

β -Adrenergic agonists and bronchial hyperreactivity: Role of β_2 -adrenergic and tachykinin neurokinin-2 receptors

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Background: β_2 -Adrenergic agonists are the most widely used bronchodilators for the treatment of asthma. On the other hand, there is concern that excessive use of β_2 -agonists may contribute to the exacerbation of asthma. However, the mechanism of such adverse effects of β_2 -agonists is not completely clear.

Objective: The aim of this study was to assess the direct influence of β_2 -agonists on airways by analyzing the effect of a β_2 -agonist, fenoterol, on airway sensitivity in an animal model and on tachykinin neurokinin-2 receptor expression in bovine tracheal smooth muscle.

Methods: We performed an acetylcholine challenge test on ovalbumin sensitized guinea pigs that were exposed to daily inhalation of ovalbumin and fenoterol. We also investigated the effects of fenoterol on neurokinin-2 receptor messenger RNA and density with Northern blot analysis and receptor binding assay.

Result: The increase of airway responsiveness and the decrease of β_2 -adrenergic receptors were found in guinea pigs that were treated with fenoterol. There were time- and dose-dependent increases of neurokinin-2 receptor mRNA and of density in tracheal smooth muscle that was treated with fenoterol.

Conclusion: This increased airway responsiveness, increased neurokinin-2 receptor expression, and decreased β_2 -adrenergic receptor density may be relevant to asthma exacerbation. (J Allergy Clin Immunol 2000;106:S104-8.)

Key words: Asthma, β_2 -adrenergic receptor, neurokinin-2 receptor

β_2 -Adrenergic agonists are effective bronchodilators that are widely used for the treatment of asthma.¹⁻³ The inhaled formulations are preferred because of the convenience and ease of use.

On the other hand, there is concern that β_2 -agonists may contribute to the exacerbation of asthma or to death from asthma.⁴ Long-term treatment with β_2 -agonists can lead to an increase of bronchial hyperreactivity, and the regular use of inhaled β_2 -agonists may be associated with an increased risk of death or near-death from asthma.⁵⁻⁷ However, the mechanisms of these adverse effects of β_2 -agonists are not completely understood.

The β_2 -agonists also have adverse effects on the cardiovascular system, which might be 1 cause of a higher mortality rate in β_2 -agonist users.^{8,9} To assess the direct influence of β_2 -agonists on the airways, we analyzed the effect of the β_2 -agonist, fenoterol, on airway sensitivity in an animal model and on tachykinin neurokinin-2 receptor expression in bovine tracheal smooth muscle.

METHODS

Effect of fenoterol on bronchial sensitivity in guinea pigs

Acetylcholine challenge test. Three-week-old male Hartley guinea pigs were sensitized with ovalbumin by injection and inhalations. The animals were exposed to daily inhalation of ovalbumin (12 minutes each) for 10 days with a nebulizer (DeVilbiss Health Care Inc.). During this period, the animals were also treated with 10-second inhalations of fenoterol hydrobromide 3 times each day (Fig 1). Before and after exposure to ovalbumin, airway reactivity to acetylcholine was analyzed by a pressure-sensitive body plethysmograph (Fig 2). Respiratory resistance was determined from the intrachamber pressure, and respiration volume readings were given by the machine. The experimenter who measured pulmonary resistance was blinded to the animal treatment.

Radioligand binding assay. Guinea pigs were killed 6 hours after the acetylcholine test, and the lungs were frozen in liquid nitrogen. The frozen lungs were minced in SSB (5 mmol/L sucrose, 5 mol/L Tris · HCl buffer [pH 7.4], 1 mmol/L MgCl₂) and homogenized with a homogenizer (Polytron Omega electric type SM-3) in a total volume of 20 mL. The homogenate was centrifuged at 1000g for 10 minutes at 4°C to remove debris, and the supernatant was then centrifuged at 40,000g for 36 minutes at 4°C. The resulting pellet was resuspended in 2.5 mL of 50/10 buffer (50 mmol/L Tris · HCl [pH 7.4], 10 mmol/L MgCl₂, 5 mmol/L EDTA) and was used as the membrane fraction. The diluted membrane fraction (100 μ L: 200–300 μ g/mL protein) was incubated with 50 μ L of various concentrations of [¹²⁵I]cyano pindolol (up to 8 nmol/L, approximately 1.5×10^6 cpm) at 37°C for 60 minutes. The membrane-bound radioactivity was collected on filters (Whatmann glass microfiber filters, Whatmann International Ltd., Tokyo, Japan) with a cell harvester and was counted with a gamma counter.

Effect of fenoterol on neurokinin-2 receptor expression in bovine tracheal smooth muscle

Tissue preparation. Fresh bovine tracheas were obtained from the abattoir, and the smooth muscle layer was dissected after the epithelium and mucosa were stripped off. The smooth muscle layer was cut into small pieces and incubated at 37°C in T-75 flasks con-

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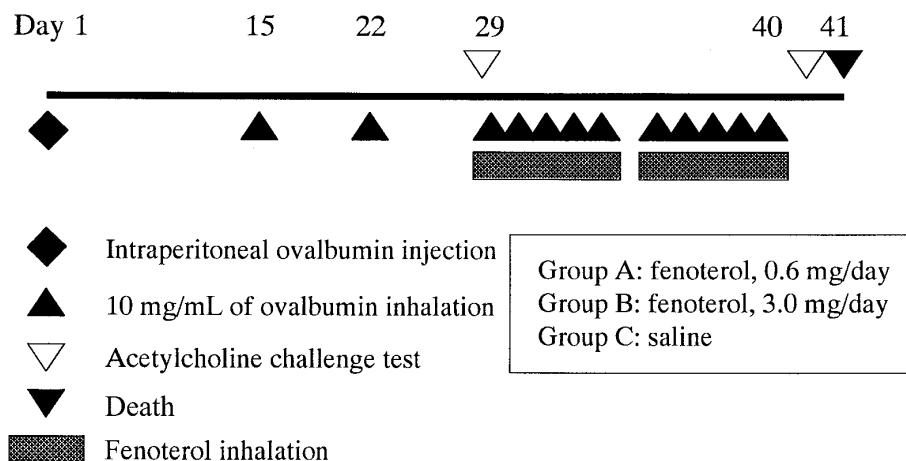


FIG 1. Study design.

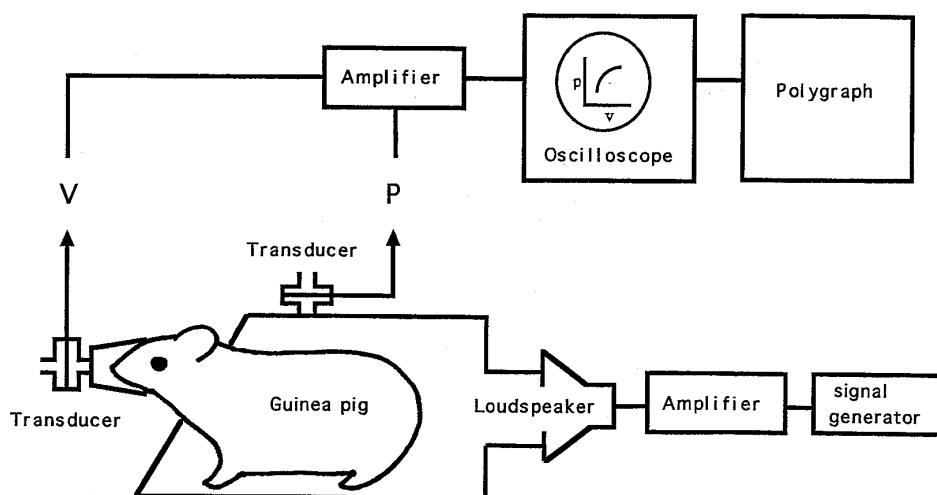


FIG 2. Diagram of the pressure-sensitive body plethysmograph used in this study.

taining Dulbecco's modified Eagle's medium with or without 10^{-9} to 10^{-5} mol fenoterol for the indicated period. Then the tissue was frozen and kept at -70°C for RNA extraction and membrane preparation. To examine whether the effect of fenoterol was mediated by receptors, smooth muscle was preincubated with vehicle or 10^{-7} mol propranolol (a nonselective β -adrenergic receptor antagonist), ICI 118551 (a selective β_2 -adrenergic receptor antagonist), or CGP 20712A (a selective β_1 -adrenergic receptor antagonist) for 1 hour before the addition of 10^{-7} mol fenoterol for a further 6 hours.

RNA isolation. A single-step method with guanidinium thiocyanate-phenol-chloroform extraction was used to isolate total RNA from bovine tracheal smooth muscle.¹⁰ Poly (A)⁺ RNA was prepared with a PolyAtract messenger RNA isolation kit (system IV; Promega, Southampton, UK) according to the manufacturer instructions. Samples of mRNA were size-fractionated on 1% agarose/formaldehyde gel and blotted onto a nylon membrane.

Northern blot analysis. Random primer labeling was performed with the 891 base pair EcoRI and SacI fragment from the human neurokinin-2 receptor complementary DNA (provided by Dr J.E.

Krause, St Louis, Mo), and the 1.3-kb PstI fragment from the rat glyceraldehyde-3-phosphate dehydrogenase cDNA with [α - ^{32}P]dCTP (3000 Ci/mmol; Amersham, UK). The blot was prehybridized for 5 hours in 50% formamide, $5 \times$ standard saline citrate, $5 \times$ Denhardt solution, 0.1% sodium dodecyl sulfate, 10 mmol/L NaH_2PO_4 , and 100 mg/mL sonicated denatured salmon sperm DNA, and then hybridized with ^{32}P -labeled cDNA probes for 12 to 16 hours at 42°C . After hybridization, the filter was washed at high stringency in $0.1 \times$ standard saline citrate and 0.1% sodium dodecyl sulfate at 55°C for 30 minutes. Finally, the filter was exposed to film (OMAT XS; Kodak) at -70°C for 1 to 3 days.

Radioligand binding assay. Frozen bovine tracheal smooth muscle pieces were ground under liquid nitrogen, suspended in 10 volumes of ice-cold 50 mmol/L Tris $\cdot$ HCl buffer (pH 7.4) containing 0.32 mol sucrose, and homogenized with a Polytron homogenizer. The homogenate was centrifuged at $1000g$ for 10 minutes at 4°C to remove debris, and the supernatant was then centrifuged at $40,000g$ for 20 minutes at 4°C . The resulting pellet was washed and recentrifuged at the same speed, after which the final pellet was resus-

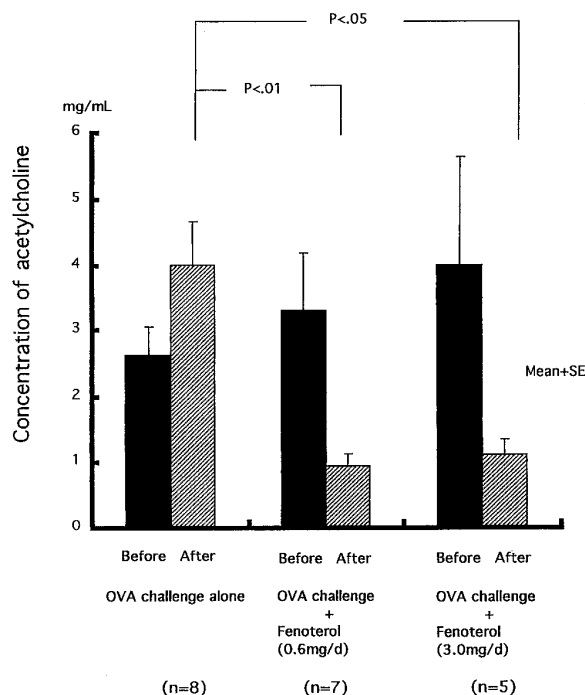


FIG 3. Airway responsiveness in guinea pigs that were treated with fenoterol. Guinea pigs were sensitized with ovalbumin (OVA) and exposed to ovalbumin inhalation for 2 weeks. During the exposure period, the animals were either untreated or treated with fenoterol (0.6 mg/d or 3 mg/d) with a nebulizer. The concentration of acetylcholine that increased the airway resistance by 50% is indicated on the y-axis.

pendent in 50 mmol/L Tris · HCl (pH 7.4). Protein concentrations were determined by the method of Lowry et al.¹¹ Neurokinin-2 receptor density and affinity were assessed from saturation isotherms with 0.5 to 10 nmol/L [³H]SR48968 (25.7 Ci/mmol; DuPont NEN, Hounslow, UK) in a final volume of 0.5 mL of assay buffer containing approximately 300 to 400 mg of membrane protein in 50 mmol/L Tris · HCl (pH 7.4), 0.4 mg/mL bovine serum albumin, 3 mmol/L MnCl₂, 0.04 mg/mL bacitracin, and 0.004 mg/mL chymostatin. Nonspecific binding was determined in the presence of 10⁻⁶ mol SR48968. After incubation at 25°C for 30 minutes, bound and free radioactivity was separated through Whatman GF/C glass-fiber filters. The filters were washed 3 times with 5 mL of ice-cold buffer and were placed in vials with 4 mL of scintillation mixture (Filtron X; National Diagnostics, Manville, NJ) for counting with a liquid scintillation counter (2200 CA model; Packard Instruments, Downers Grove, Ill.). Specific binding was determined by subtraction of the nonspecific binding from the total binding (it usually accounted for 71%-88% of total binding). The maximal binding capacity, and the dissociation constant (kilodalton) were analyzed with the computerized nonlinear regression program Ligand.¹²

RESULTS

Bronchial sensitivity in guinea pigs

The airway responsiveness was expressed as the concentration of acetylcholine, which increased the airway

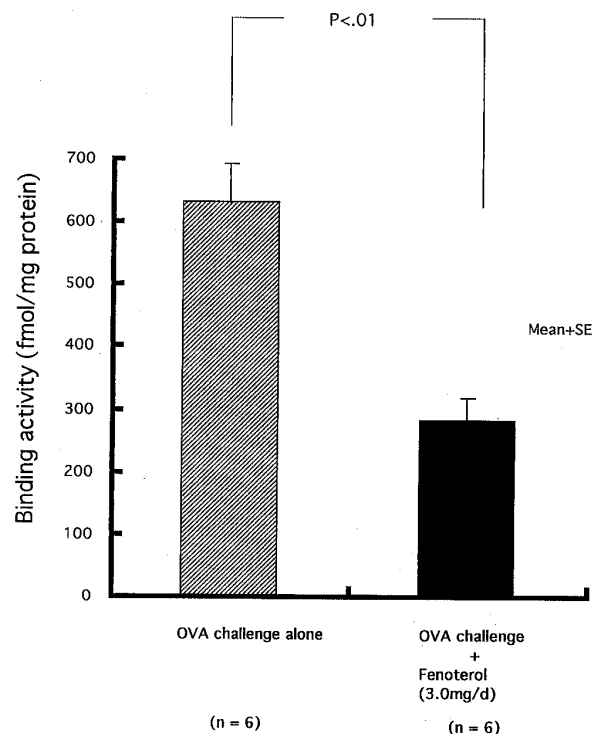


FIG 4. The β_2 -adrenoceptor density in lung membrane fractions. OVA, Ovalbumin.

resistance by 50%. As shown in Fig 3, airway responsiveness was increased when animals were treated with fenoterol at a dose of either 0.6 mg/day or 3 mg/day, although the responsiveness did not change significantly in untreated animals. This suggested that the regular use of fenoterol could paradoxically increase nonspecific airway responsiveness in guinea pigs. In animals that were treated with fenoterol, the density of β_2 receptors decreased to 60% of that in untreated control animals (Fig 4).

Neurokinin-2 receptor expression in tracheal smooth muscle

There was a time-dependent increase of neurokinin-2 receptor mRNA after 4 hours of incubation with 10⁻⁷ mol fenoterol (Fig 5). After 6 hours, even 10⁻⁹ mol of fenoterol caused an increase of neurokinin-2 receptor mRNA (Fig 6). The β -adrenergic receptor antagonist, propranolol, ICI 118551, and CGP 20712A, had no effect on the expression of neurokinin-2 receptor mRNA when used alone. However, propranolol or ICI 118551 (a selective β_2 -adrenergic receptor antagonist) completely blocked the effect of fenoterol, but CGP 20712A (a selective β_1 -adrenergic receptor antagonist) did not (Fig 7), which suggests that the effect of fenoterol on neurokinin-2 receptor mRNA was likely to be mediated by the β_2 -adrenergic receptor. The [³H]SR48968 binding assay showed a significant increase in the number of binding sites after 12 hours and 18 hours of incubation with fenoterol (Fig 8). There were no significant differences of kilodalton values.

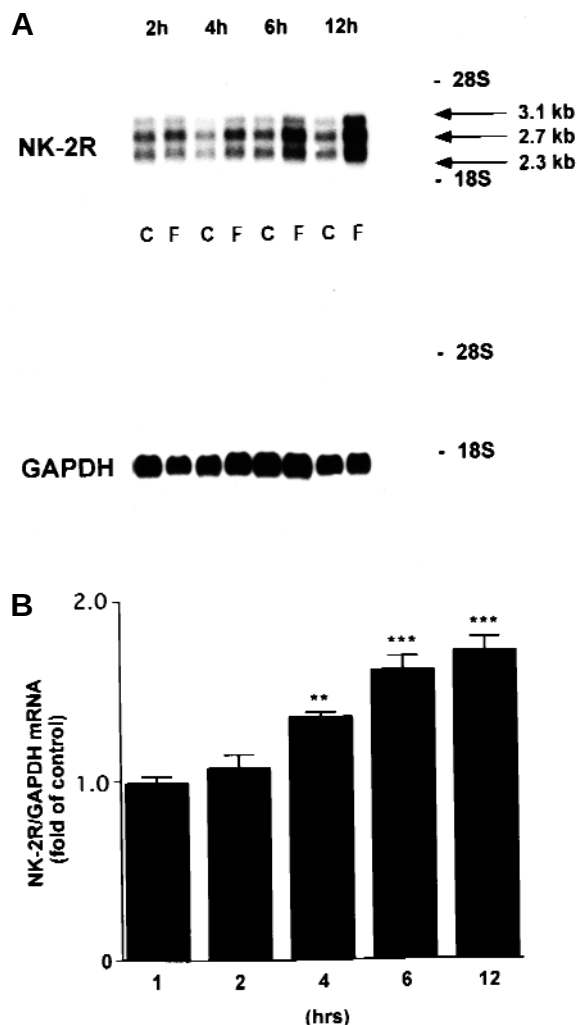


FIG 5. Time course of fenoterol-induced neurokinin-2 receptor (NK-2R) mRNA expression in bovine tracheal smooth muscle. The fenoterol concentration was 10^{-7} mol. **A**, Northern blot analysis with cDNA for neurokinin-2 receptor and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). **B**, Densitometric measurements of neurokinin-2 R mRNA. $n = 3$; $^{*}P < .01$; $^{***}P < .001$. (Modified from Katsunuma T, Roffel AF, Elzinga RS, Zaagsma J, Barnes PJ, Mak JW. β_2 -adrenoceptor agonist-induced up-regulation of tachykinin NK-2 receptor expression and function in airway smooth muscle. *Am J Respir Cell Mol Biol* 1999;21:409-17. Official Journal of the American Thoracic Society.© American Lung Association.)

DISCUSSION

We showed that regular inhalation of fenoterol increased the airway reactivity in ovalbumin-sensitized guinea pigs and that fenoterol increased neurokinin-2 receptor expression by bovine tracheal smooth muscle in vitro. β_2 -Agonists are the most effective bronchodilators presently available for the treatment of asthma. However, there is still concern that excessive use of β_2 -agonists may contribute to the exacerbation of asthma.

In our first experiments, 2 weeks of treatment with fenoterol caused the airway responsiveness to acetylcholine to increase by approximately 2-fold. The

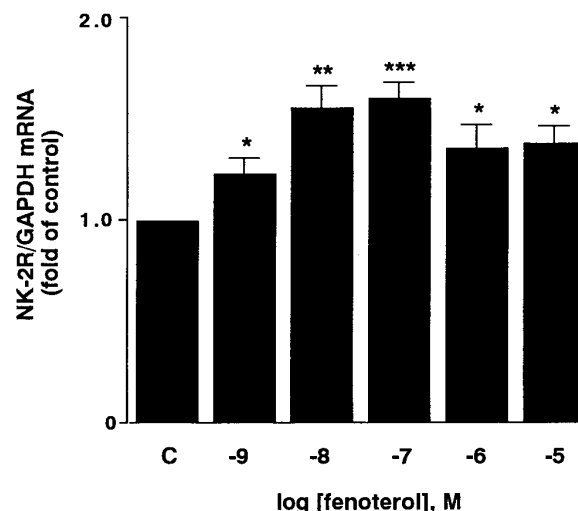


FIG 6. Dose-response of fenoterol-induced neurokinin-2 receptor (NK-2R) mRNA expression in bovine tracheal smooth muscle that was incubated in the absence (C) or presence of increasing doses of fenoterol for 6 hours. $n = 3$; $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$. (Modified from Katsunuma T, Roffel AF, Elzinga RS, Zaagsma J, Barnes PJ, Mak JW. β_2 -adrenoceptor agonist-induced up-regulation of tachykinin NK-2 receptor expression and function in airway smooth muscle. *Am J Respir Cell Mol Biol* 1999;21:409-17. Official Journal of the American Thoracic Society.© American Lung Association.)

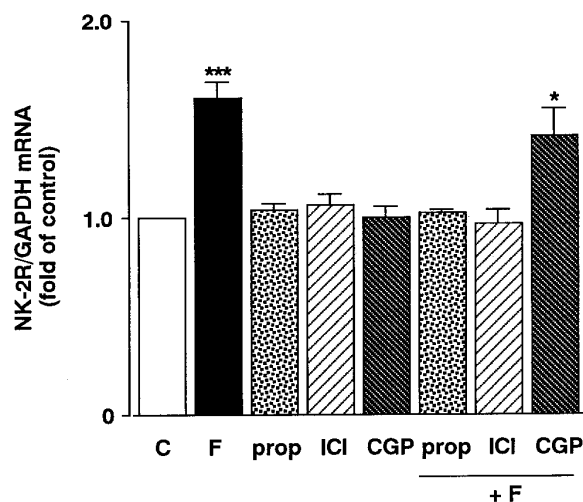


FIG 7. Effect of the nonselective and selective β -antagonists, propranolol (*prop*; 10^{-7} mol), ICI 118551 (*ICI*; 10^{-7} mol), and CGP 20712A (*CGP*; 10^{-7} mol) on fenoterol (*F*)-induced neurokinin-2 receptor (NK-2R) mRNA in bovine tracheal smooth muscle. $n = 3$; $^{*}P < .05$; $^{***}P < .001$. (Modified from Katsunuma T, Roffel AF, Elzinga RS, Zaagsma J, Barnes PJ, Mak JW. β_2 -adrenoceptor agonist-induced up-regulation of tachykinin NK-2 receptor expression and function in airway smooth muscle. *Am J Respir Cell Mol Biol* 1999;21:409-17. Official Journal of the American Thoracic Society.© American Lung Association.)

fenoterol-treated animals showed a decrease of β_2 receptor expression in lung tissue compared with untreated challenged animals, so their increased responsiveness to acetylcholine may have been related to the decrease of β_2 receptors. A decrease in β_2 -receptor density might reduce the protective effect by endogenous catecholamines,

which are released after bronchoconstriction is induced by acetylcholine inhalation.

In the second experiment, our results suggested that fenoterol increases neurokinin-2 receptor expression in the airways. Another β_2 -agonist, salbutamol, also increased neurokinin-2 receptor mRNA expression in bovine tracheal smooth muscle (data not shown). As shown in Fig 5, mRNA isolated from the smooth muscle featured 3 hybridization bands with estimated sizes of approximately 3.1, 2.7, and 2.3 kb, in both control and fenoterol-treated tissues; results were identical to previous findings.¹³

The neurokinin-2 receptor may be relevant to the pathophysiologic features of asthma. Inhalation of neurokinin A induces bronchoconstriction in normal humans¹⁴ and in patients with asthma.¹⁵ Neurokinin A induces the contraction of human isolated airway in vitro.¹⁶ In fact, neurokinin A inhalation enhances airway responsiveness to acetylcholine in rats.¹⁷ Studies with selective neurokinin-2 receptor antagonists have also suggested the importance of the neurokinin-2 receptor in airway reactivity. SR48968 blocks hyperpnea-induced bronchoconstriction¹⁸ and reduces allergen-induced bronchoconstriction in guinea pigs.¹⁹ SR48968 also decreases IL-5-induced bronchial hyperreactivity.²⁰ These results suggest that neurokinin A may play a role in bronchial hyperreactivity by way of the neurokinin-2 receptor, even for nonspecific stimuli.

We found that fenoterol increased neurokinin-2 receptor mRNA density as well as receptor numbers. The increase of neurokinin-2 receptor density was associated with an increased contractile response of tracheal smooth muscle to neurokinin-2 receptor agonists, because incubation with fenoterol for 18 hours led to significantly increased contraction on exposure to a neurokinin-2 receptor agonist.²¹ The increase of neurokinin-2 receptor density and the decrease in the density of β_2 -adrenergic receptors may also occur in human airway smooth muscle in vivo. Such changes might well contribute to the adverse effects suggested to be related to the regular or sustained use of β_2 -agonists.

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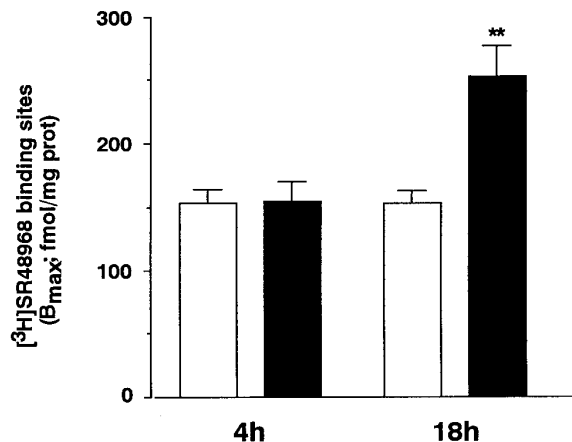


FIG 8. The time course of changes in the number of neurokinin-2 receptors, determined by binding of [^{3} H]SR48968, in bovine tracheal smooth muscle exposure to fenoterol. Means $\pm$ SEM of 3 separate experiments are shown. B_{max} , Maximal binding capacity. ** $P < .01$. (Modified from Katsunuma T, Roffel AF, Elzinga RS, Zaagsma J, Barnes PJ, Mak JW. β_2 -adrenoceptor agonist-induced up-regulation of tachykinin NK-2 receptor expression and function in airway smooth muscle. *Am J Respir Cell Mol Biol* 1999;21:409-17. Official Journal of the American Thoracic Society © American Lung Association.)