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Salmeterol, a β2-receptor agonist, attenuates lipopolysaccharide-induced lung inflammation in mice

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Salmeterol, a β2-receptor agonist, was administered as a β2-receptor agonist in asthma and COPD because of its potent bronchodilatory properties (37). After pulmonary instillation of LPS, activated neutrophils adhere to the vascular endothelium and migrate toward the alveolar and interstitial spaces and the role of CD11b herein.

METHODS

Materials. LPS (Escherichia coli O55:B5) was obtained from Sigma (St. Louis, MO). Monoclonal purified rat anti-mouse CD11b (clone M1/70) and isotype-matched control antibody (purified rat IgG2b) were purchased from Pharmingen (San Diego, CA). Propranolol (1 mg/ml) was obtained from Astra Zeneca (Zoetermeer, The Netherlands).

Animals. Female C57BL/6 mice were purchased from Harlan Sprague-Dawley (Horst, The Netherlands). At the start of the experiments mice were 8 wk old and randomly distributed in groups of eight. All experiments were approved by the Animal Care and Use Committee of the Academic Medical Center (Amsterdam, the Netherlands).

Experimental procedure. All compounds were administered in a nonblinded fashion. LPS was administered intranasally according to previously described methods (22). LPS was diluted in 0.9% sterile
saline (50 μl) to a final concentration of 200 μg/ml (10 μg per mouse). Control mice received 50 μl of sterile saline. Mice were pretreated (at −30 min) with either salmeterol (0.005–5 mg/kg, dissolved in 200 μl of sterile saline) or sterile saline (200 μl) intraperitoneally, which was repeated after 12 h. In some experiments these treatments were combined with intravenous injection of anti-CD11b or control antibody (2 mg/kg) at −30 min. Propranolol (10 mg/kg) was injected intraperitoneally 30 min before salmeterol injection and repeated every 2 h to block β-adrenergceptors (1). Inhalation of saline (1 ml) or salmeterol (1 mg/ml or 2.4 mM) (3) was achieved by attaching a plastic chamber (5 l) containing eight conscious mice to an Aeroneb pro-nebulizer (Medicare, Uitgeest, the Netherlands). Salmeterol was aerosolized (airflow 5 l/min) for 3 min, after which mice were maintained in the chamber for an additional 7 min.

Bronchoalveolar lavage. Bronchoalveolar lavage (BAL) was performed as described (22). No significant differences in returned lavage volume (>80%) were measured between the groups and time points studied. The supernatant was collected and stored at −80°C until used for cytokine and chemokine measurements, and the pellet was resuspended in fluorescence-activated cell sorting (FACS) buffer (PBS supplemented with 0.5% BSA, 0.01% NaN 3 , and 0.35 mM EDTA). Total cell numbers were counted in each sample with a Burker-Turk chamber. Bronchoalveolar lavage fluid (BALF) differential counts were done on cytopsin preparations stained with Giemsa.

Preparation of lung cell suspensions. After the BAL procedure, lungs were removed and lung cell suspensions were obtained with an automated disaggregation device (Medimachine system; Dako, Glostrup, Denmark) as described (22). Neutrophil numbers were determined as described for BALF.

Histological analyses. In a separate experiment, lungs for histological examination were collected at 24 h after LPS administration, fixed in 4% formalin, and embedded in paraffin. Sections of 4 μm were stained with hematoxylin and eosin. Granulocyte staining was done with fluorescein isothiocyanate-labeled anti-mouse Ly-6G MAb (Pharmingen) (28).

FACS analysis. Expression of CD11b on neutrophils in BALF and lung cell suspensions was determined by flow cytometric analysis as described previously, using rat anti-mouse CD11b PE and Gr-1 FITC (Ly-6G) antibodies (Pharmingen) (28).

ELISAs. Tumor necrosis factor (TNF)-α, macrophage inflammatory protein (MIP)-2, and cytokine-induced neutrophil chemotactant (KC) were measured by specific ELISAs according to the manufacturer’s instructions (all R&D Systems, Minneapolis, MN). The detection limits were 5.1 pg/ml (TNF-α), 1.5 pg/ml (MIP-2), and 2.0 pg/ml (KC). The coefficient of variation of all ELISAs was <10%.

Statistical analysis. Values are expressed as means ± SE. Differences between groups were analyzed by Mann-Whitney U-test, and dose-response data were analyzed by one-way ANOVA. P < 0.05 was considered statistically significant.

RESULTS

Salmeterol inhibits LPS-induced neutrophil recruitment. Intranasal administration of LPS resulted in a profound rise in the number of total cells recovered from BALF 6 h postchallenge (P < 0.05 vs. control, Table 1, n = 8 per group). The LPS-induced increase in total cell counts was primarily due to a rise in the number of neutrophils (Table 1 and Fig. 1A; n = 8, respectively, 16 per group). Salmeterol dose-dependently inhibited this recruitment of neutrophils (Table 1), with the strongest effect observed at the highest dose tested (5 mg/kg). Further experiments were done with this salmeterol dose.

LPS administration also elicited a marked increase in the number of neutrophils in lung tissue, as reflected by rises in neutrophil counts in total lung cell suspensions (Fig. 1B). Salmeterol strongly diminished this response (P < 0.05 vs. LPS only, n = 16 per group). Of note, the effect of salmeterol on neutrophil accumulation in BALF and lungs shown in Fig. 1 was studied in two independent experiments (both with eight mice per group), since we wished to confirm our first finding (with n = 8 per group) that salmeterol inhibits neutrophil influx in this model. As both experiments yielded similar results, the data were pooled. The inhibitory effect of salmeterol on neutrophil migration was further confirmed by histological analysis. Twenty-four hours after LPS administration a clear interstitial influx of granulocytes was observed (Fig. 2B). This granulocytic infiltration was strongly reduced when mice were pretreated with salmeterol (Fig. 2C). These data were confirmed by immunostaining for granulocytes (data not shown).

Effect of salmeterol on chemokine and cytokine levels in BALF. CXC chemokines play an important role in the recruitment of neutrophils to sites of inflammation, including the lung (30, 40, 41). Therefore, we considered it of interest to assess the effect of salmeterol on LPS-induced release of MIP-2 and KC into BALF (Table 2, n = 8 per group at each time point). Salmeterol reduced the early release of KC into BALF after LPS administration (6 h, P < 0.05 vs. LPS only), while enhancing KC production at 24 h (P < 0.05 vs. LPS only). MIP-2 concentrations increased significantly 6 h after LPS but were not altered by pretreatment with salmeterol. These modest effects of salmeterol on chemokine levels contrasted with a strong inhibition of LPS-induced TNF-α release into BALF, revealing an ~10-fold reduction in peak TNF-α concentrations 6 h postchallenge (Table 2, P < 0.05 vs. LPS only, n = 8 per group).

Salmeterol reduces CD11b expression on neutrophils. The accumulation of neutrophils in the airways in response to local administration of LPS requires β-integrins, of which CD11b/CD18 is an example (12, 44). We were therefore interested in the effect of salmeterol on CD11b expression on neutrophils in the pulmonary compartment early after the administration of LPS. At 6 h post-LPS, CD11b expression was reduced in salmeterol-treated mice compared with mice treated with LPS.

Table 1. Dose-dependent effect of salmeterol on cellular composition of BALF

<table>
<thead>
<tr>
<th>LPS in</th>
<th>−</th>
<th>+</th>
<th>+ 0.05</th>
<th>+ 0.5</th>
<th>+ 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmeterol ip. mg/kg</td>
<td>0</td>
<td>0</td>
<td>0.005</td>
<td>0.05</td>
<td>0.5</td>
</tr>
<tr>
<td>TCC (×10^6/ml BALF)</td>
<td>2.0±0.8</td>
<td>22.5±8.5*</td>
<td>19.7±7.0*</td>
<td>16.3±5.9*</td>
<td>9.5±3.6*,†</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>0.01±0.004</td>
<td>21.2±8.0*</td>
<td>18.6±6.6*</td>
<td>14.9±5.4*</td>
<td>9.1±3.8*,†</td>
</tr>
<tr>
<td>Macrophages</td>
<td>2.0±0.8</td>
<td>1.22±0.41</td>
<td>1.1±0.4</td>
<td>1.4±0.5</td>
<td>1.41±0.51</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>0.009±0.007</td>
<td>0.08±0.03</td>
<td>0.032±0.011</td>
<td>0.036±0.013</td>
<td>0.017±0.006</td>
</tr>
</tbody>
</table>

Data are means ± SE of 8 mice per group. Mice were intranasally inoculated with LPS (10 μg) at t = 0. Salmeterol (0.005, 0.05, 0.5, and 5 mg/kg) or saline was administered intraperitoneally at t = −30 min. BALF, bronchoalveolar lavage fluid; TCC, total cell count. *P < 0.05 vs. saline/saline and †P < 0.05 vs. LPS/saline.
only, both on neutrophils recovered from BALF and on neutrophils recovered from whole lung cell suspensions (both \( P < 0.05 \) for the difference between LPS/salmeterol and LPS only, \( n = 8 \) per group, Fig. 3).

Role of CD11b in salmeterol effect on neutrophil recruitment. Because CD11b has been implicated in LPS-induced neutrophil recruitment to the lungs, salmeterol could inhibit neutrophil influx by reducing CD11b expression on these cells. To evaluate this possibility, we determined the effect of salmeterol on LPS-induced neutrophil influx in BALF in mice treated with an anti-CD11b MAb or an irrelevant control antibody (Fig. 4, \( n = 8 \) per group). In mice treated with the control antibody, salmeterol strongly reduced the number of neutrophils recovered from BALF 6 h after LPS administration (\( P < 0.05 \) vs. LPS only), confirming the results presented in Fig. 1. As expected, anti-CD11b treatment also profoundly inhibited neutrophil recruitment into BALF (\( P < 0.05 \) vs. control antibody). Combining anti-CD11b and salmeterol almost completely prevented these cells from entering the alveolar compartment (\( P < 0.05 \) vs. anti-CD11b only). Treatment with anti-CD11b did not influence the concentration of KC, MIP-2, or TNF-\( \alpha \) in BALF of mice treated with LPS with or without salmeterol (data not shown). These experiments confirmed the strong inhibitory effect of salmeterol on LPS-induced TNF-\( \alpha \) release in BALF (data not shown).

Role of the \( \beta \)-adrenoceptor in inhibition of neutrophil influx and TNF-\( \alpha \) production by salmeterol. To determine whether salmeterol-induced inhibition of neutrophilic influx and TNF-\( \alpha \) production was dependent on an effect on \( \beta \)-adrenergic rece-
tors, we treated mice with salmeterol (5 mg/kg) with or without the β-receptor antagonist propranolol (10 mg/kg, n = 8 per group). Propranolol only partially reversed the effect of salmeterol on neutrophil influx after LPS inhalation (Fig. 5A, P < 0.05 for the difference between salmeterol and saline and for the difference between salmeterol-propranolol and saline). In contrast, TNF-α production was normalized by pretreatment with propranolol to levels seen in mice challenged with LPS only (Fig. 5B, P < 0.05 for the difference between salmeterol and saline and for the difference between salmeterol and salmeterol-propranolol). These data suggest that salmeterol modulates LPS-induced pulmonary inflammation either via the β-adrenoreceptor (TNF-α) or via an effect bypassing this receptor (neutrophil influx).

Effects of aerosolized salmeterol on LPS-induced pulmonary inflammation. Next we assessed the effect of salmeterol administered via the Airways on neutrophil influx and TNF-α production in mice challenged with LPS intranasally (Fig. 6, n = 8 group). Inhaled nebulized salmeterol exerted effects on these inflammatory responses that were similar to those of intraperitoneally administered salmeterol. Both neutrophil count (Fig. 6A) and TNF-α production (Fig. 6B) were significantly decreased by nebulized and inhaled salmeterol (P < 0.05 vs. LPS only).

DISCUSSION

In our studies we demonstrate that salmeterol strongly reduced the accumulation of neutrophils in the pulmonary com-

Table 2. Effect of salmeterol on KC, MIP-2, and TNF-α concentrations in BALF

<table>
<thead>
<tr>
<th>Chemokine/Cytokine</th>
<th>LPS/Saline</th>
<th>LPS/Salmeterol</th>
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<tbody>
<tr>
<td>KC, pg/ml</td>
<td>6 h</td>
<td>24 h</td>
</tr>
<tr>
<td>MIP-2, pg/ml</td>
<td>6 h</td>
<td>24 h</td>
</tr>
<tr>
<td>TNF-α, pg/ml</td>
<td>6 h</td>
<td>24 h</td>
</tr>
</tbody>
</table>

Data are means ± SE of 8 mice per group at each time point. Mice were intranasally inoculated with LPS (10 μg) at t = 0, salmeterol (5 mg/kg) or saline was administered at t = −30 min and t = 12 h. KC, cytokine-induced neutrophil chemoattractant; MIP, macrophage inflammatory protein. *P < 0.05 vs. LPS/saline at the corresponding time point.

Fig. 4. Salmeterol further increases the inhibitory effect of anti-CD11b on LPS-induced neutrophil influx. Salmeterol (5 mg/kg, open bars) or saline (solid bars) was given intraperitoneally 30 min before intranasal administration of LPS (10 μg/mouse); anti-CD11b (2 mg/kg) or control antibody was given intravenously 30 min before LPS administration. Data are means ± SE of 8 mice per group (6 h after LPS administration). *P < 0.05 vs. LPS/saline without anti-CD11b; +P < 0.05 vs. LPS/salmeterol without anti-CD11b; ++P < 0.05 vs. LPS/salmeterol without anti-CD11b and LPS/saline with anti-CD11b.

Fig. 5. The effect of salmeterol on neutrophil influx is β-receptor independent, whereas TNF-α is inhibited in a receptor-dependent fashion. Saline (solid bars), salmeterol (5 mg/kg, open bars), or salmeterol and propranolol (10 mg/kg, hatched bars) were given intraperitoneally before intranasal administration of LPS (10 μg/mouse). Saline and salmeterol were given intraperitoneally 30 min before LPS; propranolol was given intraperitoneally 1 h before LPS and at 1, 3, and 5 h after LPS. Data are means ± SE of 8 mice per group (6 h after LPS administration). A: neutrophil counts in BALF; B: TNF-α in BALF. *P < 0.05 vs. LPS/saline; +P < 0.05 vs. propranolol-salmeterol.
inhibit the expression of CD11b/CD18 on the surface of neutrophils (11), which is relevant in the context of LPS-induced neutrophil recruitment to the lungs, considering that this inflammatory response is dependent on neutrophil CD11b/CD18 (12, 44). In accordance with this, treatment with dibutyryl cAMP or rolipram, which elevates intracellular cAMP levels by different mechanisms, inhibited the influx of neutrophils in BALF of mice exposed to LPS via the airways (14, 15). These combined in vitro and in vivo data led us to hypothesize that salmeterol would inhibit LPS-induced neutrophil recruitment to the lung by a mechanism that involves inhibition of CD11b expression by neutrophils. The experiments described here provide some evidence that, indeed, this is the case. Mice treated with salmeterol displayed markedly less neutrophil numbers in their BALF and lung tissue after intranasal delivery of LPS, and these lung neutrophils expressed significantly less CD11b at their surface. Inhibition of CD11b function in another way, namely by administration of a blocking antibody, also reduced neutrophil influx upon LPS challenge. Importantly, salmeterol modestly enhanced this anti-CD11b-mediated inhibitory effect. Together, these data suggest that salmeterol attenuates the migration of neutrophils to the pulmonary compartment upon exposure to LPS via a mechanism that may be in part but not exclusively mediated by a reduction of neutrophil CD11b expression by this β2-receptor agonist. Of note, in vitro studies have indicated that stimulation of β2-adrenergic receptors on neutrophils may have a bimodal effect on chemotaxis, i.e., terbutaline increased neutrophil chemotaxis to formyl-methionyl-leucyl-phenylalanine at low concentrations, whereas it inhibited neutrophil chemotaxis at higher concentrations (8). We did not find evidence for such a bimodal effect of salmeterol in the current investigation, although doses <5 μg/kg (which is ~0.1 μg per mouse) were not investigated (Table 1). Our data confirm and extend previous observations in guinea pig lungs, in which formoterol and salmeterol inhibited neutrophil accumulation upon LPS administration (45). Previously, other β2-receptor agonists have been studied in inflammatory models and found to be effective in attenuating neutrophil influx. In a model of carrageenan-induced pleurisy in mice, salbutamol strongly inhibited neutrophil migration (29), whereas in a rat model of endotoxemia both liver and lung showed only minimal neutrophil influx in animals treated with terbutaline 30 min before LPS challenge (46). These data show that different β2-receptor agonists are able to interfere with neutrophil migration in vivo.

Theoretically, one additional mechanism by which salmeterol could influence neutrophil migration is by an effect on the local concentrations of CXC chemokines, of which MIP-2 and KC are prominent examples in rodents (26). Indeed, this subfamily of small chemotactic proteins plays a major role in selectively attracting neutrophils to sites of inflammation, including the lung (30, 40, 41). In the current investigation, salmeterol treatment caused a modest but not significant reduction in MIP-2, whereas KC production in BALF was inhibited early (6 h) and enhanced at a later time point (24 h) post-LPS. Although we do not have a firm explanation for this finding, it should be noted that human monocytes released more IL-8, the prototypic human CXC chemokine, upon stimulation with LPS in the presence of β-receptor agonists (21, 43). Earlier studies have documented the anti-inflammatory effects of β-receptor stimulation on the cytokine network. Indeed, β-receptor agonists potently inhibited the production of tumor necrosis factor (TNF-α) in vitro (3). The results of the present study lend support to this observation.

Fig. 6. Inhibition of neutrophil influx and TNF-α release by nebulized salmeterol administered via the airways. Saline (solid bars) or salmeterol (1 mg/ml, open bars) was nebulized and inhaled by mice 30 min before intranasal administration of LPS (10 μg/mouse). Data are means ± SE of 8 mice per group (6 h after LPS administration). A: neutrophil counts in BALF; B: TNF-α in BALF. ∗P < 0.05 vs. LPS/saline.
of TNF-α by monocytes in vitro and in mice and normal humans in vivo (5, 32, 33, 38, 42, 46). In addition, β-receptor stimulation resulted in an enhanced release of the anti-inflammatory cytokine IL-10 in these models (36, 38, 42, 46). In mice, salmeterol attenuated TNF-α release after systemic administration of LPS, which was associated with an improved survival in galactosamine-sensitized mice (32). Here we add to this finding that salmeterol potently inhibited the release of TNF-α into BALF upon local exposure to LPS. An increase in IL-10 levels in BALF upon administration of salmeterol could not be demonstrated (data not shown). It is unlikely that the reduced BALF concentrations of TNF-α contributed to a significant extent to the strong inhibitory effect of salmeterol on LPS-induced neutrophil recruitment to the lung, since this response is largely TNF-α independent (24, 34).

Salmeterol is a potent and selective β2-adrenoceptor agonist and the effects of which at least in part can be blocked by β-receptor antagonists like propranolol (3). Of note, β-blockers have been shown to be ineffective in reversing the anti-inflammatory effects of salmeterol on, for instance, airway macrophages and blood monocytes (2), mast cells (9), and eosinophils (13). In this study we show that the effect of salmeterol on neutrophil influx is likely to be largely receptor independent, since propranolol had only a marginal influence on this salmeterol effect. In contrast, inhibition of TNF-α release by salmeterol was completely reversible by repeated administration of propranolol and therefore β-adrenoceptor dependent. In line with this is the observation that inhibition of neutrophilic influx was achieved at higher doses of salmeterol than reduction in TNF-α levels. This discrepancy may be explained by the presence of an alternative binding site (10, 16) that cannot be blocked by propranolol and is activated by higher doses of β2-adrenoceptor agonists. This exosite has been shown to interact with the lipophilic tail of salmeterol, which is able to modulate signal transduction probably by interfering with G protein function (31). Together, these data suggest that the β2-adrenoceptor dependency of salmeterol effects may relate to the specific inflammatory effect studied.

In conclusion, we show here that salmeterol exerts dose-dependent anti-inflammatory effects in the lungs of mice exposed to LPS via the airways. Salmeterol diminished LPS-induced accumulation of neutrophils in the lungs, which is associated with a profound reduction in pulmonary TNF-α concentrations. Further studies are warranted to confirm these effects in humans.

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REFERENCES


