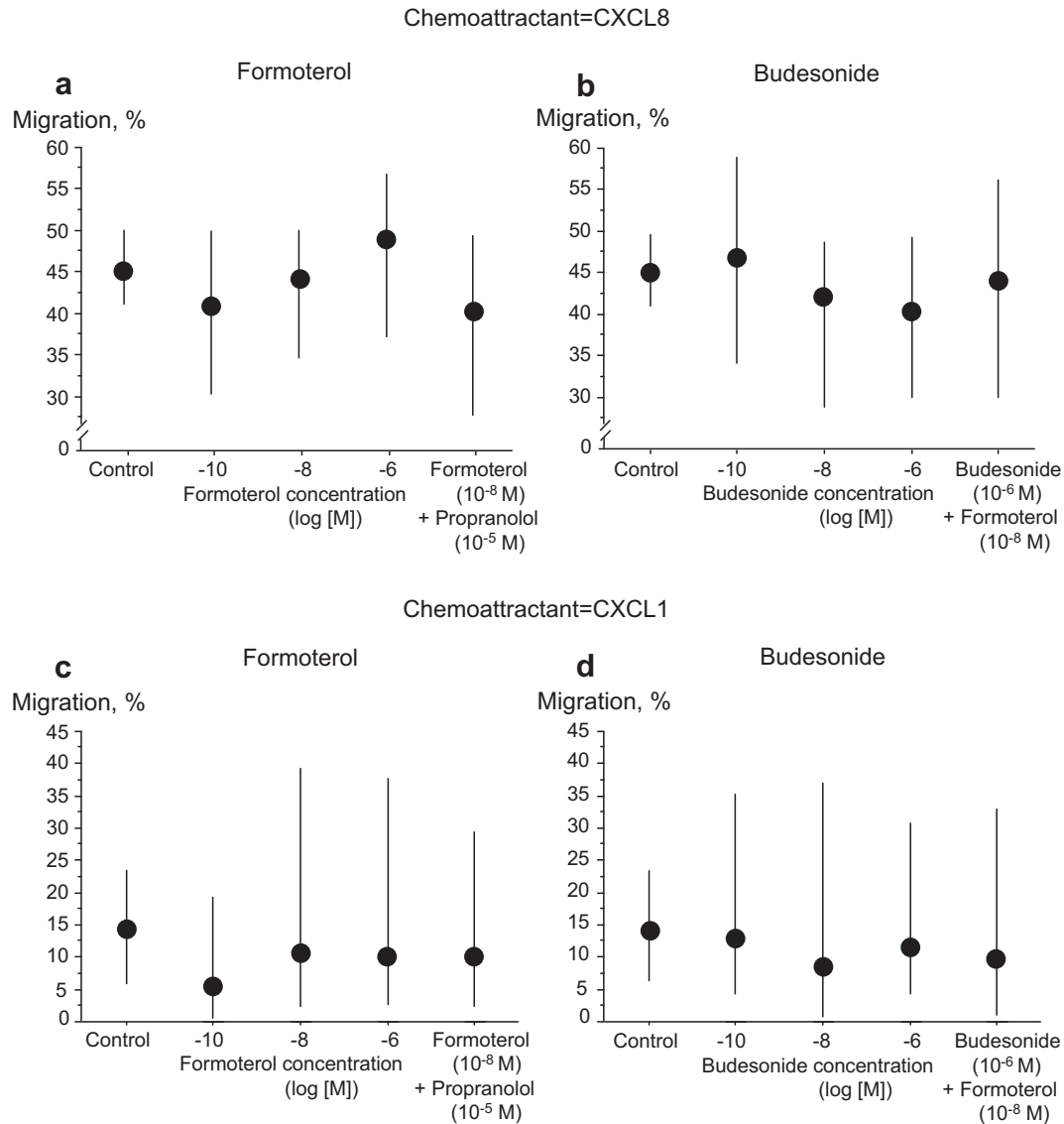


Fig. 4. Effect of 20 min pre-incubation with formoterol and budesonide on neutrophil chemotaxis during 60 min with CXCL8 (100 ng/ml) and CXCL1 (100 ng/ml) as chemoattractants. Propranolol was used for blocking of the formoterol effects. Neutrophil migration is expressed as percent of maximum migration (cells added directly to the bottom of the wells) and presented as median and 25th–75th percentiles. No significant drug effects on chemotaxis were observed.

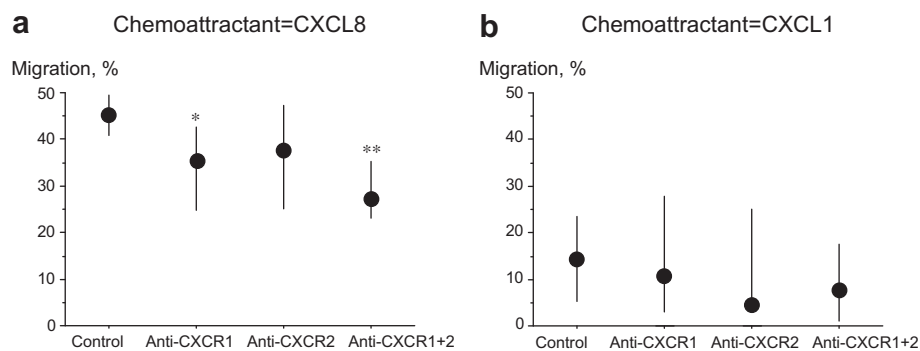


Fig. 5. Effect of 20 min pre-incubation with antibodies against CXCR1, CXCR2 or both, on neutrophil chemotaxis during 60 min. CXCL8 (100 ng/ml) and CXCL1 (100 ng/ml) were used as chemoattractants. Neutrophil migration is expressed as percent of maximum migration (cells added directly to the bottom of the wells) and presented as median and 25th–75th percentiles. * $P < 0.05$, ** $P < 0.01$ indicate comparison with control.

neutrophil migration. Based on previous findings of an inhibitory effect only in neutrophils from females [27], it could not be excluded that we, by not separating analyses of female and male neutrophils, have missed a gender dependent effect. However, separate analysis of women and men in our small study did not reveal a gender difference, or even a trend towards a difference (data not shown).

In the present study we used CXCL8 as chemoattractant and an autologous down-regulation of the CXCR1 and CXCR2 receptors was demonstrated. It is reasonable to believe that the lack of drug effect on cell migration is a result of autologous, CXCL8-induced down-regulation of CXCR2 receptors which is possibly counteracted by β_2 -agonist- and steroid-induced CXCR2 up-regulation.

There was a tendency towards a (non-significant) inhibitory effect on IL-6, CXCL8 and CXCL1 release of propranolol, beyond the effect of LPS alone. It could be argued that this may indicate a contamination of LPS or serum (FCS) with agents that may stimulate production or mimic the effect of cAMP. According to the manufacturer, FCS does not contain compounds that may stimulate cAMP production. Furthermore, as FCS was used in all protocols a possible effect of the medium of LPS in this respect would be identical in the controls situation and when adding the drugs and would thus not influence the interpretation of the main results.

In a study by Hauser et al. it was shown that CXCR2 is more responsive to CXCL8 than is CXCR1 and the authors suggested that CXCR2 acts as a “priming” receptor for CXCR1. This would likely be of importance *in vivo* as circulating CXCL8 levels appear to be too low to stimulate CXCR1 directly, but do appear to be sufficient to stimulate CXCR2 [33]. In the present study, the up-regulation of CXCR1 expression by CXCL1 was abolished by formoterol and/or budesonide. Considering that CXCR1 (but not CXCR2) is thought to be important in the respiratory burst and the activation of phospholipase D at inflammatory sites [34], this finding might be of clinical importance. It may be worth mentioning that we found a tendency towards a small (non-significant) up-regulation of CXCR1 by CXCL1. The implication of this is unclear but the phenomenon has been demonstrated also by others [33].

It is not clear whether the agonists (CXCL8, CXCL1) bind to the same epitopes on the chemokine receptors as do the anti-CXCR1 and anti-CXCR2 antibodies used in the present study. If receptor binding competition is the case, this might explain the decreased receptor antibody-labelling observed after incubation with CXCL8 or CXCL1. Previous binding experiments with a radiolabelled ligand showed that the rate of down-regulation of the CXCL8 receptor is very rapid (within minutes), and that the receptor internalization is completely inhibited at 4 °C [35]. In the present study, incubation with the receptor agonists was performed at 37 °C while incubation with anti-CXCR1 and anti-CXCR2 antibodies was performed at low temperature (samples kept on ice in the refrigerator), thereby probably preventing the internalized receptors from recycling back to the cell surface. Although it seems most likely that the attenuation of chemokine receptors reflects an autologous down-regulation caused by the agonists it can not definitely be excluded that there is a competitive inhibition of CXCR1 and CXCR2 induced by CXCL8 and CXCL1.

Apart from the effects on CXCL1 release from LPS-activated neutrophils where formoterol added to the inhibitory effect of budesonide, no additive or synergistic effects of the β_2 -agonist and the steroid were found. This is in concordance with previous results where no additive or synergistic effect of formoterol and budesonide on IL-6 and CXCL8 release from primary bronchial epithelial cells was found [23]. Although synergistic effects between β_2 -agonists and glucocorticosteroids have been shown [10,20,21], there are other *in vitro* findings rather supporting antagonistic actions of β_2 -agonists and glucocorticosteroids, both on molecular

and cellular levels [36,37]. An additive inhibitory effect with formoterol and budesonide was observed on GM-CSF, but not on CXCL8 production in IL-1 β -stimulated human lung fibroblasts [38]. Previous studies have thus come to different results and whether an additive/synergistic or even an antagonistic effect between β_2 -agonists and glucocorticosteroids is obtained may differ in different cell types and is likely depending on which inflammatory markers have been assessed.

In the present *in vitro* study, we have clearly demonstrated that a β_2 -agonist and a glucocorticosteroid influence cytokine release and chemokine receptor regulation in neutrophils. The clinical implication of these findings remains unclear since neutrophils from healthy subjects were used, and the results of the present study may not reflect the situation in disease. The concentrations of formoterol and budesonide used in the present study are similar to those found in the airways during treatment in a clinical setting [39–41]. Formoterol has been shown to reduce sputum CXCL8 and neutrophil numbers in mild asthmatic patients [42] but to what extent this is due to a direct effect on the neutrophils, or whether it is an indirect effect via other effector cells taking part in the inflammatory reaction, needs further investigation. The expression of CXCR1 and CXCR2 receptors can be regulated by immunomodulators such as bacterial lipopolysaccharide (LPS) and pro-inflammatory cytokines such as tumour necrosis factor (TNF) [43]. LPS causes down-regulation of CXCR1 and CXCR2 [7,43], but has been shown both to inhibit and enhance chemokine-induced neutrophil migration [44–46]. The interplay between receptor down-regulation and neutrophil migration is thus complicated and controversial results have also been reported with regard to CXCRs expression in COPD. Thus, in COPD increased expression of CXCR1 [47], decreased expression of CXCR2 (but not CXCR1) [48], and a lack of difference between CXCR1 and CXCR2 expression on peripheral blood neutrophils [2] have been demonstrated. Cigarette smoke contains LPS and causes release of chemoattractants which may cause receptor down regulation, as a basis for the reduced maximal responses to CXCL8 and CXCL1 observed in patients with COPD [2]. It could be speculated that one of the reasons of the favourable clinical effect observed with the combination of β_2 -agonists and glucocorticoids in treatment of COPD, is an up-regulation of CXCR1 and CXCR2, thereby counteracting the detrimental effect of cigarette smoke on neutrophil function.

In conclusion, we have shown that formoterol stimulated and budesonide inhibited IL-6, CXCL8 and CXCL1 release from LPS-stimulated neutrophils *in vitro*. Though an up-regulation of CXCR1 and CXCR2 receptors was induced by formoterol and budesonide, no influence on neutrophil chemotaxis could be demonstrated, possibly due to an autologous, chemokine-induced down-regulation of CXCRs.

Acknowledgement

The authors wish to thank the volunteers who generously participated in the study as blood donors. The study was supported by grants from Swedish Heart and Lung Foundation.

References

- [1] Kobayashi Y. The role of chemokines in neutrophil biology. *Front Biosci* 2008;13:2400–7.
- [2] Nicholson GC, Tennant RC, Carpenter DC, Sarau HM, Kon OM, Barnes PJ, et al. A novel flow cytometric assay of human whole blood neutrophil and monocyte CD11b levels: upregulation by chemokines is related to receptor expression, comparison with neutrophil shape change, and effects of a chemokine receptor (CXCR2) antagonist. *Pulm Pharmacol Ther* 2007;20:52–9.
- [3] Tarlowe MH, Kannan KB, Itagaki K, Adams JM, Livingston DH, Hauser CJ. Inflammatory chemoreceptor cross-talk suppresses leukotriene B4 receptor

- 1-mediated neutrophil calcium mobilization and chemotaxis after trauma. *J Immunol* 2003;171:2066–73.
- [4] Richardson RM, Marjoram RJ, Barak LS, Snyderman R. Role of the cytoplasmic tails of CXCR1 and CXCR2 in mediating leukocyte migration, activation, and regulation. *J Immunol* 2003;170:2904–11.
- [5] Chung KF. Cytokines in chronic obstructive pulmonary disease. *Eur Respir J Suppl* 2001;34:50s–9s.
- [6] Hogan SP, Rosenberg HF, Moqbel R, Phipps S, Foster PS, Lacy P, et al. Eosinophils: biological properties and role in health and disease. *Clin Exp Allergy* 2008;38:709–50.
- [7] Parker LC, Whyte MK, Vogel SN, Dower SK, Sabroe I. Toll-like receptor (TLR)2 and TLR4 agonists regulate CCR expression in human monocytic cells. *J Immunol* 2004;172:4977–86.
- [8] Romagnoli M, Caramori G, Braccioni F, Ravenna F, Barreiro E, Siafakas NM, et al. Near-fatal asthma phenotype in the ENFUMOSA Cohort. *Clin Exp Allergy* 2007;37:552–7.
- [9] Lamblin C, Gosset P, Tillie-Leblond I, Saulnier F, Marquette CH, Wallaert B, et al. Bronchial neutrophilia in patients with noninfectious status asthmaticus. *Am J Respir Crit Care Med* 1998;157:394–402.
- [10] Adcock IM, Maneechotesuwan K, Usmani O. Molecular interactions between glucocorticoids and long-acting beta2-agonists. *J Allergy Clin Immunol* 2002;10:S261–8.
- [11] Green RH, Brightling CE, Woltmann G, Parker D, Wardlaw AJ, Pavord ID. Analysis of induced sputum in adults with asthma: identification of subgroup with isolated sputum neutrophilia and poor response to inhaled corticosteroids. *Thorax* 2002;57:875–9.
- [12] Larsson K. Aspects on pathophysiological mechanisms in COPD. *J Intern Med* 2007;262:311–40.
- [13] Calverley PM, Anderson JA, Celli B, Ferguson GT, Jenkins C, Jones PW, et al. Salmeterol and fluticasone propionate and survival in chronic obstructive pulmonary disease. *N Engl J Med* 2007;356:775–89.
- [14] Calverley PM, Boonsawat W, Cseke Z, Zhong N, Peterson S, Olsson H. Maintenance therapy with budesonide and formoterol in chronic obstructive pulmonary disease. *Eur Respir J* 2003;22:912–9.
- [15] Celli BR, Thomas NE, Anderson JA, Ferguson GT, Jenkins CR, Jones PW, et al. Effect of pharmacotherapy on rate of decline of lung function in chronic obstructive pulmonary disease: results from the TORCH study. *Am J Respir Crit Care Med* 2008;178:332–8.
- [16] Greening AP, Ind PW, Northfield M, Shaw G. Added salmeterol versus higher-dose corticosteroid in asthma patients with symptoms on existing inhaled corticosteroid. Allen & Hanburys Limited UK Study Group. *Lancet* 1994;344:219–24.
- [17] Pauwels RA, Lofdahl CG, Postma DS, Tattersfield AE, O'Byrne P, Barnes PJ, et al. Effect of inhaled formoterol and budesonide on exacerbations of asthma. Formoterol and Corticosteroids Establishing Therapy (FACET) International Study Group. *N Engl J Med* 1997;337:1405–11.
- [18] Szafranski W, Cukier A, Ramirez A, Menga G, Sansores R, Nahabedian S, et al. Efficacy and safety of budesonide/formoterol in the management of chronic obstructive pulmonary disease. *Eur Respir J* 2003;21:74–81.
- [19] Korn SH, Jerre A, Brattsand R. Effects of formoterol and budesonide on GM-CSF and IL-8 secretion by triggered human bronchial epithelial cells. *Eur Respir J* 2001;17:1070–7.
- [20] Pang L, Knox AJ. Synergistic inhibition by beta(2)-agonists and corticosteroids on tumor necrosis factor-alpha-induced interleukin-8 release from cultured human airway smooth-muscle cells. *Am J Respir Cell Mol Biol* 2000;23:79–85.
- [21] Eickelberg O, Roth M, Lox R, Bruce V, Rudiger J, Johnson M, et al. Ligand-independent activation of the glucocorticoid receptor by beta2-adrenergic receptor agonists in primary human lung fibroblasts and vascular smooth muscle cells. *J Biol Chem* 1999;274:1005–10.
- [22] Linden A. Increased interleukin-8 release by beta-adrenoceptor activation in human transformed bronchial epithelial cells. *Br J Pharmacol* 1996;119:402–6.
- [23] Strandberg K, Palmberg L, Larsson K. Effect of budesonide and formoterol on IL-6 and IL-8 release from primary bronchial epithelial cells. *J Asthma* 2008;45:201–3.
- [24] Ek A, Larsson K, Siljerud S, Palmberg L. Fluticasone and budesonide inhibit cytokine release in human lung epithelial cells and alveolar macrophages. *Allergy* 1999;54:691–9.
- [25] Larsson K, Tornling G, Gavhed D, Muller-Suur C, Palmberg L. Inhalation of cold air increases the number of inflammatory cells in the lungs in healthy subjects. *Eur Respir J* 1998;12:825–30.
- [26] Frevert CW, Wong VA, Goodman RB, Goodwin R, Martin TR. Rapid fluorescence-based measurement of neutrophil migration in vitro. *J Immunol Methods* 1998;213:41–52.
- [27] de Coupade C, Gear RW, Dazin PF, Sroussi HY, Green PG, Levine JD. Beta 2-adrenergic receptor regulation of human neutrophil function is sexually dimorphic. *Br J Pharmacol* 2004;143:1033–41.
- [28] Straub RH, Mayer M, Kreutz M, Leeb S, Scholmerich J, Falk W. Neurotransmitters of the sympathetic nerve terminal are powerful chemoattractants for monocytes. *J Leukoc Biol* 2000;67:553–8.
- [29] Koyama S, Rennard SI, Leikauf GD, Shoji S, Von Essen S, Claassen L, et al. Endotoxin stimulates bronchial epithelial cells to release chemotactic factors for neutrophils. A potential mechanism for neutrophil recruitment, cytotoxicity, and inhibition of proliferation in bronchial inflammation. *J Immunol* 1991;147:4293–301.
- [30] Tjabringa GS, Ninaber DK, Drijfhout JW, Rabe KF, Hiemstra PS. Human cathelicidin LL-37 is a chemoattractant for eosinophils and neutrophils that acts via formyl-peptide receptors. *Int Arch Allergy Immunol* 2006;140:103–12.
- [31] Silvestri M, Oddera S, Lantero S, Rossi GA. beta 2-agonist-induced inhibition of neutrophil chemotaxis is not associated with modification of LFA-1 and Mac-1 expression or with impairment of polymorphonuclear leukocyte antibacterial activity. *Respir Med* 1999;93:416–23.
- [32] Sadowska AM, Manuel-Keenoy B, De Backer WA. Inhibition of in vitro neutrophil migration through a bilayer of endothelial and epithelial cells using beta2-agonists: concomitant effects on IL-8 and elastase secretion and impact of glucocorticosteroids. *Pulm Pharmacol Ther* 2005;18:354–62.
- [33] Hauser CJ, Fekete Z, Goodman ER, Kleinstein E, Livingston DH, Deitch EA. CXCR2 stimulation primes CXCR1 [Ca²⁺]_i responses to IL-8 in human neutrophils. *Shock* 1999;12:428–37.
- [34] Jones SA, Wolf M, Qin S, Mackay CR, Baggiolini M. Different functions for the interleukin 8 receptors (IL-8R) of human neutrophil leukocytes: NADPH oxidase and phospholipase D are activated through IL-8R1 but not IL-8R2. *Proc Natl Acad Sci U S A* 1996;93:6682–6.
- [35] Samanta AK, Oppenheim JJ, Matsushima K. Interleukin 8 (monocyte-derived neutrophil chemotactic factor) dynamically regulates its own receptor expression on human neutrophils. *J Biol Chem* 1990;265(1):183–9.
- [36] Adcock IM, Stevens DA, Barnes PJ. Interactions of glucocorticoids and beta 2-agonists. *Eur Respir J* 1996;9:160–8.
- [37] Nielson CP, Hadjokas NE. Beta-adrenoceptor agonists block corticosteroid inhibition in eosinophils. *Am J Respir Crit Care Med* 1998;157:184–91.
- [38] Spoelstra FM, Postma DS, Hovenga H, Noordhoek JA, Kauffman HF. Additive anti-inflammatory effect of formoterol and budesonide on human lung fibroblasts. *Thorax* 2002;57:237–41.
- [39] Esmailpour N, Hogger P, Rabe KF, Heitmann U, Nakashima M, Rohdewald P. Distribution of inhaled fluticasone propionate between human lung tissue and serum in vivo. *Eur Respir J* 1997;10:1496–9.
- [40] Kerrebijn KF. Long-term drug treatment of asthma in children. *Lung* 1990;168 (Suppl):142–53.
- [41] Van den Bosch JM, Westermann CJ, Aumann J, Edsbacker S, Tonnesson M, Selroos O. Relationship between lung tissue and blood plasma concentrations of inhaled budesonide. *Biopharm Drug Dispos* 1993;14:455–9.
- [42] Maneechotesuwan K, Essilife-Quaye S, Meah S, Kelly C, Kharitonov SA, Adcock IM, et al. Formoterol attenuates neutrophilic airway inflammation in asthma. *Chest* 2005;128(4):1936–42.
- [43] Khandaker MH, Mitchell G, Xu L, Andrews JD, Singh R, Leung H, et al. Metalloproteinases are involved in lipopolysaccharide- and tumor necrosis factor-alpha-mediated regulation of CXCR1 and CXCR2 chemokine receptor expression. *Blood* 1999;93:2173–85.
- [44] Aomatsu K, Kato T, Fujita H, Hato F, Oshitani N, Kamata N, et al. Toll-like receptor agonists stimulate human neutrophil migration via activation of mitogen-activated protein kinases. *Immunology* 2008;123:171–80.
- [45] Fan J, Malik AB. Toll-like receptor-4 (TLR4) signaling augments chemokine-induced neutrophil migration by modulating cell surface expression of chemokine receptors. *Nat Med* 2003;9:315–21.
- [46] Parker LC, Whyte MK, Dower SK, Sabroe I. The expression and roles of Toll-like receptors in the biology of the human neutrophil. *J Leukoc Biol* 2005;77:886–92.
- [47] Yamagata T, Sugiura H, Yokoyama T, Yanagisawa S, Ichikawa T, Ueshima K, et al. Overexpression of CD-11b and CXCR1 on circulating neutrophils: its possible role in COPD. *Chest* 2007;132:890–9.
- [48] Pignatti P, Moscato G, Casarini S, Delmastro M, Poppa M, Brunetti G, et al. Downmodulation of CXCL8/IL-8 receptors on neutrophils after recruitment in the airways. *J Allergy Clin Immunol* 2005;115:88–94.