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LPS-induced bronchoalveolar neutrophilia; effects of salmeterol treatment

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Summary Salmeterol has earlier been reported to have immune modulating effects on Lipopolysaccharide (LPS)-induced neutrophilic inflammation in rodents. The aim of this study was to explore whether 3 weeks regular treatment with inhaled Salmeterol would have a protective effect against neutrophilia, following an LPS inhalation as assessed by bronchoscopy with bronchial wash (BW) and bronchoalveolar lavage (BAL) in healthy subjects. Fifteen volunteers all underwent bronchoscopies with bronchial wash and BAL on three occasions, each being 3 h after inhalation provocation. The initial inhalation was with saline (dilutant) as a reference and the two following with LPS 50 μg diluted in saline. After the saline inhalation the subjects were randomised to treatment with Salmeterol 50 μg twice daily and placebo in a double-blind double-dummy crossover design. Compared to saline inhalation, the LPS inhalations resulted in a two-fold increase in neutrophils both in BW and BAL, respectively ($P \leqslant 0.01$). The neutrophilia was present irrespective of the LPS inhalation was preceded by placebo or Salmeterol. This experimental study could not confirm any modulating effect of Salmeterol on LPS-induced airway neutrophilia.

Introduction

Lipopolysaccarid (LPS) endotoxin, is normally found in the cell walls of Gram-negative bacteria. Apart from mediating effects during G^- bacterial infections, LPS also plays a role for respiratory health following inhalation of organic dust in cotton mills, poultry houses, swine confinement buildings, sawmills and many other places. Workers in these enviroments have an increased prevalence of airway symptoms, predominantly cough and phlegm[1] but wheeze and chest tightness have also been observed.[2,3] Several studies have shown a chronic airway inflammation as assessed by bronchoalveolar lavage (BAL) with an increased number of neutrophils in the airways of swine farmers[4–6] as well as in farmers inhaling grain dust.[7] In experimental studies in man, inhaled LPS has been found to induce fever and chills, combined by transient bronchoconstriction. Bronchial hyperreactivity and a decrease in carbon monoxide transfer factor have also been shown.[8–10] The local airway effects of inhaling pure LPS have been evaluated experimentally with bronchoscopy technique in one previous study. In that study healthy non-smoking subjects were investigated with bronchoalveolar lavage (BAL) after inhalation provocation with 100 μg LPS.[11] Compared with inhaled saline, LPS-induced

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prominent airway inflammation with approximately 100-fold increase in neutrophils and a tripling in number of lymphocytes in BAL fluid.

Salmeterol, an inhaled long-acting β_2-agonist has in several clinical studies been shown to give excellent asthma control when added to inhaled corticosteroids. We recently investigated whether the high degree of asthma control seen with inhaled salmeterol was only due to its bronchodilating capacity or modulation of asthmatic airway of inflammation. We demonstrated salmeterol capable of improving asthma control in parallel with suppression of the airway inflammatory condition in particular with regard to mast cell effects.[12] A suppressive effect of salmeterol has been suggested both in animal and in vitro studies.[13–16]

In two animal studies, Whelan et al. demonstrated salmeterol to inhibit LPS-induced neutrophil accumulation in guinea pig lung.[13,14] Salmeterol has been reported to suppress LPS-induced cytokine production and to have a protective effect in a galactosamine/LPS model of endotoxin shock.[15] Furthermore, endothelial permeability has been shown to be contracted by pretreatment of salmeterol in in vitro models.[16]

The aim of the study was to evaluate whether regular salmeterol treatment in healthy subjects would have a protective effect against airway neutrophilia induced by LPS inhalation. If such an effect could be confirmed, this would broaden the base for understanding the capability of salmeterol to interact with inflammatory airway events. Additionally, it would give support for a potential treatment effect in individuals experiencing adverse respiratory effects from inhaled LPS from organic dust in their work environments.

Methods

Subjects

Fifteen healthy non-smoking volunteers, seven women, mean age 25 years (range 21–38 years) with no history of allergy or asthma participated in the sudy. No upper or lower respiratory infection was permitted within at least 4 weeks before the start as well as during the study.

Study design

After a screening visit the volunteers all underwent inhalations with saline alone, serving as a reference for subsequent inhalations, when saline was used as a diluent. After the saline inhalation the subjects were randomised to treatment with salmeterol 50 μg and placebo twice daily, in a double-blind double-dummy crossover design. During the end of each of the 3-week treatment periods an inhalation with LPS 50 μg diluted in saline was given. The interval between the inhalation challenges was at least 3 weeks. Three hours after each of the three inhalation challenges a bronchoscopy with airway sampling was performed. The time point was chosen to reflect an early established LPS-induced inflammation. The last dose of the 3 weeks treatment of salmeterol/placebo was given in the morning 1–2 h before the LPS challenges. The Ethics Committee of Umeå University had approved the study and each subject gave written informed consent.

LPS-inhalation

LPS-exposure was performed with LPS from *Escherichia coli* (*E.coli* serotype 026:B6. prepared using a phenol extraction procedure, Sigma Chemical. St. Louis. USA). One mg was suspended in 10 ml NaCl to a dilution of 100 μg/ml. This solution was then further diluted to a final concentration of 25 μg/ml and 2 ml (50 μg) was inhaled for a period of 10-15 min from a Pari–Boy nebulizer. The dose was chosen after previous experiments to give a clearly defined neutrophil inflammation in the bronchi as described in Sandström. et al.[11] In previous experiments using the Pari Boy nebulizer it has been shown to produce a particle size with a mass median diameter of 3.5 μm (range 0.5–5.5 μm). The dose delivered to the lower airways has been calculated to be approximately 50%.[8] The initial reference inhalation consisted of 2 ml of the saline dilutant alone.

Lung function test

A standard dry bellows spirometer (Vitalograph) was used throughout the study to measure forced expiratory volume at 1 s (FEV_1). The highest of at least three measurements was recorded. FEV_1 was measured before inhalation (0 h) and before bronchoscopy (3 h).

Fiberoptic bronchoscopy

The bronchoscopy premedication consisted of atropine (0.5–1.0 mg) s.c. Lidocaine was used for topical anaesthesia. The subjects were examined in supine position using an Olympus BF T10 or BF T20 fiberoptic bronchoscope (Olympus Optical Co.

Tokyo. Japan). After wedging the bronchoscope in a segmental bronchus of the lingula or the middle lobe a bronchial wash and a BAL were performed. For the bronchial wash two 20 ml aliquots of sterile phosphate buffered saline was installed and gently suctioned back followed by the BAL in 3 aliquots of 60 ml each. The saline was prewarmed to 37°C.

The BAL fluid was kept on ice during the transport to the laboratory. After filtration through a nylon filter (pore diameter 100 μm. Syntab Product AB. Malmö. Sweden) the fluid was centrifuged at 400 G for 15 min at 4°C. The cells were immediately processed and the supernatant frozen at −80°C. The cells were resuspended in a balanced salt solution adjusted to pH 7.0 to a concentration of 10^6 inflammatory cells/ml. The total number of cells was counted with a Bürker chamber. Slides for cytological studies were made using a Cytospin 3^R (Shandon Southern Instruments Inc. Sewickly, PA, USA) at 1000 rpm for 5 min. The non-epithelial cells on each slide were stained with May–Grünwald Giemsa before differential counting which was based on at least 400 cells per slide.

Albumin and total protein were analysed at the Department of Clinical Chemistry at the University Hospital.

Data analysis

The nonparametric Wilcoxon's rank sum test was used to compare changes in BAL and BW parameters after saline inhalation (baseline), with LPS exposure preceded by salmeterol (LPS + Salm) or by placebo treatment (LPS + Placebo). FEV_1 was analysed with the same statistical method to identify a potentional decrease in lung function after LPS inhalation.

A P-value < 0.05 was considered statistically significant.

Results

Data from the bronchoalvelolar lavage is given in Table 1. LPS inhalation after treatment with either salmeterol or placebo resulted in a significant increase in both the percentage and the total amount of neutrophils in the bronchial wash and the BAL compared with the saline inhalation at the study start. The percentage of neutrophils increased in the bronchial wash from 16.5 ± 13.5 after saline to 41.2 ± 17.6 after LPS + Salm, ($P < 0.001$ vs. saline) and 36.5 ± 14.3 after LPS + Placebo, ($P < 0.01$ vs.saline). In the BAL similar results was found. The percentage of neutrophils in BAL increased significantly from 2.8 ± 1.2 to 9.3 ± 8.8 after LPS + Salm, ($P < 0.01$ vs. saline) and to 6.8 ± 3.8 after LPS + placebo, ($P < 0.01$ vs. saline). There was no signficant differences between pre-treatment with salmeterol and placebo. The increase in neutrophils was paralleled by a decrease in the percentage of macrophages. No effects on total cells, lymphocytes, albumin or total protein were detected after LPS inhalation, compared with saline.

The morning FEV_1 increased after 3 weeks of inhaled salmeterol treatment compared with after placebo, representing a small but significant bronchodilation in the study population (Table 2). No decrease in FEV_1 was detected after LPS inhalation.

Discussion

In this study LPS inhalation in healthy volunteers was demonstrated to induce a significant increase in airway neutrophils as reflected in both BW and BAL. This increase was seen irrespectively of whether three weeks pre-treatment with inhaled salmeterol 50 μg twice daily or placebo had been given or not.

The benefit of adding a long-acting β_2-agonist rather than doubling the dose of inhaled corticosteroid has been well established in asthma both when assessing pulmonary function and symtoms.[17,18] In two recent studies with bronchial biopsies in asthmatics, salmeterol treatment was associated with suppressive effects on eosinophils and mast cells.[12,19] We have also demonstrated similar effects of another long-acting inhaled β_2-agonist, formoterol, on steroid naïve asthmatic subjects.[20] Salmeterol has also been shown to have the capability to reduce neutrophils in the bronchial mucosa of mild asthmatic subjects, accompanied by suppressed myeloperoxidase and E-selectin levels in serum.[21] The suppressive effects on asthmatic airway inflammation may represent links to favourable clinical effects of long-acting β_2-agonists, and the underlying mechanisms mediating these effects are currently subject for further studies. The observations, of additional antiinflammatory effects on LPS-induced inflammation by salmeterol treatment in animal and in-vitro models, triggered the question on whether similar effects would be present in humans exposed to LPS.

The present study was designed to produce a less pronounced neutrophil influx after LPS inhalation compared to our preceding study.[11] After pilot experiments the dose of LPS was decreased to 50 μg

Table 1 Bronchoalveolar lavage (BAL) and bronchial wash (BW) components after saline inhalation, and after LPS inhalation preceded by 3 weeks treatment with inhaled salmeterol or placebo.

	After saline inhalation at study start	After LPS + saline inhalation, during salmeterol treatment	After LPS + saline inhalation, during placebo treatment
Total cells $\times 10^9$/l			
BW	14.3 ± 11.3	12.7 ± 11.3	15.2 ± 14.2
BAL	10.7 ± 3.1	11.5 ± 5.4	12.2 ± 4.4
Neutrophils %			
BW	16.5 ± 13.5	41.2 ± 17.6***	36.5 ± 14.3**
BAL	2.8 ± 1.2	9.3 ± 8.8**	6.8 ± 3.8**
Neutrophils $\times 10^9$/l			
BW	1.9 ± 1.2	4.4 ± 3.0**	5.6 ± 6.5***
BAL	0.3 ± 0.2	1.0 ± 0.8**	0.9 ± 0.5**
Lymphocytes %			
BW	3.3 ± 3.0	2.2 ± 2.0	2.6 ± 1.8
BAL	12.5 ± 4.4	10.5 ± 4.8	14.7 ± 7.0
Lymphocytes $\times 10^9$/l			
BW	0.6 ± 0.9	0.3 ± 0.3	0.4 ± 0.4
BAL	1.4 ± 0.7	1.3 ± 1.0	1.9 ± 1.2
Macrophages %			
BW	79.7 ± 13.1	56.4 ± 17.1***	60.6 ± 14.2**
BAL	84.3 ± 4.5	80.1 ± 8.7	78.1 ± 9.0*
Macrophages $\times 10^9$/l			
BW	11.6 ± 10.0	7.9 ± 9.9	9.2 ± 8.2
BAL	9.0 ± 2.5	9.3 ± 4.7	9.4 ± 3.4
Eosinophils %			
BW	0.33 ± 0.59	0.24 ± 0.39	0.20 ± 0.51
BAL	0.34 ± 0.54	0.16 ± 0.15	0.29 ± 0.43
Albumin mg/l†			
BW	34.7 ± 22.8	28.4 ± 19.7	38.6 ± 21.5
BAL	48.0 ± 32.0	42.4 ± 24.1	51.2 ± 22.0
Total protein mg/l†			
BW	45.7 ± 30.9	39.9 ± 27.1	51.6 ± 33.8
BAL	49.3 ± 36.1	45.8 ± 30.9	55.0 ± 27.4

*$P<0.05$ **$P<0.01$ ***$P<0.001$ compared with saline inhalation.
Data are given as mean ± SD. $n=15$ except †indicating $n=14$.

Table 2 FEV_1 at 0 and 3 h after inhalation. Inhalation with saline at start and with LPS diluted in saline after pre treatment with salmeterol or placebo.

FEV_1 (L)†		Before inhalation time 0 h		Before bronchoscopy time 3 h	
		No		No	
At start	Saline	(14)	4.26 ± 0.96	(10)	3.90 ± 0.64
Salm	LPS + Saline	(15)	4.40 ± 0.86*	(15)	4.41 ± 0.89
Placebo	LPS + Saline	(15)	4.19 ± 0.90	(13)	4.30 ± 0.88

†Values are expressed as mean ± SD.
*$P=0.003$ salmeterol vs placebo.

compared with the previously used 100 μg. In the present study the neutrophil numbers were doubled in BW and tripled in BAL after LPS inhalation compared to after saline inhalation. Despite this moderate but significant LPS induced increase in neutrophils salmeterol was not demonstrated to inhibit this migration of neutrophils. The differing outcome in the present study in human volunteers compared to the in vitro and animal studies can have several explanations.

Firstly, there could be species differences and differing functions in in vitro systems, that could cause contrasting results.

Secondly, the salmeterol dose. In animal studies, relatively high doses have been given per kg to small animals, in contrast to the standard 50 μg twice daily given to humans.[12,13]

Thirdly, the effect of a single dose of salmeterol could differ from repeated doses. In a study by Giannini et al.[22] the protective effect of a single dose of salmeterol, against allergen induced early asthmatic reaction (EAR), was lost after regular treatment with salmeterol for 1 week.

Fourthly, the effect of salmeterol could potentially differ between healthy subjects and those with allergic asthma and/or rhinitis. Salmeterol has recently been demonstrated to have the capability to reduce neutrophils in the bronchial mucosa of mild asthmatic subjects, which supports the possibility for differentiating effects.[21] It could be speculated that the mechanisms for neutrophil recruitment could be at least partly different in a chronic disease as asthma as compared with LPS challenge. In addition to the suppression of asthmatic airway inflammation, salmeterol has also been shown to reduce vascular permeability in subjects with allergic rhinitis.[23]

In our previous LPS study an increase in lymphocytes was shown in lavage fluid.[11] This could not be seen in the current samples. The reason for this could be the lower dose of LPS used in the present study or possibly due to an earlier time point for the bronchoscopy, 3 h after inhalation. No decrease in lung function was detected after LPS inhalation. This is in contrast to some other studies[8,10] and probably reflects the outcome of the lower provocative dose of LPS given. Instead a modest and not clincially significant increase in FEV_1 was detected after 3 weeks treatment with salmeterol compared with placebo. This increase in lung function after salmeterol treatment reflects a minor bronchodilation by the β_2-agonist in the investigted non-asthmatic subjects This isn't a surprising result given the subjects are healthy volunteers.

It is concluded that with the present experimental model and study design no protective effect could be identified by 3 weeks treatment with inhaled salmeterol 50 μg twice daily, as compared to placebo, against LPS induced airway inflammation, in a population of healthy subjects.

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