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Short communication

Characterization of tolerance to the anti-leakage effect of formoterol in rat airways

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Abstract

These experiments addressed the question of whether the anti-plasma leakage action of β_2-adrenoceptor agonists in rat airways is subject to tolerance. Pathogen-free F344 rats were pretreated with the highly selective, long-acting β_2-adrenoceptor agonist, formoterol (0, 0.1, 1, 10 μg/kg, i.p.) for 7 days; 24 h later the effectiveness of acute doses of formoterol (0, 0.1, 1, 10 μg/kg, i.v.) was tested against substance P-induced plasma leakage. The anti-leakage effect of formoterol was not subject to tolerance with the low or intermediate pretreatment dose. Pretreatment with 10 μg/kg formoterol reduced the effectiveness of the 1 μg/kg acute dose but not the 10 μg/kg acute dose. We conclude that tolerance to the anti-leakage effect of formoterol can occur, but airway vessels are likely to retain functionally coupled receptors even after high dose pretreatment. © 1997 Elsevier Science B.V.

Keywords: β_2-Adrenoceptor agonist; Plasma leakage; Substance P; Trachea; Vascular permeability

1. Introduction

β_2-adrenoceptor agonists are useful in the treatment of reversible obstructive airway diseases because of their action on several cellular targets. β_2-adrenoceptor agonists act directly on airway smooth muscle to induce relaxation and hence bronchodilatation (Avner and Noland, 1978; Cheung et al., 1992), on mast cells to inhibit degranulation produced by allergen challenge (Chong et al., 1995) and on endothelial cells of the microvasculature to reduce plasma leakage produced by inflammatory mediators (Baluk and McDonald, 1994; Bowden et al., 1994; Sulakvelidze and McDonald, 1994).

The effects of β_2-adrenoceptor agonists are subject to the development of tolerance in that they can decrease with repeated exposure [4]. In humans, tolerance to the inhibitory effect on mast cell degranulation develops rapidly with continued treatment (Cheung et al., 1992; Chong et al., 1995), but tolerance to the bronchodilator effect is usually small and self-limited (Lipworth et al., 1989; Cheung et al., 1992). It is unclear whether the anti-plasma leakage effect of β_2-adrenoceptor agonists on the airway microvasculature is subject to tolerance. Although tolerance to the anti-leakage effect of clenbuterol, a long acting β_2-adrenoceptor agonist, develops in mouse skin after 4 days (Bottcher et al., 1988), none is seen in guinea-pig lung after 5 days of treatment with salmeterol, another long acting β_2-adrenoceptor agonist (Whelan et al., 1993). In this regard, the development of tolerance may depend on the particular drug, dosage, duration of

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[4] Here we use the term tolerance to mean the aggregate of mechanisms leading to diminished response to chronically administered β_2-adrenoceptor agonists. The term tachyphylaxis, a specific form of tolerance, has been used with different meanings in different disciplines. In pharmacological studies, tachyphylaxis represents homologous desensitization that occurs over minutes to hours. However, tachyphylaxis has been used in the clinical literature to describe decreased effects occurring over days, weeks, or months.

treatment and tissue or cell type (Avner and Noland, 1978; Lipworth et al., 1989).

Formoterol is a potent, selective, long acting β_2-adrenoceptor agonist (Anderson, 1993) that can inhibit plasma leakage induced in the airway mucosa by a variety of inflammatory mediators, including substance P, platelet activating factor and bradykinin (Baluk and McDonald, 1994; Sulakvelidze and McDonald, 1994). This effect is mediated by β_2 adrenoceptors and is independent of the agent used to cause plasma leakage (Bowden et al., 1994). This functional antagonism appears to result from the inhibition of endothelial gap formation in postcapillary venules and collecting venules (Baluk and McDonald, 1994). The anti-leakage effect occurs in the airways of rats with systemic doses as small as 0.1 μg/kg and is maximal at 10 μg/kg, with no greater effect at 100 μg/kg (Baluk and McDonald, 1994).

The present study had two objectives: (1) to determine whether the anti-leakage effect of formoterol in the airway mucosa is subject to the development of tolerance; and (2) if so, to determine the relationship between the dose of formoterol and the amount of tolerance that develops.

2. Materials and methods

2.1. Animals

The 114 pathogen-free male F344 rats (Simonsen Laboratories, Gilroy, CA, USA) used in the study were 12 weeks old, weighed 250–275 g and were housed in plastic microisolator cages under barrier conditions.

2.2. Experimental protocols

The rats were pretreated daily between 9.00 and 10.00 a.m. for 7 days with formoterol (eformoterol, 2′-hydroxy-5′-[(*RS*)-1-hydroxy-2-[[(*RS*)-*p*-methoxy-α-methylphenethyl]amino]ethyl] formanilide, prepared as the fumarate dihydrate salt, Novartis Pharmaceuticals, Basel, Switzerland) at a dose of 0.1, 1, or 10 μg/kg per day i.p., or with vehicle (0.9% NaCl, 1 ml/kg per day i.p.). Between 10.00 a.m. and 1.00 p.m. on the eighth day, the rats were anaesthetized with sodium pentobarbital (50 mg/kg i.p., Nembutal®, Abbott Laboratories, North Chicago, IL, USA) and given an acute dose of formoterol (0.1, 1, or 10 μg/kg i.v.) or vehicle (1 ml/kg i.v.). Five minutes later, the rats were injected intravenously with Evans blue (30 mg/kg in 0.9% NaCl, EM Science, Cherry Hill, NJ, USA) followed by substance P (5 μg/kg in 0.9% NaCl containing 5 mM acetic acid, Peninsula Laboratories, Belmont, CA, USA). Five minutes after the substance P, the rats were perfused via the left ventricle with 1% paraformaldehyde in 50 mM citrate buffer, pH 3.5, for 2 min at 120–140 mmHg to remove the intravascular Evans blue (Sulakvelidze and McDonald, 1994). The 4 pretreatment groups, each divided into 4 acute treatment groups, constituted a total of 16 groups ($n = 6$ rats per group).

The baseline amount of Evans blue leakage (no substance P) was measured in 6 rats that received 7 daily injections of vehicle and then were anaesthetized, received injections of vehicle and Evans blue, and were perfused 5 min later.

In addition, the effect of the daily injections was examined in two groups (6 rats per group) by comparing the amounts of substance P-induced plasma leakage after 7 daily injections of vehicle with that after no pretreatment. The amount of substance P-induced plasma leakage in the rats that had daily injections (51 ± 4 ng/mg trachea) was not significantly different from that in rats that had no handling (57 ± 5 ng/mg trachea).

2.3. Measurement of plasma leakage

After the perfusion, the trachea was removed, blotted and weighed, and the Evans blue was extracted with Suramin (FBA Pharmaceuticals Division, Mobay Chemical, NY, USA) and measured by spectrophotometry (Beckman DU-400, Beckman Instruments, Fullerton, CA, USA) (Sulakvelidze and McDonald, 1994). The concentration of Evans blue was calculated from the absorption at a wavelength of 610 nm and expressed as nanograms of dye per milligram of trachea (mean ± standard error of the mean, S.E.). Groups were compared by two-way analysis of variance and Dunnett's test. Differences were considered significant when $P < 0.05$.

3. Results

The amount of substance P-induced leakage of Evans blue varied with the doses of formoterol used for the pretreatment and for the acute treatment. In rats that received vehicle both for the pretreatment as well as for the acute treatment, substance P increased the Evans blue content of the trachea some 10-fold, from 6.6 ± 0.6 ng/mg in the baseline state to 68 ± 4.5 ng/mg after substance P.

In the rats pretreated with vehicle for 7 days, the acute formoterol caused a dose-dependent reduction in the amount of substance P-induced leakage. Doses of 0.1, 1 and 10 μg/kg caused 7, 33 and 49% reductions, respectively, in the amount of leakage (Fig. 1). The reductions caused by the two higher doses were significant.

In rats that were pretreated for 7 days with the 0.1 μg/kg dose of formoterol, the response to the acute formoterol was similar to that found after vehicle pretreatment (Fig. 1). The 0.1 μg/kg acute dose of formoterol had no significant effect, but the 1 and 10 μg/kg acute doses produced reductions of 29 and 55%, respectively ($P < 0.05$). Similarly, in the rats pretreated for 7 days with the 1 μg/kg dose of formoterol, the three acute doses caused reductions of 14, 46 and 55%, respectively (Fig. 1).

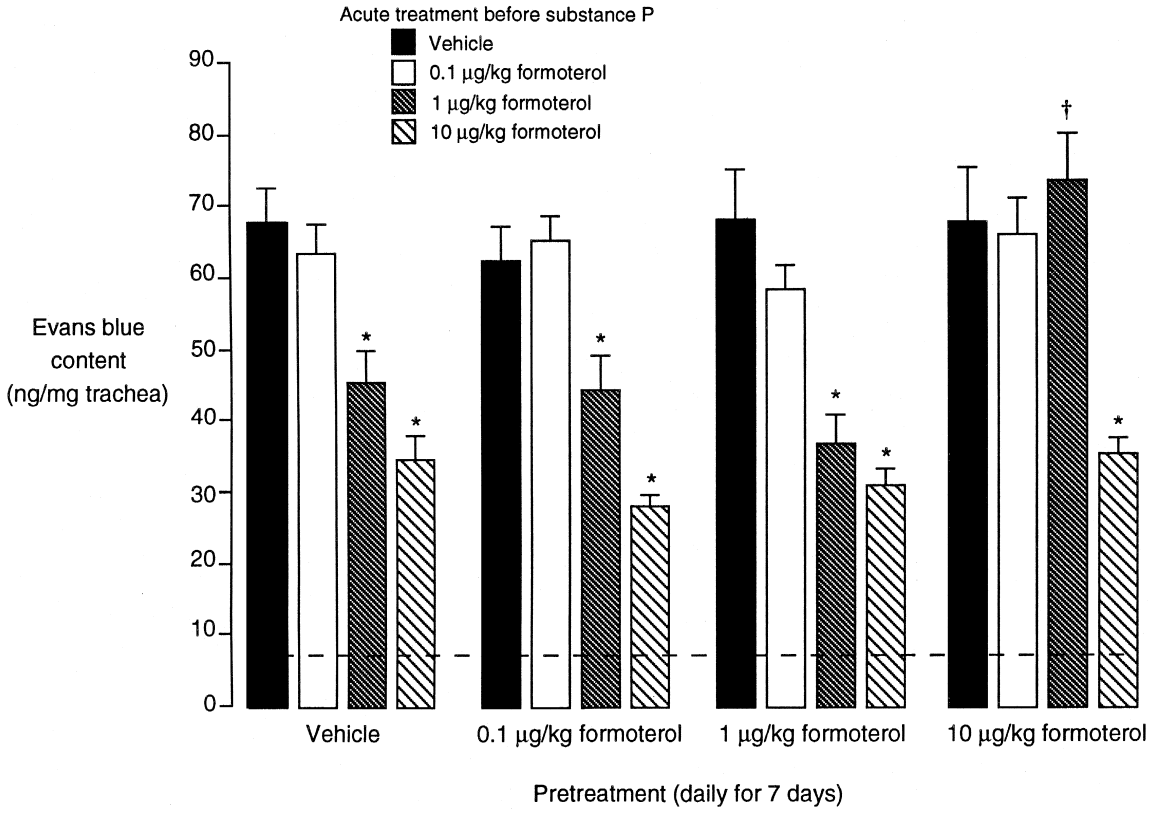

Fig. 1. Effect of 7-day pretreatment with formoterol or vehicle on anti-leakage effect of an acute dose of formoterol. Evans blue content of tracheas was measured 5 min after substance P (5 μg/kg i.v.), which was injected 10 min after the acute dose of formoterol or vehicle and 24 h after the last pretreatment. Dashed line indicates baseline Evans blue content of tracheas (no substance P, 7 ± 0.7 ng/mg). * Significantly different from corresponding value for acute vehicle (within group differences). †Significantly different from corresponding value in other pretreatment groups (between group differences) treated with the same acute dose, $P < 0.05$. Values are means ± S.E., 6 rats per group.

Again, this dose-dependent reduction in plasma leakage did not differ significantly from that found in the vehicle pretreated group.

In rats pretreated for 7 days with the 10 μg/kg dose of formoterol, the acute dose of 0.1 or 1 μg/kg had no significant effect on the amount of substance P-induced leakage (Fig. 1). The ineffectiveness of the 0.1 μg/kg acute dose fit with the results of the other pretreatment groups. The amount of leakage after the 1 μg/kg acute dose (74 ± 6 ng/mg) resembled that after acute vehicle (68 ± 8 ng/mg) and was significantly greater than the 1 μg/kg acute dose values after pretreatment with 0.1 μg/kg (44 ± 5 ng/mg) or 1 μg/kg (37 ± 4 ng/mg) formoterol or with vehicle (46 ± 4 ng/mg) (Fig. 1). The loss of response to the 1 μg/kg acute dose indicates a degree of tolerance. In contrast, the acute dose of 10 μg/kg caused a 48% decrease in substance P-induced leakage (35 ± 2 ng/mg compared to 68 ± 8 ng/mg after an acute dose of vehicle; $P < 0.05$). This decrease was as great as was found in the other pretreatment groups, indicating that the maximal effect was not attenuated.

The 7-day formoterol pretreatment had no detectable residual anti-leakage effect, as shown by a comparison of the baseline amounts of substance P-induced leakage (no acute formoterol) 24 h after the 7-day pretreatment with 0.1, 1 or 10 μg/kg of formoterol (62.7 ± 4.7, 68.2 ± 7.0, 68.0 ± 7.6 ng/mg trachea, respectively) or with vehicle (68.1 ± 4.5 ng/mg trachea).

4. Discussion

The present study sought to determine whether the anti-leakage action of formoterol is subject to the development of tolerance. The pretreatment consisting of 7 daily doses of 0.1, 1, or 10 μg/kg tested the likely therapeutic range of the drug. On the day after the pretreatment, we compared the anti-leakage effects of the same three doses of formoterol. The highest dose is known to produce maximal inhibition and reduce the amount of substance P-induced plasma leakage by approximately 60% (Baluk and McDonald, 1994; Bowden et al., 1994; Sulakvelidze and McDonald, 1994).

Pretreatment for 7 days with a daily dose of formoterol of 0.1 or 1.0 μg/kg had no effect on the drug's anti-leakage action. Although pretreatment with the 10 μg/kg dose abolished the subsequent anti-leakage effect of the intermediate acute dose of formoterol, it did not reduce the

effect of the high acute dose. In other words, the tolerance associated with the highest pretreatment dose of formoterol was detectable only when lower doses were used subsequently to test the anti-leakage action. These data contrast with the complete and long-lasting tolerance to the anti-leakage action of clenbuterol in mouse skin (Bottcher et al., 1988).

4.1. Experimental design: Dose and duration of pretreatment

The doses of formoterol used were based on previous experiments that documented the drug's anti-leakage effect over the dosage range of 0.1 to 10 μg/kg (Baluk and McDonald, 1994; Sulakvelidze and McDonald, 1994).

We administered the formoterol pretreatment intraperitoneally rather than by inhalation to avoid variations in dosage due to breathholding or changes in breathing pattern, and to avoid the stress of restraining unanesthetized animals for repeated delivery of nebulized drugs. Moreover, we tested the acute effects of formoterol via the intravenous route to ensure uniform contact with the tracheal microvasculature, as formoterol apparently exerts its anti-leakage effect through a direct effect on endothelial cells (Baluk and McDonald, 1994). Although intravenously administered formoterol can increase heart rate, blood pressure and tracheal blood flow (discussed in Bowden et al., 1994), such effects should favor plasma leakage and thus would not explain the drug's anti-leakage effect.

The extent of tolerance to β-adrenoceptor agonists is dependent on the dose and duration of treatment. For example, tolerance to isoprenaline-induced bronchodilatation occurs within one day after treatment with a relatively high dose (5 mg/kg), but occurs after 3 days of treatment with a lower daily dose (1 mg/kg) (Avner and Noland, 1978). Moreover, in clinical studies tolerance to the bronchodilator effect of long acting β_2-adrenoceptor agonists is evident within 1–4 days (Bhagat et al., 1995). Therefore, we considered 7 days a reasonable period to test the development of tolerance to the anti-leakage effect. The failure to demonstrate tolerance to the anti-leakage effect of salmeterol in one previous study (Whelan et al., 1993) may have been due to an insufficient duration of pretreatment or, more likely, to an insufficient dose of pretreatment. Because we found no evidence of tolerance to low doses, a logical next step in future studies would be to examine the effect of more prolonged treatment or more frequent dosing.

To standardize any cumulative effects of the 7-day pretreatment, we spaced the doses 24 h apart and then waited 24 h after the final pretreatment to determine whether an acute dose of formoterol still had an anti-leakage effect. This interval was based on the long duration of action of formoterol (approximately 12 h) (Anderson, 1993). No anti-leakage effect of the formoterol pretreatment was found 24 h after the final dose. All animals were studied between the hours of 10 a.m. and 1 p.m. to minimize the effects of diurnal variations. The stress of daily injections apparently did not influence the outcome of our experiments, because there was no change in the amount of substance P-induced plasma leakage in rats that received daily injections of vehicle.

4.2. Mechanism of tolerance

Formoterol exerts its anti-leakage effect by inhibiting the formation of endothelial gaps through an action on β_2 adrenoceptors on endothelial cells of postcapillary venules (Baluk and McDonald, 1994; Bowden et al., 1994). Although we did not specifically examine changes in the β_2 adrenoceptors on endothelial cells, several lines of evidence suggest that the number of these receptors would be reduced by chronic dosing with formoterol. A 14-day infusion of formoterol in guinea-pigs reduces the number of functional β_2 adrenoceptors identified in the lung by autoradiography (Kompa et al., 1995). Nonetheless, no technique currently available offers sufficient resolution to determine whether these changes occur specifically on venular endothelial cells, the presumptive site of action of formoterol in this model of plasma leakage (Baluk and McDonald, 1994). Our data are consistent with down-regulation of venular β_2 adrenoceptors, causing a rightward shift in the dose response curve but no decrease in maximal effect.

Several mechanisms could explain the loss of cell-surface G protein coupled receptors (Collins et al., 1992; Barnes, 1995). Acute exposure of β_2 adrenoceptors to an agonist results in receptor phosphorylation by the kinases β-adrenergic receptor kinase and protein kinase A and subsequent internalization into endosomes (Menard et al., 1996). β_2 adrenoceptor phosphorylation leads to an uncoupling of stimulatory G_s from adenylyl cyclase that, along with the loss of cell surface receptors due to internalization, accounts for rapid desensitization or tachyphylaxis. Exposure of β_2 adrenoceptors to agonists over a longer period, such as in the current study, could lead to a reduction in the synthesis of receptors by inhibition of transcription of β_2 adrenoceptor mRNA or to post-translational instability or degradation of mRNA (Nishikawa et al., 1994).

The data from the present study suggest that tolerance to the anti-leakage effect of β_2-adrenoceptor agonists in the airways of normal animals does not develop with standard doses, but can develop with repeated administration of a high dose over 7 days. Additional studies will be necessary to determine whether these results are predictive of the anti-leakage effect of β_2-adrenoceptor agonists in diseased airways.

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