

Bronchoprotection by salmeterol: cell stabilization or functional antagonism? Comparative effects on histamine- and AMP-induced bronchoconstriction

M. Solèr, L. Joos, C.T. Bolliger, S. Elsasser, A.P. Perruchoud

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ABSTRACT: Salmeterol provides bronchoprotection against a number of constrictor stimuli for more than 12 h after a single dose. This effect could be due either to functional antagonism at the level of airway smooth muscle or to cell-stabilizing effects of the compound.

In this study, we attempted to clarify this mechanism by comparing the effects of salmeterol (50 µg), salbutamol (200 µg) and placebo on the airway responsiveness to histamine (to assess functional antagonism), and to adenosine 5'-monophosphate (AMP) (to assess additional cell-stabilizing effects), 14 h after drug treatment. Thirteen patients with mild allergic asthma were studied in a double-blind, randomized protocol on 6 days, at least 48 h apart. Forced expiratory volume in one second (FEV₁) was measured before and 15 min after inhalation of the study medication. Then, 14 h later (8 a.m. the following morning), a bronchoprovocation test with histamine or AMP was performed.

We found that 14 h after inhalation, salmeterol still had a significant effect on FEV₁ in comparison to placebo and salbutamol. The provocative dose producing a 20% fall in FEV₁ (PD₂₀histamine) was significantly increased after salmeterol, whilst the increase in PD₂₀AMP did not reach significance. The shift in PD₂₀ (in doubling dose steps) induced by salmeterol pretreatment was not different between histamine and AMP.

We conclude that the prolonged protective effect of salmeterol occurs *via* an extended bronchodilating and functional antagonistic action and not *via* a cell-stabilizing effect.

Eur Respir J., 1994, 7, 1973-1977.

Pulmonary Division, Dept of Internal Medicine, University Hospital, Basel, Switzerland.

Correspondence: M. Solèr
Pulmonary Division
Dept of Internal Medicine
University Hospital
CH-4031 Basel
Switzerland

Keywords: Adenosine-5'-monophosphate
asthma
bronchoprotection
histamine
salmeterol

Received: February 11 1994
Accepted after revision July 8 1994

Supported by Swiss National Science Foundation, Grant No. 32-30154.90 and Glaxo, Switzerland.

Long acting β_2 -agonists, *i.e.* salmeterol and formoterol, have recently been introduced as a potent new treatment for bronchial asthma. Several clinical studies have indicated a continuous bronchodilating effect of these drugs when inhaled twice daily. This treatment was superior to short acting β_2 -agonists, not only for night-time symptoms [1], but also for the long-term control of the disease [2-4].

In addition to their bronchodilating effect, long-acting β_2 -agonists provide protection against induced bronchoconstriction for prolonged periods. Bronchoprotection by salmeterol has been reported against histamine [5, 6], methacholine [7], and cold air challenge [8], for up to 12 h after a single inhalation, *i.e.* for the full duration of the usual dosing interval. Functional antagonism at the level of airway smooth muscle might well explain these findings. However, other mechanisms, such as stabilization of bronchial inflammatory cells and prevention of mediator release may also be involved.

A cell-stabilizing effect would be compatible with

in vitro data, showing that salmeterol prevented the antigen-induced release of histamine and other mediators from human lung tissue for up to 20 h [9]. This phenomenon was reported as an "anti-inflammatory" effect [10], an expression still lacking a clear definition in the context of asthma. We prefer to use the term "cell-stabilization" for these β_2 -agonist effects on mediator release *in vitro*. *In vivo*, O'CONNOR *et al.* [11] found a significantly greater protective effect of terbutaline against adenosine-5' monophosphate(AMP) than against methacholine, 20 min after inhalation of the drug. Because AMP is thought to cause bronchoconstriction mainly by releasing histamine and other mediators from immunologically preactivated bronchial mast cells [12, 13], the greater protection against AMP was interpreted to be a consequence of a mast cell-stabilizing effect of terbutaline [11].

In this study, we therefore assessed whether the cell-stabilizing effect of salmeterol, as demonstrated *in vitro*, could also be confirmed *in vivo*, 14 h after a single

inhaled dose. We hypothesized that in case of a persistent cell-stabilizing effect, the influence of salmeterol on the provocative dose causing a 20% fall in forced expiratory volume in one second (PD_{20}) to a mast cell-activating stimulus, such as AMP, should be more pronounced than on PD_{20} to a direct stimulus, such as histamine.

Methods

Subjects

Thirteen volunteers (6 females and 7 males) with mild allergic asthma to various antigens participated in the study (table 1). The participants had not received treatment with corticosteroids, theophylline, cromolyn sodium or nedocromil sodium for at least 3 months. Furthermore, in the 2 months preceding the study, no airway infections and no asthma exacerbations had occurred. Occasional symptoms were controlled with inhaled short-acting β_2 -agonists. Such treatment was withheld for at least 12 h on study days.

Written informed consent was obtained from each subject. The protocol was approved by the Ethics Committee of the Department of Internal Medicine, University Hospital, Basel, Switzerland.

Study design

Baseline assessment included spirometry (Masterlab, Jaeger, Würzburg, Germany) with full flow-volume loop, and bronchoprovocation tests with AMP and histamine on two different days at 8 a.m. Baseline forced expiratory volume in one second (FEV_1) had to be above 70% predicted, $PD_{20}FEV_1$ for histamine <0.8 mg, and PD_{20} for AMP <80 mg nebulized dose.

The study had a double-blind, cross-over design with

6 study days in randomized order at least 48 h apart. The three study medications were supplied by Glaxo GmbH (Bad Oldesloe, Germany) in identical metered-dose inhalers, containing placebo, salbutamol (100 $\mu\text{g}\cdot\text{puff}^{-1}$), and salmeterol (25 $\mu\text{g}\cdot\text{puff}^{-1}$). The drugs were inhaled using a large volume spacing device (Volumatic, Glaxo) at a dose of two puffs.

The six study days started with a visit at 6 p.m., when FEV_1 was measured and had to be within $\pm 10\%$ of the initially measured baseline value. Then two puffs of study medication were inhaled and FEV_1 was measured after 15 min. On the following morning at 8.00 a.m. (14 h later) FEV_1 was measured, followed by a bronchoprovocation test with histamine or AMP. Each of the three study medications was inhaled on two of the six study days, and bronchoprovocation tests to AMP and to histamine were performed once after each treatment.

Bronchoprovocation tests

Bronchial provocation tests were performed using a Mefar dosimeter system with calibrated nebulizers, adjusted to an output of 10 $\mu\text{l}\cdot\text{breath}^{-1}$. Inhalations were performed during an inspiratory capacity manoeuvre followed by a 5 s breathhold. The tests were discontinued if FEV_1 fell by more than 20% of the postsolvent value. $PD_{20}FEV_1$ was calculated by linear interpolation on a semi-logarithmic plot.

Histamine-dihydrochloride (Merck ABS, Basel, Switzerland) was dissolved in phosphate-buffered saline (PBS), at concentrations of 0.5, 2.5, 10 and 40 $\text{mg}\cdot\text{ml}^{-1}$ and inhaled in doubling dose steps from 0.01–6.4 mg cumulative nebulized dose.

Fresh solutions of AMP (disodium-salt, Fluka-Chemie, Buchs, Switzerland) in PBS were prepared daily at doubling dilutions from 400–3.125 $\text{mg}\cdot\text{ml}^{-1}$. Inhalations were performed in doubling dose steps from 0.156–159.8 mg cumulative nebulized dose.

Table 1. – Characteristics of the subjects included in the study

Pat. No.	Age yrs	Sex	FEV_1 l	FEV_1 % pred	PD_{20} hist mg	PD_{20} AMP mg	Allergen
1	31	M	3.5	91	0.35	2.05	HDM
2	35	F	3.2	103	1.94	1.40	HDM
3	43	F	2.9	115	0.16	1.29	Cat
4	31	F	3.2	91	0.33	2.00	HDM
5	24	M	4.5	104	0.24	2.18	HDM
6	23	M	3.9	83	0.17	2.79	HDM
7	46	F	2.7	92	0.43	5.69	HDM
8	25	F	2.9	91	0.05	0.18	HDM
9	31	M	3.5	95	0.24	0.84	Cat
10	24	M	4.1	101	0.93	1.60	HDM
11	18	M	5.7	115	0.32	12.49	Birch
12	41	F	2.2	73	0.20	7.13	HDM
13	38	M	4.8	115	0.05	3.60	HDM

Pat: patient; M: male; F: female; FEV_1 : forced expiratory volume in one second; PD_{20} : provocative dose producing a 20% fall in FEV_1 ; % pred: percentage of predicted value; hist: histamine; AMP: adenosine-5'-monophosphate; HDM: house dust mite.

Statistical analysis

Changes in FEV₁ (in % predicted, European Community for Steel and Coal [14]; and in % change from the baseline value of each test day measured at 6.00 p.m.) were analysed by a repeated measures analysis of variance (ANOVA), followed by pairwise comparisons using the Student's paired t-test. Bronchial responsiveness, expressed as PD₂₀FEV₁, was compared between the three study days with a repeated measures ANOVA, followed by pairwise comparisons. In addition, changes in PD₂₀ (differences in doubling dose steps) were compared using the Student's paired t-test and Wilcoxon's signed rank test. The statistical power to demonstrate a 1 doubling dose difference in the shift of PD₂₀ between AMP and histamine at the 0.05 significance level using the Student's t-test was calculated to be 91%, based on a standard deviation of 1. However, at the standard deviation found in this study (2.7) it decreased to 23%. Values of $p < 0.05$ were considered to indicate statistical significance. Values in the text are given as arithmetic means \pm SD, except for fig. 1 where SEM given.

Results

Baseline values of FEV₁ on the six study days were not significantly different (mean values in the range of 94–97% predicted) and individual values were within $\pm 10\%$ of the baseline FEV₁ measured at the first screening visit.

Drug effects on FEV₁

Fifteen minutes after inhalation of salbutamol, FEV₁ increased by $6.5 \pm 3.4\%$ (average of the two treatment days), which was significantly greater than after salmeterol ($3.9 \pm 3.0\%$, $p < 0.05$ versus placebo: $0.8 \pm 1.9\%$).

Fourteen hours after inhalation of salmeterol there was a persistent bronchodilating effect (fig. 1). This change was significantly different from that seen after salbutamol or placebo, where the FEV₁ in the morning tended to be lower than the evening before ($p = NS$). Therefore the bronchoprovocation tests with histamine and AMP after salmeterol pretreatment started at slightly higher absolute FEV₁ values, due to the persistent bronchodilating effect.

Drug effects on PD₂₀

The values for PD₂₀histamine and PD₂₀AMP after the three treatments are given in figure 2. PD₂₀histamine was significantly increased after salmeterol (geometric mean 0.35 mg) when compared to the other two treatments (0.17 mg after placebo, 0.13 mg after salbutamol, $p < 0.05$). PD₂₀AMP after salmeterol (geometric mean 6.90 mg) was not significantly different from the values after placebo (4.41 mg) or after salbutamol (2.07 mg).

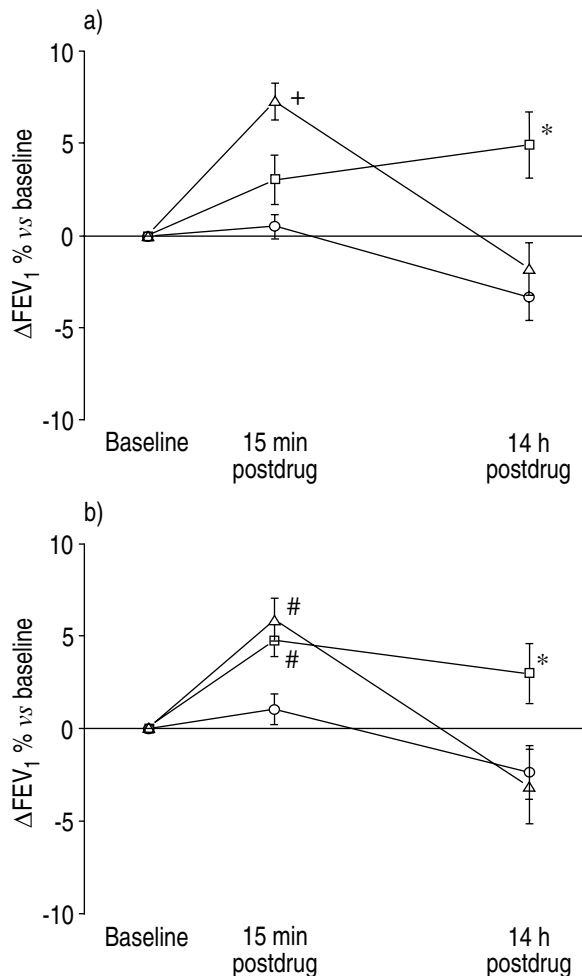


Fig. 1. — Changes in FEV₁ (Δ FEV₁) over time (in percent vs baseline) at 15 min and 14 h after inhalation of the study medication, a) for the three study days with histamine provocation; b) for the days with AMP provocation. Values are mean \pm SEM, $n = 13$. +: $p < 0.05$ vs placebo and salmeterol; #: $p < 0.05$ vs salbutamol and placebo; *: $p < 0.05$ vs placebo. FEV₁: forced expiratory volume in one second; AMP: adenosine-5'-monophosphate. —○—: placebo; —△—: salbutamol; —□—: salmeterol.

To correct for the large interindividual differences in PD₂₀ for both stimuli, the changes in PD₂₀ induced by the study medication as compared to placebo were calculated (difference in doubling dose steps (DD)). Using this approach, salmeterol caused a significantly greater change in PD₂₀histamine ($+1.06 \pm 0.72$ DD) and PD₂₀AMP ($+0.64 \pm 2.39$ DD) than salbutamol (-0.35 ± 1.26 and -1.09 ± 1.67 DD, respectively). There was no difference, however, in the extent of bronchoprotection by salmeterol against histamine- and AMP-induced bronchoconstriction (fig. 2).

Correlation of effects on PD₂₀ and FEV₁

There was a trend towards lower PD₂₀ values after salbutamol pretreatment (fig. 2). To assess the degree to which this was explained by variations of FEV₁ at the beginning of the bronchoprovocation tests, we

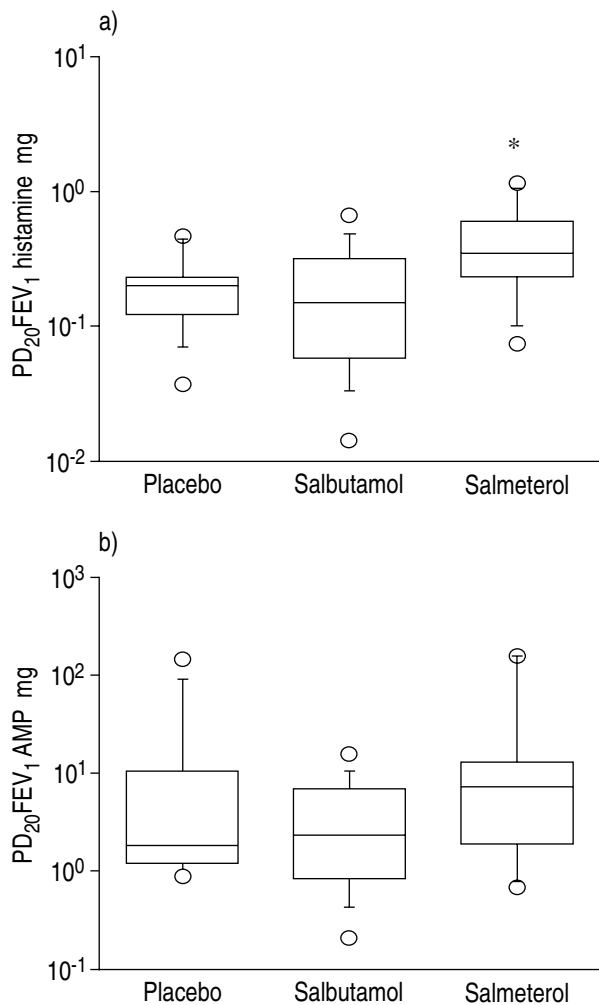


Fig 2. – Box-plots of the PD₂₀ values for: a) histamine; and b) AMP after the three study medications (n=13). PD₂₀histamine was significantly increased (p<0.05) after salmeterol pretreatment, as compared to placebo or salbutamol. PD₂₀ for AMP was not significantly different on the three occasions. Box: interquartile-range with median as horizontal line; whiskers: 10th and 90th percentile; ○: 5th and 95th percentile. PD₂₀: provocative dose producing a 20% fall in FEV₁. For further abbreviations see legend to figure 1. *: p<0.05 versus placebo and salbutamol.

compared the differences in starting-FEV₁ between the placebo and the treatment day with the differences observed in PD₂₀ (in doubling doses). After salbutamol, we found significant correlations of the change in FEV₁ and the change in PD₂₀ both for histamine (r=0.62) and AMP (r=0.57) bronchoprovocation tests. After salmeterol, however, there was no such correlation, indicating that an additional mechanism had to be involved.

Discussion

This study attempted to better define the mechanism of bronchoprotection by salmeterol, a new long-acting β₂-agonist. Fourteen hours after inhalation of a single dose of 50 μg salmeterol, there was a persistent bronchodilating effect (FEV₁), a significant increase in

PD₂₀histamine and a trend for an increase in PD₂₀AMP. In direct comparison, the influence of salmeterol on PD₂₀AMP was not significantly different in magnitude from its effect on PD₂₀histamine. This argues against a cell-stabilizing effect of salmeterol at this time after inhalation, and in favour of a persisting bronchodilating and functional antagonistic effect at the level of airway smooth muscle.

For this study, we selected individuals with mild and stable asthma on the basis of a pronounced hyperresponsiveness to AMP and histamine, such that a drug-induced increase in PD₂₀ could still be quantitated. In order to minimize the influence of spontaneous variation of airway responsiveness over time, we performed all measurements within the shortest possible period (3–4 weeks, 6 weeks in two subjects) and demonstrated stable FEV₁ baseline values on the six study days. However, the variability of the PD₂₀ values, inherent in this type of measurement, was still considerable (screening to placebo day: mean difference 0.6±1.2 doubling dose steps for histamine and 1.0±2.3 for AMP). Therefore, the power to detect differences in bronchoprotection between histamine and AMP was low, thereby limiting the inference of the present negative finding.

The mechanism of action of inhaled AMP is not clearly understood, but it is thought to cause bronchoconstriction mainly by releasing mediators from pre-activated mast cells in the bronchial mucosa [12, 15, 16]. Histamine release seems to be the predominant pathway, as pretreatment with antihistamines, such as terfenadine or astemizole, blocks AMP-induced bronchoconstriction by more than 80% [12]. If salmeterol prevented this histamine release in addition to functionally antagonizing the consecutive constriction of airway smooth muscle, we would have expected a more pronounced shift in the PD₂₀AMP than in the PD₂₀histamine after salmeterol treatment. This was not the case, however.

Prevention of mediator release by a β₂-agonist *in vivo* was the mechanism suggested by O'CONNOR *et al.* [11] when interpreting the significantly greater degree of protection against AMP- than methacholine-induced bronchoconstriction 20 min after inhalation of terbutaline. This phenomenon was demonstrated at around the time of maximal bronchodilation. It remains possible, therefore, that salmeterol might have a similar effect at an earlier time-point. CHEUNG *et al.* [17] have recently demonstrated that, after regular treatment with salmeterol over 8 weeks, the bronchodilating effect is maintained, whilst there is a tachyphylaxis for the protective effect against methacholine-induced bronchoconstriction in day 28 and 56 as compared to day 1. These measurements were taken 1 h after inhalation of salmeterol [17]. A recent study by BOOTH *et al.* [18], assessing PD₂₀ to methacholine 12 h after inhalation of salmeterol before and during 8 weeks of treatment, found no tachyphylaxis for its bronchoprotective or dilating effects. However, half of their patients were also on inhaled steroids. As the mechanism of bronchoprotection is poorly understood, it is difficult to speculate on the reasons for these different findings.

A functional downregulation of β_2 -receptors on inflammatory cells was suggested by O'CONNOR *et al.* [11] when interpreting the disappearance of the difference in bronchoprotection between the direct (methacholine) and the indirect (AMP) stimulus after two weeks of regular terbutaline treatment. In the light of our data, it may be speculated that β_2 -receptors on airway inflammatory cells might demonstrate functional downregulation within hours, if permanently stimulated. This is supported by the finding that the tachyphylaxis of β_2 -receptors on peripheral blood lymphocytes evolves within hours of permanent stimulation *in vivo* [19]. Because we have demonstrated a persisting bronchodilating effect for 14 h after inhalation of salmeterol, airway mast cells can be expected to be permanently exposed to β_2 -stimulation over this period. A downregulation of β_2 -receptors on airway mast cells within this time-period could explain the lack of a differential protection against the direct and indirect constrictor stimulus in our study, and would also be consistent with the findings of CHEUNG *et al.* [17] and BOOTH *et al.* [18].

In conclusion, our data indicate that the persistent bronchoprotective effect of salmeterol against different stimuli is due mainly to persisting bronchodilation and functional antagonism at the level of airway smooth muscle. There is no evidence, however, of a persisting mast cell-stabilizing effect 14 h after salmeterol inhalation. Even though the statistical power of this study is limited, our results indicate that the drug should be regarded as a long-acting bronchodilator, without evidence of an anti-inflammatory effect of salmeterol *in vivo*.

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