In Vitro Effect of β₂-Agonists on Bacterial Killing and Superoxide Anion (O₂⁻) Release From Alveolar Macrophages of Patients With Chronic Bronchitis*

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A new class of long-acting β₂-adrenoceptor agonists has been studied in the last few years. Apparently, they display an important anti-inflammatory activity with an inhibition of different cellular functions. This study was carried out to compare a long-acting β₂-agonist, formoterol, with a conventional short-acting one, salbutamol, on the release of superoxide anion (O₂⁻) and bacterial killing by alveolar macrophages obtained with bronchoalveolar lavage (BAL) from 20 patients with chronic bronchitis. The O₂⁻ production in basal conditions was not affected by β₂-agonists. On the contrary, after phagocytosis of opsonized zymosan 10⁻⁴ M formoterol significantly affected the phagocytic index (difference between stimulated and basal O₂⁻ release): 7.9 ± 2.0 nM O₂⁻/10⁶ AM/10 min vs 16.8 ± 2.5, p<0.0007. Bacterial killing was inhibited by the two drugs in a dose-dependent way, but the effect of formoterol was more evident than that of salbutamol. After blocking β₂-receptors with propranolol, we observed a prevention of the β₂-agonist effects on both O₂⁻ release and bacterial killing. The inhibition of the alveolar macrophage functions considered in this study is evident for both β₂-agonists, but it is significantly more pronounced for formoterol. Our data can be interpreted as one possible mechanism of the anti-inflammatory effect described for long-acting β₂-agonists. On the other hand, also a potential suppression of pulmonary antibacterial defenses must not be overlooked, particularly in chronic bronchitis, a disease characterized by recurrent airways infections. Whether current therapeutic dosages are sufficient to achieve anti-inflammatory or microbicidal suppressive effects of clinical relevance has not been demonstrated so far. (Chest 1993; 104:491-86)

**Adrenergic receptors are widely distributed in human tissues and blood cells. In particular, β₂-adrenergic receptor effects on lymphocytes and granulocytes have been probed extensively.** Recently, Liggett² identified and characterized β₂-adrenergic receptors in human alveolar macrophages responding to specific stimulation with an increase of intracellular cyclic adenosine monophosphate (cAMP).

This increase in cAMP has striking inhibitory effects on alveolar macrophage functions such as lysosomal content release,³ migration,⁴ phagocytosis,⁵ calcium-dependent Na⁺/K⁺ pump,⁶ and oxygen-free radical production.⁷ Most of these studies were carried out on alveolar macrophages from animals. Few reports on human lung macrophage activities have been published and the results are often controversial.

The defensive role of alveolar macrophages is of primary importance, but an excessive release of inflammatory mediators can cause damage to lung tissues. The understanding of what may condition the predominance of favorable vs autoaggressive effects remains incomplete.

A new class of long-acting β₂-agonists such as formoterol and salmeterol has been studied in the recent years. They show a prolonged bronchodilator effect and a significant ability to prevent exercise-induced asthma.¹²,¹³ Preliminary studies have shown a more pronounced receptor affinity and stronger anti-inflammatory effects, in vivo and in vitro, than the conventional short-acting β₂-agonists, such as salbutamol.¹²

Those effects could be very important in the treatment of asthma, but also for patients affected by chronic bronchitis and emphysema, who may regularly use inhaled bronchodilators. Chronic obstructive airways diseases are characterized by local inflammation, which may be present also in clinically stable phase.¹⁴ On the other hand, inflammation is a naturally occurring defense mechanism and to what extent its inhibition may be useful is at present unknown.

In this article, we compared the effects of salbutamol and formoterol on superoxide anion production and bacterial killing of human alveolar macrophages from patients affected by chronic bronchitis. The goal of the study was to evaluate the effects of conventional and new long-acting β₂-agonists on macrophage activ-

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ities related to inflammation and antimicrobial defenses in particular.

**Methods**

Alveolar cells were obtained by diagnostic bronchoalveolar lavage (BAL) from 20 patients (5 female and 15 male, mean age 55.6 ± 14.7 [SD] years, mean FEV, 67.7 ± 22.4 [SD] percent of predicted) affected by chronic bronchitis diagnosed according to the American Thoracic Society standard criteria.14 All patients were in a clinically stable phase and completely free of pharmacologic treatment (including inhaled bronchodilators) for at least 10 days; 6 were nonsmokers and 14 were ex-smokers for more than 3 years. Occupational exposure to inorganic dust and/or recurrent airway infection in a nonimmunocompromised condition could be recognized as risk factors for chronic bronchitis in nonsmoker subjects.

Bronchoalveolar lavage was performed during fiberoptic bronchoscopy, injecting a subsegment of the middle lobe or of the lingula with three 50-ml boluses of sterile saline solution at 37°C.15 Each aliquot was recovered and analyzed separately for cellularity and cell differentials as previously described.16 Briefly, BAL fluids were filtered through a monolayer of sterile gauze. Before centrifugation, cytocentrifuge (Cytospin II, Shandon, London, UK) slide preparations on native fluids were made in duplicate and stained with May-Grünwald-Giemsa. A total amount of at least 500 cells per each slide were examined at a 1,000× magnification to determine the cell differentials. Bronchoalveolar cells were separated from fluids by refrigerate (4°C) centrifugation (Beckman TJ 6, Beckman, Fullerton, Calif), 400 g for 15 min, and resuspended in cell culture medium RPMI 1640 (Boehringer Mannheim GmbH, Mannheim, Germany) at a concentration of 10^6/ml alveolar macrophages.

First recoveries (with neutrophils usually >5 percent) were discarded to avoid excessive granulocyte contamination. The final neutrophil percentage in the tested samples never exceeded 3 percent. Cell viability was evaluated with trypan blue dye exclusion test. Macrophages were preincubated at 37°C in atmosphere of 5 percent CO₂ for 5 min without drugs as control and with 3 different concentrations of formoterol (supplied by Ciba-Geigy, Origgio, VA, Italy) and salbutamol (all reagents and drugs from Sigma Chemicals Co, St Louis, Mo, unless specified differently) 10⁻¹, 10⁻², and 10⁻³ M.

To evaluate whether the effects of the drugs were dependent on β₂-receptor activation, five tests were repeated with a β-receptor blocker, propranolol at a concentration of 10⁻³ M. All tests were performed at least twice.

**Superoxide Anion Measurement**

A modified assay of Bellavite et al17 was used for superoxide anion evaluation. Four sterile test tubes labeled 1A, 1B, 2A, and 2B were prepared for each control and drug-challenged macrophage suspension with 400 μl of a Krebs-Ringer-phosphate buffer solution, containing 6.25 mM/L of cytochrome C and 5 mM/L of glucose. Ten microliters of superoxide anion dismutase solution, 2.5 mg/ml (3,000 U/mg protein) were added in the tubes labeled as "B." Tubes 2A and 2B contained zymosan, 1 g/L, opsonized with AB group human sera (10 percent) in water bath at 37°C for 15 min to evaluate the phagocytic activity. The 1A and 1B tubes, without zymosan, were used to evaluate the basal production of superoxide anion. We added 100 μl of alveolar macrophage suspension and incubated in water bath for 10 min at 37°C under continuous shaking (about 100 rpm). The reaction was stopped with 2 ml of ice-cold Krebs-Ringer-phosphate buffer. The tubes were centrifuged at 1,500 g for 10 min. The absorbance of cell-free supernatants was measured at 550 nm and data were multiplied by the dilution factor and divided by the extinction coefficient μmol/L of cytochrome C determined at 550 nm (0.0198). The difference between the values of O₂⁻ production with and without zymosan is reported as a "phagocytic index." O₂⁻ production was expressed in nM O₂⁻/10⁶ HAM/10 min.

The influence of β₂-agonists on cytochrome C was excluded with control tests without macrophages.

**Bacterial Killing Evaluation**

The bacterial killing was evaluated as previously described.18 Briefly, a set of sterile test tubes was prepared with 500 μl of RPMI 1640 suspension containing about 20×10⁸ Staphylococcus aureus ATCC 6538 opsonized at 37°C for 15 min with 10 percent of pooled normal human sera. The concentration of bacteria was determined in a culture broth of 18 h (Brain Heart Infusion Broth, Difco, Detroit, Mich) with a spectrophotometric assay based on a dilution curve of the culture at a wavelength of 628 nm.

One tube was used as control without phagocytes; 500 μl of RPMI 1640 containing about 1×10⁶ alveolar macrophages challenged or not with β₂-agonists were incubated in 5 percent CO₂ atmosphere and agitation for 20 min at 37°C; the ratio bacteria/macrophages was 10:1. The cells were resuspended and lysed in distilled water to release the surviving bacteria. The lysates containing live bacteria and cellular debris were incubated on agar plates (Brain Heart Infusion Agar, Difco, Detroit, Mich) and incubated at 37°C for 24 h. Surviving bacteria were evaluated by counting the number of colony forming units.

We excluded the antibacterial effects of β₂-agonists on staphylococci with control tests.

**Statistical Analysis**

All values are reported as mean ± standard error, unless otherwise specified. Means were compared using Student's t test for paired data and analysis of variance as appropriate. Analyses were performed using a microcomputer (Macintosh SE) and software (Statview + Graphics, Abacus Concept).

**Results**

Bronchoalveolar lavage was well tolerated by all the patients. The mean recovery was 84.3 ml ± 20.6 (SD). Cellularity was 372.1 ± 206.1×10⁶ (mean ± SD). The percentages (weighed mean of the three recoveries ± SD) of individual alveolar cell populations were 88.7 ± 6.7, 6.8 ± 5.8, 3.0 ± 2.4, 1.4 ± 1.7, and 0.1 ± 0.1 for macrophages, lymphocytes, neutrophils, eosinophils, and basophils, respectively. The viability of alveolar macrophages was always >90 percent and we did not observe any modifications after challenge with β₂-agonists. Superoxide anion production (Table 1) in

| Table 1 — Superoxide Anion Production by Human Alveolar Macrophages (nM O₂⁻/10⁶ AM/10 min)* |
|---------------------------------|-----------------|-----------------|
|                                | Basal           | Zymosan         |
| Control                        | 11.4 ± 2.8      | 28.2 ± 4.3      |
| Salbutamol                     |                 |                 |
| 10⁻¹ M                         | 10.8 ± 2.0      | 23.5 ± 4.4      |
| 10⁻² M                         | 11.6 ± 2.0      | 27.2 ± 5.0      |
| 10⁻³ M                         | 13.2 ± 2.2      | 28.4 ± 5.6      |
| Formoterol                     |                 |                 |
| 10⁻¹ M                         | 12.2 ± 2.1      | 20.1 ± 3.4      |
| 10⁻² M                         | 13.8 ± 2.1      | 25.3 ± 3.9      |
| 10⁻³ M                         | 12.3 ± 2.6      | 27.6 ± 5.3      |

*Comparison of formoterol with salbutamol effects on superoxide anion release by human alveolar macrophages in basal conditions and after stimulation with opsonized zymosan.
basal conditions was not affected by salbutamol or formoterol. After stimulation with opsonized zymosan, an increase in O$_2^-$ release was observed in all test tubes (Table 1) but the macrophages challenged with 10$^{-5}$ and 10$^{-7}$ M formoterol showed a significantly lower increase of O$_2^-$ production (phagocytic index: 7.9±2.0 and 11.4±2.7 vs 16.8±2.5, control value, p=0.005 and p=0.022, respectively). Also, 10$^{-5}$ M salbutamol affected O$_2^-$ production after zymosan phagocytosis but not significantly (12.7±2.9 vs 16.8±2.5, p=0.08) (Fig 1).

With a β-agonist, propranolol, we observed a significant recovery of the supernatant anion release by phagocytes challenged with 10$^{-5}$ M formoterol (6.1±4.8 vs 13.1±4.0, p<0.01) after zymosan phagocytosis (Table 2). This was a definitive demonstration that depression of phagocytosis was due to β$_1$-receptor stimulation.

Both formoterol and salbutamol affected bacterial killing, the former more strongly, with a dose-dependent trend (Fig 2).

Killing of S aureus by alveolar macrophages of control test tubes (35.8±3.8 percent) was in fact decreased significantly after challenge with 10$^{-5}$ M (20.1±3.0, p<0.006) and 10$^{-7}$ M (25.5±3.7, p<0.03) formoterol and 10$^{-5}$ M salbutamol (23.4±3.3, p<0.03) (Fig 2).

After incubation with propranolol, we observed prevention of the killing depression induced by 10$^{-5}$ M (17.7±4.8 vs 39.8±5.5, p<0.001) and 10$^{-7}$ M (28.3±8.3 vs 42.4±4.9, p<0.01) formoterol and 10$^{-5}$ M salbutamol (22.8±4.3 vs 45.6±4.8, p<0.005) (Table 3, Fig 3). We did not observe statistically significant differences between equimolar dosages of formoterol and salbutamol.

![Table 2](https://example.com/table2.png)

**Table 2—Superoxide Anion Production by Human Alveolar Macrophages (nM O$_2^-$/10$^6$ AM/10 min)**

<table>
<thead>
<tr>
<th>Phagocytic Index</th>
<th>Without Propranolol</th>
<th>With Propranolol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.1±4.0</td>
<td>11.9±3.2</td>
</tr>
<tr>
<td>Salbutamol</td>
<td>10$^{-5}$ M</td>
<td>11.2±4.1</td>
</tr>
<tr>
<td></td>
<td>10$^{-7}$ M</td>
<td>10.5±4.4</td>
</tr>
<tr>
<td></td>
<td>10$^{-8}$ M</td>
<td>14.4±4.0</td>
</tr>
<tr>
<td>Formoterol</td>
<td>10$^{-5}$ M</td>
<td>6.1±4.8†</td>
</tr>
<tr>
<td></td>
<td>10$^{-7}$ M</td>
<td>10.6±5.7</td>
</tr>
<tr>
<td></td>
<td>10$^{-8}$ M</td>
<td>14.7±6.9</td>
</tr>
</tbody>
</table>

*Effect of 10$^{-4}$ M propranolol on the phagocytic index decrease induced by β$_2$-agonists.
†p<0.01, formoterol 10$^{-4}$ M vs formoterol 10$^{-5}$ M + propranolol.

![Table 3](https://example.com/table3.png)

**Table 3—Bacterial Killing: Percent of Bacteria Killed by Human Alveolar Macrophages**

<table>
<thead>
<tr>
<th>Without Propranolol</th>
<th>With Propranolol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>47.0±8.7</td>
</tr>
<tr>
<td>Salbutamol 10$^{-5}$ M</td>
<td>22.8±4.3†</td>
</tr>
<tr>
<td>10$^{-7}$ M</td>
<td>39.4±7.2</td>
</tr>
<tr>
<td>10$^{-8}$ M</td>
<td>46.3±8.8</td>
</tr>
<tr>
<td>Formoterol 10$^{-5}$ M</td>
<td>17.7±4.8†</td>
</tr>
<tr>
<td>10$^{-7}$ M</td>
<td>26.3±8.3‡</td>
</tr>
<tr>
<td>10$^{-8}$ M</td>
<td>40.5±9.7</td>
</tr>
</tbody>
</table>

*Effect of 10$^{-4}$ M propranolol on the bacterial killing depression induced by β$_2$-agonists.
†p<0.005, salbutamol 10$^{-4}$ M vs salbutamol 10$^{-4}$ + propranolol.
‡p<0.001, formoterol 10$^{-4}$ M vs formoterol 10$^{-4}$ + propranolol.
§p<0.01, formoterol 10$^{-7}$ M vs formoterol 10$^{-7}$ + propranolol.
the two drugs with regard to $O_2^-$ production in basal condition and after opsonized zymosan phagocytosis. On the contrary, $10^{-7}$ and $10^{-9}$ M formoterol affected bacterial killing more than equimolar salbutamol (25.5 ± 3.7 vs 30.6 ± 3.6, p<0.004, and 31.8 ± 4.1 vs 37.2 ± 3.5, p<0.02, respectively) (Fig 3).

FIGURE 3. Human alveolar macrophage killing of Staphylococcus aureus ATCC 6538: prevention of the $\beta_2$-agonist inhibitory effect with propranolol. Mean values, standard error and statistics are reported in Table 3.

DISCUSSION

Biochemistry, molecular structure, function, and regulation of $\beta_2$-adrenergic receptors have been studied extensively. An $\beta_2$-adrenergic receptors are linked to the adenylate cyclase system and their stimulation induces an increase of intracellular cAMP and a modification of different cellular functions.

Long-acting $\beta_2$-agonists such as formoterol and salmeterol possess a long side chain and are highly selective for $\beta_2$-adrenoceptors. An inhibitory influence of these drugs has been demonstrated on histamine release and degranulation of basophils. Long-acting $\beta_2$-agonists have an inhibiting effect on oxygen-free radical production, intracellular calcium mobilization, thromboxane generation by stimulated animal and/or human eosinophils, monocytes, and neutrophils. The inhibition is generally prevented by $\beta_2$-antagonists.

Anti-inflammatory effects have also been demonstrated for short-acting $\beta_2$-adrenoceptor agonists though to a less remarkable extent than long-acting compounds. Studies on human alveolar macrophages are few and apparently long-acting $\beta_2$ agonists are able to inhibit thromboxane-B$_2$ generation. The stimulation of these receptors with higher-affinity long-acting $\beta_2$-agonists could explain an anti-inflammatory effect in vivo. On the other side, the same bronchodilator effect as short-acting agents can be obtained with lower dosages, unable to affect phagocytic functions according to our data, and probably not associated with anti-inflammatory effects. Similar results have been obtained with theophylline that induces an increase of intracellular cAMP comparable to that caused by $\beta_2$-receptor stimulation. It is curious to observe that the potentially negative effects on lung defenses were underlined without any indications of possible anti-inflammatory effects.

In the present study, we observed an inhibitory effect on the superoxide anion release following phagocytosis of opsonized zymosan, more evident for formoterol than for salbutamol at the highest concentration tested ($10^{-8}$ M). Since the basal release was not affected, we can speculate that $\beta_2$-agonists are able to reduce $O_2^-$ production through an inhibition of phagocytosis. It has been demonstrated that phagocytosis induces a marked increase of oxidative metabolism and oxygen radical release, which derive from cell membrane only for 5 percent, the 95 percent of increased oxidative activity being mitochondrial and the most important for killing. Such data can contribute to understand bacterial killing results, a dose-dependent decrease of alveolar macrophage ability to kill staphylococci, this being probably explained by a reduced competence to phagocytose bacteria and to release lysosomal enzymes and $O_2^-$ into phagosomes.

In summary, one possible mechanism to explain the effects of $\beta_2$-agonists on alveolar macrophage antibacterial activities is the induction of an intracellular cAMP increase with an inhibition phagocytosis, lysosomal enzyme and $O_2^-$ release and consequently of intracellular killing.

These effects are certainly correlated with $\beta_2$-receptors because the use of propranolol prevents them. Mita and Shida measured the cAMP produced after $\beta_2$-receptor stimulation in guinea-pig lung membranes and their data show that formoterol is 200 times more potent than salbutamol in activating adenyl-cyclase system. Whether there may be a concentration-dependent trend in cAMP effects on phagocyte antibacterial activities is not known.

Probably the mechanisms herein described are critical, at least in vitro, only at concentrations of $\beta_2$-agonists somehow higher than those of therapeutic relevance, as our data suggest. Janson has evaluated that plasma levels of salbutamol in patients treated for acute asthma with infusion of 5 $\mu$g/kg in 10 min are about $10^{-8}$ to $10^{-9}$ M. Similar concentrations have been obtained after inhalation of 0.15 $\mu$g/kg of nebulized salbutamol. These dosages are higher than those generally used in the treatment of patients with stable COPD and plasma concentrations are lower than those causing a significant effect in our in vitro experimental setting.

The inhibition of phagocyte activities may not necessarily prove to be good from a clinical point of view. Defenses against microbial agents could be impaired similarly as with theophylline, unless mechanisms of adaptation such as tachyphylaxis appear in...
**vitro** on long-term administration. If not, negative consequences might be expected for those patients predisposed to recurrent airways infections, particularly in chronic bronchitis. In patients with asthma, the inhibitory effects of long-acting β₂-agonists on the release of inflammatory mediators could represent a positive aspect for the reduction of bronchial hyperreactivity but also the consequences of an eventual decline in phagocyte functions should be correctly evaluated in **vitro**.

**In vitro** studies alone are far from elucidating the possible anti-inflammatory role of β₂-agonists in **vitro** and its clinical value. An in **vitro** study carried out in guinea pigs demonstrated that both salbutamol and formoterol promptly inhibit plasma protein exudation into bronchial Airways induced by bradykinin provocation. In sensitized guinea pigs, formoterol has anti-exudative effects also in allergen-induced inflammation. In that article, a longer duration of action was reported, too, for formoterol. Studies in humans focused on local anti-inflammatory effects are lacking. Apart from ethical considerations, difficulties arise from the incomplete knowledge of asthma pathogenesis, which renders it problematic to decide the biologic parameters to be considered most appropriate. A proper evaluation of β₂-agonist efficacy is best conducted first of all on a clinical basis, even independently from a supposed anti-inflammatory activity.

In conclusion, conventional and long-acting β₂-agonists can cause in **vitro** a depression of alveolar macrophage antimicrobial function. Whether this can be important in **vitro** in terms of anti-inflammatory effect or suppression of immune defenses has to be demonstrated, but it is improbable that drug concentrations achievable in **vitro** may be responsible for effects such as those described in **vitro**.

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