In vitro and in vivo effects of salbutamol on neutrophil function in acute lung injury

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Abstract

Background: Intravenous salbutamol (albuterol) reduces lung water in patients with the acute respiratory distress syndrome (ARDS). Experimental data demonstrate it also reduces pulmonary neutrophil accumulation/activation and inflammation in ARDS. The aim of this study was to investigate the effects of salbutamol on neutrophil function.

Methods: The in-vitro effects of salbutamol on neutrophil function determined. In the clinical study, blood and bronchoalveolar lavage (BAL) fluid was collected from 35 patients with acute lung injury (ALI)/ARDS, 14 patients at risk from ARDS and 7 ventilated controls at baseline and after 4 days treatment with placebo or salbutamol (ALI/ARDS group). Alveolar-capillary permeability was measured in-vivo by thermodilution (PiCCO). Neutrophil activation, adhesion molecule expression and inflammatory cytokines were measured.

Results: In-vitro, physiological concentrations of salbutamol had no effect on neutrophil chemotaxis, viability or apoptosis. In ALI/ARDS, there was increased neutrophil activation, adhesion molecule expression compared to at risk patients and ventilated controls. There were associations between alveolar-capillary permeability and BAL myeloperoxidase (r=0.4, P=0.038) and BAL IL-8 (r=0.38, P=0.033). In patients with ALI/ARDS, salbutamol increased circulating neutrophil numbers, but had no effect on alveolar neutrophil numbers.

Conclusion: At the onset of ALI/ARDS, there is increased neutrophil recruitment and activation. Physiological concentrations of salbutamol did not alter neutrophil chemotaxis, viability or apoptosis in-vitro. In vivo, salbutamol increased circulating neutrophils, but had no effect on alveolar neutrophil numbers or neutrophil activation. These data suggest the beneficial effects of salbutamol in reducing lung water are unrelated to modulation of neutrophil dependent inflammatory pathways.
Introduction

Acute lung injury (ALI) and the acute respiratory distress syndrome (ARDS) are major causes of respiratory failure in the critically ill patient[1]. Although controversy still exists regarding the role of neutrophil in all causes of acute lung injury[2], a recent systematic review of laboratory and clinical studies concluded that neutrophils played a central role in most cases[3]. Pathophysiologically, ARDS is characterized by intense inflammation in the alveolar space, with a predominance of neutrophils. Analysis of bronchoalveolar lavage (BAL) fluid from patients with ARDS have shown increased numbers of activated neutrophils in the early stages of ARDS[4,5]. The number of neutrophils in BAL fluid relates to the severity of lung injury[6] and the persistence of neutrophils is associated with increased mortality[5]. A study examining ARDS BAL fluid demonstrated a positive correlation between neutrophil myeloperoxidase (MPO) and oxidatively modified amino-acids suggesting an association between pulmonary neutrophil activation and oxidative protein damage[7]. Animal studies have shown that neutrophil mediated lung injury reduces alveolar fluid clearance, preventing the resolution of non-cardiogenic pulmonary oedema[8]. Neutrophil elastase inhibitors in animal models limit the degree of lung injury caused by ischaemia reperfusion[9] and lipopolysaccharide (LPS)[10], although a recent multi-centre clinical trial of the elastase inhibitor Sivelestat failed to improve outcome in a heterogeneous group of patients with ARDS[11].

β2 agonists have a number of inhibitory effects on neutrophil function[12]. In animal models of acute lung injury β2 agonists reduce pulmonary neutrophil sequestration[13,14]. In vitro β2 agonists reduce oxygen free radical production from neutrophils and other inflammatory cells[15,16] and reduces inflammatory cytokine production [17]. In humans, inhaled salmeterol (long acting β2 agonist) inhibited LPS-induced neutrophil influx, degranulation and tumor necrosis factor α release[18]. These experimental findings suggest that treatment with a β2 agonist could have a favourable effect in ARDS by reducing neutrophilic inflammation.

We recently conducted a randomised controlled trial in humans with ARDS (BALTI-1 study) which demonstrated that intravenous salbutamol (albuterol) significantly reduced extravascular lung water probably through up-regulation of alveolar fluid clearance[19]. However, the reduction in lung water was not seen until after 48 hours of the initiation of treatment suggesting some effects in addition to alveolar fluid clearance. The objective of the present study was to investigate if salbutamol modulates neutrophil function and neutrophilic inflammation at physiologically doses relevant to patients with acute lung injury. The second objective was to investigate the relationship between neutrophilic inflammation and alveolar capillary permeability in patients with ARDS.
Materials and Methods

In vitro studies

Under agarose chemotaxis, cell viability and apoptosis

Neutrophils from non smoking, healthy controls were purified from peripheral blood by discontinuous percoll density gradients[20]. The effect of salbutamol on the chemotactic activity of purified neutrophils was measured using the under-agarose method[21]. The freshly harvested neutrophils were re-suspended at $5 \times 10^7$ cells ml$^{-1}$ in RPMI 1640 culture media or $10^{-5}$ to $10^{-10}$ M salbutamol in RPMI (with or without propranolol $10^{-4}$ M) before seeding in 10µl aliquots into the central wells of a freshly prepared agarose plate. Ten µl of RPMI were placed into each of the inner wells, 10 µl of chemoattractant ($10^{-7}$ N-formyl-L-leucin-methionyl-L-phenylalanine (FMLP) in RPMI) into each of the outer wells. Negative controls contained RPMI in the inner and outer wells. The plates were incubated at $37^\circ C$ (5% CO$_2$) for 2 hours and then flooded with methanol for overnight fixation of the cells. The gel was carefully removed and the plates washed under slow running tap water. The plates were then stained with Gram Stain and left to dry. For each well the chemotactic and chemokinetic responses were read using an eye-piece graticule. The overall response to the chemoattractant (chemotactic differential) was calculated by subtracting the chemokinetic from the chemotactic response.

Purified neutrophils, suspended in RPMI at a concentration of $1 \times 10^6$ ml were incubated with $10^{-5}$ and $10^{-7}$ M salbutamol or RPMI control for 2 hours at $37^\circ C$ in humidified 5% CO$_2$. Cell proliferation was assessed by adding 20µl of CellTiter 96® AQeuous one solution (Promega, UK) to 100µl of cell suspension in a 96 well culture plate (Nunc). The reaction was allowed to proceed for 2 hours at $37^\circ C$ in 5% CO$_2$. The celltiter solution contains a tetrazolium compound which is metabolized by healthy cells to a formazan product the absorbance of which was read at 495nm on an MRX-II 96-well plate reader (Dynex Technologies, UK).

Purified neutrophils, suspended in RPMI with 10% heat-inactivated fetal calf serum (Life Technologies, Inc.) and 100 units/ml penicillin and 100 mg/ml streptomycin (Sigma) at a concentration of $1 \times 10^6$ ml were incubated with $10^{-5}$ and $10^{-7}$ M salbutamol or RPMI control for 18 hours at $37^\circ C$ in humidified 5% CO$_2$. Preliminary experiments demonstrated 18 hours to be the optimal time point for assessing apoptosis. The number of normal and apoptotic neutrophils were determined by morphological patterns. Cytospin preparations were made and stained using a commercial May-Grünwald Giemsa stain (Diff-Quick, Baxter Healthcare Products). The percentage apoptotic cells compared to total number of cells was calculated. The morphological results were confirmed using flow cytometry and the annexin V / propidium iodine apoptosis kit (Dako, UK). The percentage live (annexin and propidium iodine negative) and early apoptotic (annexin positive, propidium iodine negative) or late apoptotic/dead (annexin positive, propidium iodine positive) were calculated.
Clinical study

Mechanically ventilated adult patients enrolled in the BALTI-1 study were eligible for inclusion[19]. This study recruited patients within 48 hours of onset of ALI and ARDS and randomised them to 7 days treatment with intravenous salbutamol (15 mcg kg^{-1} hr^{-1}). ALI was defined according to the American European Consensus Conference definition[22] as: the acute onset of respiratory failure with a PaO\(_2\) : FiO\(_2\) ratio < 300 mm Hg, bilateral infiltrates on the chest radiograph in the absence of clinical evidence of left atrial hypertension. ARDS was considered present when the PaO\(_2\) : FiO\(_2\) ratio was < 200 mm Hg. The exclusion criteria were: age < 18 years; participation in other intervention trials; severe obstructive airways disease requiring nebulised or intravenous \(\beta_2\) agonist; treatment with \(\beta\) blockers within 48 hours; neutrophil count < 0.3 x 10\(^9\) L; brain stem death; treatment withdrawal within 24 hours; immunosuppression (steroids > 20mg/day, chemotherapy or other immunosuppressive agents within 2 weeks); lobectomy/pneumonectomy; burns > 40% body surface area; assent declined from the next of kin.

Patients with identifiable risk factors for ALI/ARDS, but who, at the time of recruitment had not met the criteria for ALI/ARDS were included as an at risk population. Non-smoking, age matched ventilated controls undergoing elective surgery were recruited as controls.

Biological sample collection and processing

Bronchoalveolar lavage using 150ml cold saline was performed immediately following recruitment in at risk patients and ventilated controls and at baseline and day 4 in ALI/ARDS patients. Lavage fluid was kept on ice until transferred to the laboratory for processing. Blood was simultaneously collected into lithium heparin tubes (Becton-Dickinson, Plymouth, UK) and placed immediately on ice until processed.

Broncho-alveolar lavage fluid was filtered through course surgical gauze to remove mucous and other debris. The fluid was then spun at 500g for 5 minutes in a centrifuge pre-chilled to 4°C. The supernatant was removed and immediately frozen to -80°C and stored for subsequent analysis. The cell pellet was re-suspended in 10ml phosphate buffered saline supplemented with 1% Human Serum Albumin. Total cell count was determined using a haemocytometer. Cell viability was measured by the ability of live cells to exclude Trypan blue. Cell purity was measured on a cytospin preparation stained with DiffQuick (Baxter Incorp., UK) as previously described[23].

Patient characteristics

Patient demographic characteristics were recorded at baseline. The acute physiology and chronic health evaluation II (APACHE II) and simplified acute physiology score II (SAPS II) score, predicted ICU mortality were recorded as global markers of disease severity[24]. The Murray lung injury score, PaO\(_2\) : FiO\(_2\) ratio were collected as markers of the severity of lung injury.
Alveolar capillary permeability

The single indicator transpulmonary thermodilution system (PiCCO; Pulsion Medical Systems, Munich, Germany) was used to calculate an *in vivo* alveolar-capillary permeability index. The permeability index was derived from the ratio of extravascular lung water divided by pulmonary blood volume. Previous studies have shown this index can separate cardiogenic (low permeability) and inflammatory (high permeability) causes of pulmonary oedema[25].

Flow cytometry analysis

Flow cytometry was performed using the whole blood technique[26]. One hundred microlitres aliquots of whole blood or BAL cells (suspended in 1% Human Serum Albumin (Sigma Chemicals Ltd, Poole, UK) at a final concentration of $1 \times 10^6$ ml$^{-1}$) were fixed by adding 100ul of 1% paraformaldehyde for 15 minutes. Immunofluorescent staining was performed by adding Fluorescin Isothiocyante Isomer (FITC) conjugated monoclonal antibodies (mAb) directed against: CD11b (IgG1, Dako, Ely, UK); CD18 (IgG1, Dako); CD 49 (IgG1, Serotec, Oxford, UK), CD 64 (IgG1, Serotec) and L-selectin, IgG1, Becton Dickinson). Stimulated neutrophil L-selectin expression was determined by incubating whole blood with FMLP ($10^{-6}$M) for 15 minutes prior to fixation. Controls were included using isotype matched, irrelevant antibodies directed against human IgG1 (Dako). After incubation with the appropriate monoclonal antibody for 30 minutes at room temperature (protected from light), 1.5ml of FACS brand lysing solution (Becton Dickinson, Oxford, UK) were added to each tube for 5 minutes. The cells were then washed twice with 1.5ml of wash buffer (500ml PBS, 0.1g Sodium Azide (Sigma Chemicals Ltd, Poole, UK), 5g Bovine Serum Albumin (Sigma)). Following the second wash the cell pellet was resuspended in 500ul of 1% paraformaldehyde. Samples were anlaysed on a Becton-Dickinson 440 flow cytometer. The neutrophil cell population were identified and gated from their forward and side light scatter. The median intensity of fluorescence for cells labelled with specific antibody was determined relative to the median intensity of fluoresce for cells labelled with the non-specific isotype control antibody.

TNFα, IL-8, myeloperoxidase and salbutamol assays

BAL TNFα and IL-8 were measured using a commercially available ELISA (R&D systems, Abingdon, UK) BAL myeloperoxidase was measured using the chromogenic substrate assay as previously described[23]. Salbutamol levels were measured using a commercially available ELISA (Biox diagnostics, Jemelle, Belgium).

Statistical analysis

The study was powered to detect a 60% reduction in alveolar neutrophil sequestration based on recent data demonstrating that β2 agonists reduce pulmonary neutrophil sequestration in human volunteers exposed to LPS by 60%[18]. We calculated that 9
patients would need to be recruited in each arm to detect this difference with 80% power at a significance level of 0.05. Differences between groups were examined by ANOVA or Kruskal-Wallis test. Where significant differences were identified, Tukeys test or Mann Whitney U test were used to further examine the differences. Repeated measures of neutrophil counts were non-parametric and analysed by Friedman's repeated measures test. Linear associations were tested using Pearson's correlation test. Data are expressed as mean (standard deviation) unless otherwise stated. A P value of < 0.05 was considered statistically significant.

Results

In-vitro studies

Neutrophil migration towards the chemotractant stimulus FMLP was greater than towards RPMI control (11.6 mm (1.2) vs 3.6mm (0.2), P=0.0001). Salbutamol at $10^{-4}$ M and $10^{-5}$ M significantly reduced neutrophil chemotaxis (Figure 1(i)). There was no effect on random neutrophil chemokinesis (Figure 1(ii)). The effect of salbutamol on neutrophil chemotaxis was abolished by propranolol (figure 1(iii)). Salbutamol $10^{-4}$ to $10^{-6}$ M had no effect on cellular viability (data not shown) or neutrophil apoptosis (figure 1(iv)).

Clinical studies

Patient baseline demographics

Forty patients with ALI / ARDS were initially recruited of which 35 had bronchoalveolar lavage performed at baseline and were included in this study. Fourteen patients with risk factors for ARDS but who had not developed ARDS at the time of bronchoalveolar lavage formed the at risk group. Some of the data from the at risk group (BAL myeloperoxidase levels and neutrophil counts) have previously been published[23]. Seven ventilated controls were recruited. The baseline severity of illness data and aetiology of lung injury for the ARDS and at risk patients are presented in Table 1.

Neutrophilic inflammation is present in the alveolar space in ARDS

There was evidence of increased accumulation of neutrophils and neutrophilic inflammation in the BAL fluid of patients with ARDS. Total neutrophil count, myeloperoxidase, TNFα and IL-8 were elevated in patients with ARDS compared to patients at risk from ARDS and normal ventilated controls (figure 2(i-iv)). There were significant, albeit weak, linear associations between the PiCCO alveolar capillary permeability index and myeloperoxidase ($r=0.4$, $P=0.038$) and IL-8 ($r=0.37$, $P=0.033$). Compared to normal controls, there was evidence of increased adhesion molecule expression CD11b, CD18, CD49, reduced L-selectin expression and increased neutrophil activation (CD 64) on circulating and alveolar neutrophils (Figures 3(i-ii)).
Table 1: Baseline demographics for patients at risk from ARDS and patients with established ARDS.

<table>
<thead>
<tr>
<th></th>
<th>ARDS (n=35)</th>
<th>At risk (n=19)</th>
<th>P value</th>
<th>95% CI difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>P:F ratio torr [kPa]</td>
<td>106 (92) [33.5 (12.1)]</td>
<td>155 (92) [14.9 (5.8)]</td>
<td>0.0001</td>
<td>95 – 185 [12.5 – 24.4]</td>
</tr>
<tr>
<td>Lung injury score</td>
<td>2.9 (0.6)</td>
<td>1.2 (0.3)</td>
<td>0.0001</td>
<td>1.3 – 1.9</td>
</tr>
<tr>
<td>SOFA score</td>
<td>13.4 (3.7)</td>
<td>6.7 (4.3)</td>
<td>0.0001</td>
<td>4.1 – 9.2</td>
</tr>
<tr>
<td>APACHE II</td>
<td>23.4 (6.9)</td>
<td>21.7 (8.9)</td>
<td>0.312</td>
<td>-2.1 – 6.5</td>
</tr>
<tr>
<td>SAPS II</td>
<td>52.0 (15.7)</td>
<td>54.8 (12.8)</td>
<td>0.550</td>
<td>-12.0 – 6.4</td>
</tr>
<tr>
<td>Direct lung injury</td>
<td>13 (37%)</td>
<td>6 (32%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indirect lung injury</td>
<td>22 (63%)</td>
<td>13 (68%)</td>
<td>0.771</td>
<td></td>
</tr>
</tbody>
</table>

In-vivo effects of salbutamol

From the initial group of patients randomised to salbutamol or placebo treatment, follow up blood and bronchoalveolar lavage samples were collected from 22 patients at day 4 (9 salbutamol, 13 placebo). Salbutamol was detectable by ELISA in the plasma of the treatment group at a concentration equivalent to $10^{-6}$ M (range 250 – 495 ng ml$^{-1}$). The ELISA did not perform reproducibly in BAL matrix.

Circulating neutrophils

Treatment with intravenous salbutamol significantly increased the number of circulating neutrophils (figure 4). There was a trend towards reduced L-selectin expression on unstimulated neutrophils in the salbutamol group [placebo 55(19) vs salbutamol 39(30), P=0.18]. When neutrophils were stimulated ex vivo with FMLP, neutrophils from salbutamol treated patients had significantly lower L-selectin expression (placebo 43(15) vs salbutamol 22(21), 95% CI diff 1.2–39, P=0.038)(Figure 5). There was no effect on integrin adhesion molecule expression measured at day 4 (CD 11b, CD 18 or CD 49) or expression of the neutrophil activation marker CD 64 (Figure 3(i)).
Pulmonary neutrophils and alveolar inflammation

In contrast to the plasma compartment, there was no difference in BAL fluid neutrophil count between groups at day 4 (placebo 3.5(0.6) vs salbutamol group 1.6 (1.7), P=0.4)(Figure 2(ii)). Adhesion molecule expression was also similar in placebo and salbutamol treated groups and there was no difference in the expression of the neutrophil activation marker CD 64 (Figure 3(ii)). There was no difference in L selectin expression (unstimulated cells- placebo 3 (4) vs salbutamol 4(6), P=0.753), fMLP stimulated cells - placebo 1.7(2) vs salbutamol 1.8(3), P=0.9). There were no differences in myeloperoxidase (figure 2(ii)), TNFα (figure 2(iii)) or IL-8 (figure 2(iv)).

Discussion

In a double blind randomized placebo controlled trial, we demonstrated that sustained treatment with intravenous salbutamol significantly reduced extravascular lung water in humans with ALI/ARDS[19]. In contrast to our initial hypothesis and supporting data from animal studies[27], the response was not evident until 48 hours after the initiation of treatment. The present study sought to identify if salbutamol was having an effect pulmonary neutrophil accumulation and alveolar inflammation. This study confirms previous observations that there is increased neutrophilic accumulation and inflammation in the alveolar space in ALI/ARDS. The novel finding of a positive association between the intensity of neutrophilic infiltration and an in-vivo measurement of alveolar-capillary permeability adds additional support to the hypothesis that the neutrophil is a key inflammatory mediator in ARDS. Contrary to in-vitro, animal and healthy volunteer studies, the present study found no evidence that intravenous salbutamol modulated alveolar neutrophil accumulation, activation or markers of alveolar inflammation in humans with ALI/ARDS.

Studies investigating the in vitro effects of β agonists on neutrophil chemotaxis have produced conflicting results probably due to differences in the specific β2 agonist tested, the dose used and experimental conditions. Some studies have shown a reduction in neutrophil chemotaxis [28,29] whilst others report a biphasic response with increased neutrophil chemotaxis at low concentrations of β2 agonist, and a reduction in chemotaxis when higher concentrations were used[30]. The present study reports for the first time the physiological concentration of salbutamol achieved in the plasma following an intravenous infusion of salbutamol (15 µg kg⁻¹ hr⁻¹) in patients with ALI/ARDS is 10⁻⁶ M. At this concentration, no effect on neutrophil chemotaxis was observed. Supra-physiological concentrations (10⁻⁴ and 10⁻³M) however did reduce neutrophil chemotaxis through activation of the β receptor. Moreover, physiologically relevant concentrations of salbutamol had no effect on neutrophil viability or the rate of spontaneous apoptosis measured by morphology and annexin V / propidium iodine staining.

The mechanisms regulating pulmonary neutrophil sequestration have been well characterized. Pulmonary neutrophil sequestration occurs within minutes of exposure to an inflammatory insult[31,32]. The insult causes an increase in neutrophil stiffness and reduction in deformability[33] leading to sequestration into the pulmonary capillaries
followed by emigration into the alveolar space. The process of neutrophil emigration occurs by at least two differentially regulated pathways: CD 11/18 adhesion molecule interactions determine the response to gram negative organisms, IL-1a and phorbol 12-myristate 13-acetate (PMA), whereas gram positive organisms, hyperoxia and the complement anaphylatoxins (C5a) seem to induce neutrophil emigration through a CD 11/18 independent pathway[34].

Animal models of direct[13] and indirect lung injury[14] have shown that pre-treatment with intravenous β2 agonists reduces pulmonary neutrophil sequestration by 30%. In normal human volunteers, in a placebo controlled trial, treatment with 300 µg inhaled salbutamol prevented platelet-activating factor (PAF) induced pulmonary sequestration of radio-labeled neutrophils[35]. Pretreatment with salmeterol similarly inhibited LPS-induced neutrophil influx, neutrophil degranulation (myeloperoxidase), and tumor necrosis factor α release in human volunteers{Maris, 2005 982 /id}. Reduced neutrophil-endothelial adhesion, through the down regulation of neutrophil integrin adhesion molecules expression (CD11b/18) seen with β2 agonists may in part explain this finding[36].

In the present study intravenous salbutamol significantly increased circulating neutrophil count but had no apparent effect on alveolar neutrophil counts. The increase in circulating neutrophils induced by β agonists is thought to be due to detachment of cells from the marginating neutrophil pools, rather than mobilization of neutrophils from the bone marrow[37]. Neutrophil L-selectin expression may be important in this effect due to a dominant role in the initial slowing, margination and rolling behaviour of neutrophils over the endothelium at the post capillary venule in the systemic circulation[38]. The shedding of L-selectin from the neutrophil surface allows neutrophils to break free from the vascular endothelium and return to the circulation. The mobilisation of neutrophils was not however associated with any difference in the degree of organ dysfunction between the two groups[19].

In contrast to the systemic circulation, where neutrophil sequestration usually occurs at the post-capillary venule, neutrophil migration in the lung occurs at the pulmonary capillaries, without dependence on L-selectin mediated rolling[39]. In the present study we found a trend towards reduced L-selectin expression on circulating neutrophils in patients treated with salbutamol. When the neutrophils were maximally stimulated with FMLP, neutrophils primed by treatment with salbutamol showed a significantly greater reduction in L-selectin expression than placebo. This finding is consistent with the observation that isoproterenol reduces neutrophil L-selectin expression in human volunteers[40]. Enhanced L-selectin shedding, would increase neutrophil mobilisation from the systemic circulation and may explain the finding of increased circulating neutrophils. In contrast, the absence of a measurable effect of salbutamol upon integrin adhesion molecule expression relevant to pulmonary neutrophil emigration may explain the observed lack of effect on pulmonary neutrophil sequestration.

There are several other potential explanations for the absence of a measurable effect of salbutamol in vivo on alveolar neutrophil accumulation and inflammation. Firstly,
pulmonary neutrophil emigration occurs within minutes of exposure to the toxic insult[31,32] The previous studies which report a reduction in pulmonary neutrophil sequestration with β agonists, administered the drug prior to the inflammatory insult[13,14,18,35]. In this study, salbutamol was administered many hours after the initial insult, potentially therefore too late to have an effect on pulmonary neutrophil recruitment. Although the study was relatively small, it is similar in size to previous studies [13,14,18,35] and was powered to detect a treatment effect of similar magnitude to that observed in previous work. We cannot however exclude that a smaller treatment effect would not have been observed with greater numbers, although the clinical relevance of smaller changes is unclear. Furthermore, multiple signaling pathways enhance neutrophil emigration in ARDS which may differ depending on the aetiology of ARDS. This study was not powered to investigate the effects of salbutamol on neutrophilic inflammation due to different aetiologies and so cannot exclude an effect in some causes of ARDS. Finally, alveolar neutrophil recruitment was only studied at baseline and 4 days after the initiation of treatment (up to 6 days after the onset of ARDS). In both treatment and placebo groups, neutrophil counts were significantly lower at day 4 than at the point of initial recruitment. Therefore, we cannot exclude that salbutamol may have had an effect earlier in the course of the disease, which by virtue of the timing of alveolar sampling, may have been missed.

**Conclusion**

At the onset of ALI/ARDS, there is increased pulmonary neutrophil recruitment and activation in addition to positive correlations between IL-8, MPO and alveolar-capillary permeability suggesting an association between neutrophil activation and the development of lung injury. *In vitro*, physiological concentrations of salbutamol failed to demonstrate an effect on neutrophil chemotaxis, viability or apoptosis. Treating patients with ALI/ARDS with intravenous salbutamol increased the number of circulating neutrophils, but had no effect on alveolar neutrophil numbers or on neutrophil activation or alveolar inflammation. The beneficial effects of salbutamol reducing extravascular lung water seem unrelated to modulation of neutrophilic inflammatory pathways.

**Acknowledgement**

This study was supported by a grant from West Midlands Intensive Care Society

**Competing interests**

GDP, DFM and DT have received payment to attend scientific meetings and given talks for pharmaceutical companies that manufacture beta agonists (Astra Zeneca, GlaxoSmithKline). NN and FG have no competing interests.

**Ethical approval**

This study was approved by the East Birmingham Research Ethics Committee (Reference SJR/LMH/0522).
Figure legends

Figure 1: (i) Salbutamol $10^{-5}$ M and $10^{-4}$ M significantly reduced neutrophil chemotaxis towards the chemotracant stimulus fMLP. (ii) There was no effect on neutrophil chemokineses. (iii) The effect of salbutamol on neutrophil chemotaxis was blocked by incubation with $10^{-4}$M propranolol. (iv) Salbutamol ($10^{-6}$ and $10^{-4}$ M) had no effect on the rate of apoptosis at 18 hours (apoptosis determined by morphology (grey bars) and flow cytometry (black bars)). Data shown are mean (standard error) from 6 experiments.

Figure 2: (i) BAL Neutrophil count (ii) myeloperoxidase (iii) TNFα (iv) IL-8 and (v) IL-10 in normals, patients at risk from ALI/ARDS and patients with ALI/ARDS at baseline and after 4 days treatment with intravenous salbutamol.

Figure 3: (i) Circulating and (ii) alveolar neutrophil adhesion molecule (CD 11b, CD 18, CD 49) and activation maker (CD 64) expression in normal volunteers and patients with ALI/ARDS at baseline and after 4 days treatment with intravenous salbutamol. Data shown are mean (standard error).

Figure 4: Treatment with intravenous salbutamol significantly increased circulating neutrophil count ($P=0.004$). Data are displayed as mean (standard error) for salbutamol (squares / grey dotted line) and placebo (circles / solid line).

Figure 5: Effect of systemic treatment with intravenous salbutamol on resting and fMLP stimulated neutrophil L-selectin expression. Data shown are mean (standard error).
Reference List


Figure 1(i)

Chemotaxis (mm)

Control, $10^{-10}$M, $10^{-9}$M, $10^{-8}$M, $10^{-7}$M, $10^{-6}$M, $10^{-5}$M, $10^{-4}$M

Salbutamol

* $P < 0.05$ compared to control
Figure 1(ii)

![Bar graph showing the effect of different concentrations of Salbutamol on Chemokinesis (mm). The y-axis represents Chemokinesis in millimeters, and the x-axis represents different concentrations of Salbutamol (from Control to $10^{-10}$M to $10^{-4}$M). The error bars indicate the standard deviation. The graph shows no significant difference in Chemokinesis across all concentrations compared to the control. * P < 0.05 compared to control.
Figure 1(iii)

Chemotaxis (mm)

Control  10^{-5}M  10^{-4}M  10^{-5}M  10^{-4}M
Salbutamol Salbutamol + Propranolol 10^{-4}M

* P < 0.05 compared to control
Figure 1(iv)

![Bar chart showing percentage apoptosis for different Salbutamol concentrations (control, 10^-6 M, 10^-4 M). The x-axis represents Salbutamol concentrations, and the y-axis represents percentage apoptosis. The bars for each concentration group are close together, indicating similar levels of apoptosis across all groups.](image-url)
Figure 2(i)

Neutrophil count $\times 10^6$

- Normal
- At risk
- ARDS day 0
- ARDS day 4 Salb
- ARDS day 4 Placebo

P-values:
- Normal vs At risk: P=0.0001
- Normal vs ARDS day 0: P=0.0001
- Normal vs ARDS day 4 Salb: P=0.009
- Normal vs ARDS day 4 Placebo: P=0.449
Figure 2(ii)

Myeloperoxidase (mg ml⁻¹)

- Normal
- At risk
- ARDS day 0
- ARDS day 4 Salb
- ARDS day 4 Placebo

Significance levels:
- P = 0.001
- P = 0.0001
- P = 0.05
- P = 0.376
Figure 2(iv)

IL8 pg ml

Normal  At risk  ARDS day 0  ARDS day 4 Salb  ARDS day 4 Placebo

P=0.013
P=0.06
P=0.53
P=0.759
Figure 3

Blood neutrophil count x 10^9 ml

Time (days)
Figure 4(i)

The graph shows the median fluorescence intensity for different groups and conditions:

- **Cd11b**
  - Normal
  - ARDS d0
  - Salbutamol d4
  - Placebo d4

- **CD18**
  - Normal
  - ARDS d0
  - Salbutamol d4
  - Placebo d4

- **CD49**
  - Normal
  - ARDS d0
  - Salbutamol d4
  - Placebo d4

- **CD64**
  - Normal
  - ARDS d0
  - Salbutamol d4
  - Placebo d4
Figure 4(ii)

The bar graph shows the median fluorescence intensity for different markers:
- **Cd11b**
- **CD18**
- **CD49**
- **CD64**

The y-axis represents the median fluorescence intensity, ranging from 0 to 80. The x-axis lists the markers. The bars are color-coded:
- **ARDS d0**
- **Salbutamol d4**
- **Placebo d4**

The error bars indicate the variability in the data.
Figure 5

Median fluorescence intensity

<table>
<thead>
<tr>
<th>Condition</th>
<th>Placebo</th>
<th>Salbutamol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated</td>
<td>50</td>
<td>40</td>
</tr>
<tr>
<td>FMLP stimulated</td>
<td>40</td>
<td>20</td>
</tr>
</tbody>
</table>

P = 0.18
P = 0.038
In vitro and in vivo effects of salbutamol on neutrophil function in acute lung injury

Gavin D Perkins, Nazim Nathani, Danny F McAuley, Fang Gao and David R Thickett

Thorax published online August 23, 2006

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