In vitro sensitization of human bronchus by β₂-adrenergic agonists

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Faisy, Christophe, Emmanuel Naline, Jean-Luc Diehl, Xavier Emonds-Alt, Thierry Chinet, and Charles Advenier. In vitro sensitization of human bronchus by β₂-adrenergic agonists. Am J Physiol Lung Cell Mol Physiol 283: L1033–L1042, 2002. First published June 10, 2002; 10.1152/ajplung.00063.2002.—Incubation of human distal bronchi from 48 patients for 15 h with 10⁻⁷ M fenoterol induced sensitization characterized by an increase in maximal contraction to endothelin-1 (ET-1) and acetylcholine (ACh). Incubation of human bronchi with 10⁻⁶, 3 x 10⁻⁶, and 10⁻⁵ M forskolin (an adenyl cyclase activator) reproduced sensitization to ET-1 and ACh. The sensitizing effect of fenoterol was inhibited by coincubation with gliotoxine (a nuclear factor-KB inhibitor), dexmethasone, indomethacin (a cyclooxygenase inhibitor), GR-32191 (a TP prostanoid receptor antagonist), MK-476 (a cysteinyl leukotriene type 1 receptor antagonist), SR-140333 (a bradykinin B₂ receptor antagonist), SB-203580 (an inhibitor of the 38-kDa mitogen-activated protein kinase, p38MAPK), or calphostin C (a protein kinase C blocker). Our results suggest that chronic exposure to fenoterol induces proinflammatory effects mediated by nuclear factor-KB and pathways involving leukotrienes, prostanoids, bradykinin, tachykinins, protein kinase C, and p38MAPK, leading to the regulation of smooth muscle contraction to ET-1 and ACh.

β₂-agonists; airway sensitization; airway smooth muscle; endothelin-1; asthma

THE FATAL ACUTE ASTHMA CASES attributable to fenoterol abuse in the 1980s in New Zealand started a controversy concerning the potential worsening of the bronchial hyperresponsiveness by the β₂-adrenoceptor agonists (7, 14, 42). Studies in animals and humans showed that chronic exposure to fenoterol or salbutamol induces a nonspecific bronchial sensitization, whereas the relaxant effects of these β₂-agonists on the airway smooth muscle are not decreased (11, 59, 60). The bronchial sensitization induced by fenoterol is similar to the sensitization provoked by ovalbumin in sensitized guinea pigs (60). Chronic administration of salbutamol at low doses to guinea pigs increases airway reactivity to histamine and methacholine (11). In humans, long-term use of salbutamol increases the bronchial hyperresponsiveness to histamine but does not cause subsensitization of β₂-adrenoceptors to salbutamol (59). In a bovine tracheal model, Katsunuma and colleagues (36) showed that prolonged incubation with fenoterol induced an increased contractile responsiveness to neurokinin A (NKA). In 1995, Peters and colleagues (47) suggested that the continuous activation of the intracellular signal transduction caused by the β₂-adrenoceptor stimulation could induce a proinflammatory process mediated by nuclear transcription factors in rat lung. A recent study in our institution showed that the transcription factor nuclear factor-KB (NF-κB) is involved in fenoterol-induced hyperresponsiveness to NKA in guinea pig isolated trachea (52).

Endothelin-1 (ET-1) is a 21-amino acid peptide recently implicated in chronic inflammatory airway diseases such as asthma and chronic obstructive pulmonary disease (25, 26, 41, 46). ET-1 is synthesized and metabolized in lung, and ET-1 receptors (ETₐ and ETₐ) are widely distributed in airway cells (21, 26, 41). ET-1 is one of the most potent contractile agents of human airway smooth muscle and can induce airway inflammation, airway hyperresponsiveness, and airway remodeling in animals and humans (25, 26, 27, 41), suggesting that ET-1 could be a major component of asthma pathophysiology (10, 22, 26, 27). The purpose of this study was to determine the sensitizing effect of fenoterol on the contraction to ET-1 of human bronchi and to investigate the role of inflammatory mediators and signal transduction pathways involved in airway sensitization to ET-1 induced by β₂-adrenoceptor agonists.

METHODS

Human bronchial tissue preparations. Bronchial tissues were surgically removed from 54 patients with lung cancer (45 men and 9 women, 62 ± 10 yr of age); all patients were

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smokers or ex-smokers. Just after resection, segments of human bronchi (1–3 mm ID) were taken as far as possible from the malignant lesion. They were placed in oxygenated Krebs-Henseleit solution composed of (in mM) 119 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 KH₂PO₄, 25 NaHCO₃, and 11.7 glucose. After removal of adhering lung parenchyma and connective tissues, rings of the same bronchus were prepared (5–7 mm long, 1–3 mm ID) and divided into two paired groups: one (control) group was placed in Krebs-Henseleit solution at room temperature (21°C) for 15 h; the other (pretreated) group was treated with 10⁻⁷ and 10⁻⁶ M fenoterol, 10⁻⁷ and 10⁻⁶ M formoterol, 10⁻⁸ M salbutamol, 10⁻⁶ M salmeterol, or a cAMP activator, i.e., forskolin (10⁻⁶, 3 × 10⁻⁶, and 10⁻⁵ M), for 15 h at room temperature. Incubation time and temperature were chosen in agreement with the work of Katsunuma and colleagues (36) and our previous work (52).

Fenoterol, formoterol, salbutamol, salmeterol, and forskolin concentrations were chosen according to Wang and colleagues (45, 52). Because SB-203580 has been shown to inhibit COX-1 and prostaglandin TP receptor antagonist, GR-32191 (10⁻⁸ and 10⁻⁷ M), 7M SR-48968, 10⁻⁸ and 10⁻⁷ M SR-140333, 10⁻⁸ and 10⁻⁷ M, 6) a nitric oxide (NO) synthase (NOS) inhibitor, nitro-L-arginine methyl ester (L-NAME, 10⁻⁶ M), 4) a prostanoid and prostaglandin TP receptor antagonist, GR-32191 (10⁻⁷ and 10⁻⁶ M), 5) a cytokinin leukotriene (CysLT₁) receptor antagonist, MK-476 (10⁻⁸ and 10⁻⁷ M), 6) a nitric oxide (NO) synthase (NOS) inhibitor, nitro-L-arginine methyl ester (L-NAME, 10⁻⁶ M), 7) a mixture of the tachykynin NK₁, NK₂, and NK₃ receptor antagonists, SR-140333 (10⁻⁷ M), SR-48968 (10⁻⁷ M), and SR-142801 (10⁻⁷ M), respectively, 8) a bradykinin B₂ receptor antagonist, HOE-140 (10⁻⁷ M), 9) an inhibitor of the 38-kDa mitogen-activated protein kinase (MAPK), SB-203580, 10) a protein kinase C (PKC) blocker, calphostin C (10⁻⁷ M), and 11) SB-203580 + calphostin C. Because SB-203580 has been shown to inhibit COX-1 and COX-2, on the one hand, and thromboxane synthase, on the other hand, with IC₅₀ for both enzymes of 2 × 10⁻⁶ M in human platelets (9) and 1.8 × 10⁻⁶ M in human airway smooth muscle (24), we studied in preliminary experiments the potential inhibitory effect of SB-203580, calphostin C, and SB-203580 + calphostin C on the contraction of human bronchus induced by [Sar⁹,Met(O₂)¹¹] substance P from Bachem (Bubendorf, Switzerland), ACh hydrochloride from Pharmacie Centrale des Hôpitaux (Paris, France), forskolin, gliotoxine, dexamethasone, indomethacin, L-NAME, and calphostin C from Sigma (St. Louis, MO), GR-32191 from Glaxo (Greenford, UK), MK-476 from Merck (Paris, France), SR-140333, SR-48968, and SR-142801 from Sanofi Research Center (Montpellier, France), HOE-140 from Peninsula (Merseyside, UK), SB-203580 from Calbiochem (San Diego, CA), and [Sar³,Met(O₂)¹¹]substance P from Bachem (Bubendorf, Switzerland). All drugs except indomethacin and SB-203580 were dissolved in distilled water; indomethacin was dissolved in pure ethanol and then diluted in Krebs solution, and SB-203580 was dissolved in pure ethanol and DMSO and then diluted in Krebs solution. The final amount of ethanol (0.03%) did not alter ACh reactivity (4). ET-1 was dissolved in water at 2.5 × 10⁻⁶ M and kept in small aliquots (200 µl) at −20°C until used. A fresh aliquot was used for each experiment.

Expression and analysis of data. Contractile responses were expressed in tension (g) compared with the basal tone recorded before the start of the concentration-response curve. Values are means ± SE. The data are expressed in terms of E_max for efficacy and −log EC₅₀ (pD₂) for potency. E_max represents the maximal contraction induced by ET-1 and ACh and is expressed in tension compared with the basal tone. ΔE_max represents the difference between E_max obtained with the pretreated bronchi and E_max obtained in paired control human bronchi. ΔE_max was expressed in grams compared with basal tone. −log EC₅₀ values were derived graphically from the logarithmic concentration-effect curves and defined as the negative logarithm of the drug concentration that caused 50% of maximal effect of ET-1 (10⁻⁷ M). Δ(−log EC₅₀) represents the difference between −log EC₅₀ obtained with the pretreated bronchi and −log EC₅₀ obtained with the paired control human bronchi. Bronchi with E_max of ET-1 <0.7 g were excluded from analysis, because we considered that a low level of E_max is a reliable marker of dysfunction of contractility. Statistical analysis of the results was performed using Student's t-test (2-tailed, for paired samples). P < 0.05 was considered significant.

RESULTS

Sample. Bronchi of 48 from 54 patients (89%) yielded an E_max of ET-1 ≥0.7 g. Incubation of these 48 bronchi for 15 h at 21°C with fenoterol (10⁻⁷ M) significantly increased their maximal contraction to ET-1 (Fig. 1) and ACh (E_max of ACh = 2.66 ± 0.18 and 1.99 ± 0.15 g in the presence and absence of fenoterol, respectively, n = 48, P < 0.01). Incubation of human bronchi with fenoterol did not change significantly the potency of ET-1 (−log EC₅₀ = 8.52 ± 0.05 and 8.41 ± 0.06 in the presence and absence of fenoterol, respectively, n = 48, not significant; Fig. 1). Because sensitization of human bronchi by fenoterol was characterized by an increase in maximal contraction to ET-1, we investigated the human bronchi with ΔE_max of ET-1 >0 after 15 h of fenoterol exposure. Among the 48 bronchi, 38 (79%)...
Effect of incubation with anti-inflammatory drugs, proinflammatory mediator receptor antagonists, or NOS inhibitor on maximal contraction to ET-1 in the absence of fenoterol. In the absence of fenoterol, incubation of human bronchi for 15 h at 21°C with gliotoxine (10^{-6} M), dexamethasone (10^{-6} M), indomethacin (10^{-6} M), GR-32191 (10^{-7} and 10^{-6} M), MK-476 (10^{-8} and 10^{-7} M), L-NAME (10^{-3} M), SR-142801 (10^{-7} M), and SR-140333 + SR-48968 + SR-142801 + HOE-140 (10^{-7} M) had no significant effect on the E_{max} of ET-1 and ACh (Table 2).

Effect of coincubation with anti-inflammatory drugs, proinflammatory mediator receptor antagonists, or NOS inhibitor on fenoterol-induced sensitization. When the same paired human bronchi were incubated with fenoterol (10^{-7} M) for 15 h at 21°C, coincubation with gliotoxine, dexamethasone, indomethacin, GR-32191 (10^{-6} M), MK-476 (10^{-7} M for ACh, 10^{-8} and 10^{-7} M for ET-1), and combinations of SR-140333 + SR-48968 + SR-142801 or SR-140333 + SR-48968 + SR-142801 + HOE-140 significantly decreased the rise of maximal response to ET-1 and ACh elicited by fenoterol (control bars, Figs. 2 and 3). Incubation with L-NAME or SR-142801 had no significant effect on the sensitization induced by fenoterol (Fig. 2). Addition of HOE-140 to SR-140333 + SR-48968 + SR-142801 did not significantly increase the inhibition of SR-140333 + SR-48968 + SR-142801 on the fenoterol-induced sensitizing effect (Fig. 3).

Effect of coincubation with MAPK inhibitor, PKC blocker, and MAPK inhibitor + PKC blocker on fenoterol-induced sensitization. In preliminary experiments, we found that 10^{-6} M, but not 10^{-7} and 3 \times 10^{-7} M, SB-203580 inhibited the contraction of nine human bronchi induced by [Sar^{9},Met^{O2}]substance P, which significantly modified by incubation with these drugs (Table 1). Forskolin, a cAMP activator, increased the maximal contraction to ET-1 and ACh in a concentration-dependent manner (Table 1). Incubation with forskolin (10^{-6}, 3 \times 10^{-6}, and 10^{-5} M) had no significant effect on potency of ET-1 (Table 1).

Effect of incubation of human bronchi with fenoterol (10^{-7} M) with forskolin on potency of ET-1 and ACh. E_{max} was statistically significant when fenoterol (10^{-7} and 10^{-6} M), forntomerol (10^{-8} and 10^{-7} M), salbutamol (10^{-6} M), or salmeterol (10^{-6} M) was added to the incubation medium of bronchi (Table 1). Potency of ET-1 (-log EC_{50}) was not significantly different from control. Analysis of data was performed from this sample of 38 human bronchi.

**Fig. 1.** Concentration-response curves for endothelin-1 (ET-1) in human bronchi after incubation for 15 h at 21°C with 10^{-7} M fenoterol. \( \Delta E_{\text{max}} \), difference between maximal contraction (maximal efficacy) to ET-1 in pretreated bronchi and maximal contraction to ET-1 in paired control bronchi. Values are means ± SE (n = 48). *P < 0.01; **P < 0.001 vs. control.

**Table 1.** Effect of incubation of human bronchi with \( \beta_{2} \)-adrenoceptor agonists or forskolin on potency of ET-1 and maximal efficacy of ET-1 and ACh

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>n</th>
<th>(-\log \log EC_{50}) ET-1, log unit</th>
<th>(\Delta E_{\text{max}}, \text{g ET-1})</th>
<th>(\Delta E_{\text{max}}, \text{g ACh})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fenoterol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^{-7} M</td>
<td>38</td>
<td>0.12 ± 0.07</td>
<td>+0.88 ± 0.09†</td>
<td>+1.06 ± 0.13‡</td>
</tr>
<tr>
<td>10^{-6} M</td>
<td>6</td>
<td>0.17 ± 0.11</td>
<td>+0.91 ± 0.19†</td>
<td>+1.11 ± 0.24‡</td>
</tr>
<tr>
<td>Formoterol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^{-8} M</td>
<td>6</td>
<td>0.36 ± 0.22</td>
<td>+1.09 ± 0.22†</td>
<td>+1.11 ± 0.22‡</td>
</tr>
<tr>
<td>10^{-7} M</td>
<td>8</td>
<td>0.08 ± 0.21</td>
<td>+1.11 ± 0.32‡</td>
<td>+1.13 ± 0.43*</td>
</tr>
<tr>
<td>Salbutamol  (10^{-7} M)</td>
<td>7</td>
<td>0.12 ± 0.19†</td>
<td>+0.55 ± 0.11†</td>
<td>+0.70 ± 0.16*</td>
</tr>
<tr>
<td>Salmeterol  (10^{-6} M)</td>
<td>6</td>
<td>0.01 ± 0.14†</td>
<td>+0.73 ± 0.25*</td>
<td>+0.90 ± 0.35*</td>
</tr>
<tr>
<td>Forskolin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^{-6} M</td>
<td>8</td>
<td>0.49 ± 0.16</td>
<td>+0.16 ± 0.31</td>
<td>+0.34 ± 0.50</td>
</tr>
<tr>
<td>3 \times 10^{-7} M</td>
<td>8</td>
<td>0.42 ± 0.12</td>
<td>+1.06 ± 0.38*</td>
<td>+0.98 ± 0.47</td>
</tr>
<tr>
<td>10^{-7} M</td>
<td>8</td>
<td>0.42 ± 0.10</td>
<td>+1.54 ± 0.58*</td>
<td>+1.94 ± 0.73*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of bronchi. Bronchi were pretreated for 15 h at 21°C. \(\Delta(-\log EC_{50})\), difference between \(-\log EC_{50}\) (potency) obtained with pretreated bronchi and \(-\log EC_{50}\) obtained with paired control human bronchi; \(\Delta E_{\text{max}}\), difference between maximal contraction (maximal efficacy) to endothelin-1 (ET-1) and to acetylcholine (ACh) in pretreated bronchi and in paired control; *P < 0.05; †P < 0.01; ‡P < 0.001 vs. control.
Table 2. Effect of incubation of human bronchi with anti-inflammatory drugs, proinflammatory mediator receptor antagonists, or NO synthase inhibitor on maximal contraction to ET-1 and ACh in absence of fenoterol

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>n</th>
<th>$\triangle E_{max}, g$ ET-1</th>
<th>$\triangle E_{max}, g$ ACh</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gliotoxine (10$^{-6}$ M)</td>
<td>12</td>
<td>+0.22 ± 0.26</td>
<td>+0.28 ± 0.33</td>
</tr>
<tr>
<td>Dexamethasone (10$^{-6}$ M)</td>
<td>12</td>
<td>-0.06 ± 0.28</td>
<td>-0.21 ± 0.34</td>
</tr>
<tr>
<td>Indomethacin (10$^{-6}$ M)</td>
<td>12</td>
<td>+0.36 ± 0.37</td>
<td>+0.25 ± 0.41</td>
</tr>
<tr>
<td>GR-32191 10$^{-7}$ M</td>
<td>12</td>
<td>+0.25 ± 0.20</td>
<td>+0.48 ± 0.21</td>
</tr>
<tr>
<td>GR-32191 10$^{-6}$ M</td>
<td>12</td>
<td>+0.18 ± 0.20</td>
<td>+0.08 ± 0.24</td>
</tr>
<tr>
<td>MK-476 10$^{-8}$ M</td>
<td>12</td>
<td>-0.01 ± 0.35</td>
<td>-0.11 ± 0.40</td>
</tr>
<tr>
<td>MK-476 10$^{-7}$ M</td>
<td>12</td>
<td>+0.54 ± 0.39</td>
<td>+0.38 ± 0.39</td>
</tr>
<tr>
<td>L-NAME (10$^{-3}$ M)</td>
<td>12</td>
<td>+0.11 ± 0.39</td>
<td>-0.12 ± 0.49</td>
</tr>
<tr>
<td>SR-142801 (10$^{-7}$ M)</td>
<td>8</td>
<td>+0.49 ± 0.37</td>
<td>+0.27 ± 0.35</td>
</tr>
<tr>
<td>SR-140333 + SR-48968 + SR-142801 (10$^{-7}$ M)</td>
<td>8</td>
<td>+0.33 ± 0.49</td>
<td>+0.38 ± 0.63</td>
</tr>
<tr>
<td>SR-203580 (10$^{-7}$ M)</td>
<td>8</td>
<td>+0.09 ± 0.25</td>
<td>+0.46 ± 0.41</td>
</tr>
<tr>
<td>Calphostin C (10$^{-7}$ M)</td>
<td>8</td>
<td>-0.04 ± 0.22</td>
<td>-0.35 ± 0.27</td>
</tr>
<tr>
<td>Calphostin C + SB-203580 (3 × 10$^{-7}$ M)</td>
<td>8</td>
<td>-0.13 ± 0.35</td>
<td>-0.23 ± 0.48</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of bronchi. Bronchi were pretreated for 15 h at 21°C. Gliotoxine, nuclear factor-κB inhibitor; GR-32191; prostanoid and prostaglandin TP receptor antagonist; MK-476, cysteinyl leukotriene receptor antagonist; l-NAME, N-nitro-l-arginine methyl ester, a nitric oxide (NO) synthase inhibitor; SR-14033, SR-48968, and SR 142801, tachykinin NK1, NK2, and NK3 receptor antagonists; HOE-140, bradykinin B2 receptor antagonist; SB-203580, 38-kDa mitogen-activated protein kinase inhibitor; calphostin C, protein kinase C blocker. No difference was statistically significant.

acts through the COX and thromboxane synthase pathway (Table 3). Therefore, we used <10$^{-6}$ M SB-203580 (i.e., 10$^{-7}$ and 3 × 10$^{-7}$ M). In control experiments (absence of fenoterol), 15 h of incubation at 21°C with the inhibitor of p38MAPK SB-203580 (10$^{-7}$ M) alone and the PKC blocker calphostin C (10$^{-7}$ M) had no significant effect on the $E_{max}$ of ET-1 and ACh (Table 2). Addition of SB-203580 (3 × 10$^{-7}$ M) to calphostin C (10$^{-7}$ M) did not modify significantly the $E_{max}$ of ET-1 and ACh (Table 2). When the same paired bronchi were sensitized to ET-1 and ACh by fenoterol for 15 h at 21°C (control bars, Fig. 4), coinocubation with SB-203580 (10$^{-7}$ and 3 × 10$^{-7}$ M), calphostin C (10$^{-7}$ M), or calphostin C + SB-203580 significantly decreased the rise of maximal response induced by fenoterol (Fig. 4).

**Effects of the anti-inflammatory drugs, the proinflammatory mediator receptor antagonists, or an NOS inhibitor on maximal contraction to ET-1 and ACh after sensitization by fenoterol.** In control experiments (absence of fenoterol), 45 min of incubation at 37°C
SENI TION OF HUMA N B R O N CHUS BY β2-A GON ISTS

Table 3. Effects of indomethacin, SB-203580, and calphostin C on contraction of human bronchi induced by [Sar9, Met(O2)11] substance P

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Contraction, % ACh</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>34 ± 5</td>
</tr>
<tr>
<td>Indomethacin (10⁻⁶ M)</td>
<td>6 ± 5*</td>
</tr>
<tr>
<td>SB-203580</td>
<td></td>
</tr>
<tr>
<td>10⁻⁷ M</td>
<td>31 ± 5</td>
</tr>
<tr>
<td>3 × 10⁻⁷ M</td>
<td>32 ± 5</td>
</tr>
<tr>
<td>10⁻⁶ M</td>
<td>33 ± 5</td>
</tr>
<tr>
<td>Calphostin C</td>
<td></td>
</tr>
<tr>
<td>10⁻⁶ M</td>
<td>26 ± 6</td>
</tr>
</tbody>
</table>
| SB-203580 (3 × 10⁻⁷ M) + calphostin C (10⁻⁷ M) | 35 ± 10

Values are means ± SE of 9 bronchi pretreated for 15 h at 21°C. SB-203580: 38-kDa mitogen-activated protein kinase inhibitor; Con-tractions induced by [Sar9, Met(O2)11] substance P (10⁻⁷ M) are expressed as percentage of maximal contraction induced by 3 × 10⁻³ M ACh. *P < 0.01 vs. control; all other values are not significant.

with L-NAME (10⁻³ M) increased significantly the maximal contraction to ET-1 and ACh (Table 4). Incuba-tion for 45 min at 37°C with indomethacin, GR-32191, MK-476, or combinations of the tachykinin NK₁ + NK₂ + NK₃ receptor antagonists did not alter the maximal contraction of the human bronchi (Table 4). Addition of HOE-140, a bradykinin B₂ receptor antagonist, to the tachykinin NK₁ + NK₂ + NK₃ receptor antagonists did not modify the Eₘₐₓ of ET-1 and ACh of the bronchi (Table 4). When the same paired bronchi were sensitized to ET-1 and ACh by fenoterol for 15 h at 21°C (control bars, Fig. 5), incubation for 45 min at 37°C with indomethacin, GR-32191, MK-476, and L-NAME did not affect the sensitizing effect induced by fenoterol (Fig. 5). In contrast, incubation for

Table 4. Effect of incubation of human bronchi with anti-inflammatory drugs, proinflammatory mediator receptor antagonists, or NO synthase inhibitor on maximal contraction to ET-1 and ACh in absence of fenoterol

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>n</th>
<th>ET-1</th>
<th>ACh</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indomethacin (10⁻⁶ M)</td>
<td>8</td>
<td>-0.12 ± 0.25</td>
<td>-0.15 ± 0.33</td>
</tr>
<tr>
<td>GR-32191 (10⁻⁶ M)</td>
<td>8</td>
<td>+0.13 ± 0.19</td>
<td>+0.48 ± 0.22</td>
</tr>
<tr>
<td>MK-476 (10⁻⁷ M)</td>
<td>8</td>
<td>+0.00 ± 0.46</td>
<td>-0.01 ± 0.47</td>
</tr>
<tr>
<td>L-NAME (10⁻³ M)</td>
<td>8</td>
<td>+0.43 ± 0.13*</td>
<td>+0.41 ± 0.13*</td>
</tr>
<tr>
<td>SR-140333 + SR-48968</td>
<td>9</td>
<td>+0.18 ± 0.18</td>
<td>+0.30 ± 0.21</td>
</tr>
<tr>
<td>SR-140333 + SR-48968 + SR-142801</td>
<td>9</td>
<td>+0.08 ± 0.23</td>
<td>+0.18 ± 0.29</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of bronchi. Bronchi were pretreated for 45 min at 37°C. *P < 0.05 vs. control.

DISCUSSION

In this study, we observed an in vitro sensitization to ET-1 and ACh of human bronchi by fenoterol. This sensitization is not specific to fenoterol, inasmuch as we found the same phenomenon with formoterol, salbutamol, and salmeterol. We then investigated the transduction pathways involved in sensitization of human bronchi by β₂-adrenoceptor agonists and showed

Fig. 4. Effect of coincubation for 15 h at 21°C with calphostin C (a protein kinase C blocker), SB-203580 (a 38-kDa mitogen-activated protein kinase inhibitor), and calphostin C + SB-203580 on Eₘₐₓ of ET-1 and ACh in the presence of 10⁻⁷ M fenoterol. Values are means ± SE (n = 8). *P < 0.05; **P < 0.01 vs. control.

Fig. 5. Effect of incubation for 45 min at 37°C with indomethacin, GR-32191, MK-476, and L-NAME on Eₘₐₓ of ET-1 and ACh after sensitization of human bronchi by 10⁻⁷ M fenoterol for 15 h at 21°C (control bars). Values are means ± SE (n = 8). Difference between pretreated groups and paired control group was not statistically significant.
that the nuclear transcription factor NF-κB and p38MAPK play a pivotal role in this event. Furthermore, several inflammatory processes appear to be involved in the sensitization of human bronchi by fenoterol.

Sensitization of human bronchi is not limited to fenoterol but is also observed with several β2-adrenoceptor agonists in a range of concentrations known to cause submaximal relaxation (45). We found that prolonged exposure of human bronchi to fenoterol affects maximal efficacy of ET-1 but not its potency. Our results are in agreement with the work reported by Wang and colleagues (60), who showed that chronic fenoterol exposure increased maximal airway response to ACh but not ACh EC_{50} in guinea pigs. Potency of an agonist depends in part on the affinity of receptors for binding the agonist and in part on the efficiency with which agonist-receptor interaction is coupled to response. Maximal efficacy of an agonist is determined by the characteristics of the receptor-effector system involved. In this way, our results suggest that chronic exposure to β2-adrenoceptor agonists involves changes in the contractile proteins of human bronchi but does not alter affinity of the receptors for ET-1. In addition, forskolin, a cAMP activator, increased the maximal response to ET-1 and ACh of human bronchi in a concentration-dependent manner. This suggests that prolonged activation of the cAMP-protein kinase A (PKA) system could cause sensitization. Indeed, prolonged activation of cAMP-PKA may induce stimulation of proinflammatory nuclear transcription factors, such as NF-κB or AP-1 (1, 33), and may enhance the expression of several types of receptors, such as bradykinin B₁ and B₂ and NK₁ and NK₂ receptors implied in nonspecific airway hyperresponsiveness in animals and humans (23, 32, 36, 52). However, short-term activation of the cAMP-PKA system may decrease the activity of proinflammatory enzymes such as constitutive phospholipase A₂ (cPLA₂), COX-2, 5-lipoxygenase, and MAPK (38, 54, 57). Eickelberg and colleagues (16) showed that incubation with salmeterol or salbutamol induced, probably via calmodulin stimulation, a ligand-independent activation of the glucocorticoid receptor in cultured human lung fibroblasts and vascular smooth muscle cells. Our results conflict with these findings, but it is not well known whether inflammatory processes may regulate activation of the glucocorticoid receptor in human bronchus. In asthma, various studies underlined that long-acting β₂-adrenoceptor agonists exhibit very small, if any, anti-inflammatory effects when given alone (37).

Katsunuma and colleagues (36) showed that dexamethasone and cycloheximide (a protein synthesis blocker) inhibited the increased bovine tracheal smooth muscle contractile response to NKA induced by fenoterol. Saulnier and colleagues (52) abolished the fenoterol-induced tracheal sensitization in guinea pigs with two transcription factor NF-κB inhibitors (glitoxine and pyrrolidine dithiocarbamate). In agreement with these authors, we found that glitoxine (an NF-κB inhibitor) and dexamethasone (an NF-κB and AP-1 inhibitor) abolished the sensitization induced by fenoterol in human bronchi. These results underline the pivotal role played by NF-κB in the process of sensitization. NF-κB is involved in the expression of proinflammatory molecules and mediators (cPLA₂, COX-2, prostanoids, and leukotrienes) implicated in cellular events in asthma (2, 4, 6, 13). Effects of β2-adrenoceptor agonists on NF-κB pathways are not well known in humans. Korn and colleagues (39) recently showed that expression of interleukin-8, a proinflammatory cytokine stimulated in part by NF-κB, was markedly increased by formoterol (10^{-10} M) in cultured human bronchial epithelial cells. In contrast, Wilson and colleagues (62) observed a reduction of NF-κB expression in mucosal eosinophils and epithelial cells in bronchial biopsies from 10 atopic asthmatic patients after 8 wk of treatment with formoterol. In this study, formoterol did not reduce the immunoreactivity for adhesion molecules and proinflammatory cytokines stimulated by NF-κB, in contrast to glucocorticosteroid treatment. We found that indomethacin, GR-32191, and MK-476 significantly decreased or abolished the sensitization induced by fenoterol, suggesting that prolonged activation of the cAMP-PKA system by fenoterol may induce an enzymatic inflammatory process (cPLA₂ and COX-2) mediated by NF-κB.

We established that a mixture of tachykinin NK₁, NK₂, and NK₃ receptor antagonists decreased the sensitization elicited by fenoterol. In contrast to results obtained previously in the guinea pig trachea by Saulnier and colleagues (52), we found that the NK₃ receptor antagonist SR-142801, when used alone, did not significantly reduce the fenoterol-induced sensitization. NK₃ receptors seem to be involved in airway sensitization in guinea pigs (43), but its role is not well known in humans. However, SR-142801 has been shown to inhibit interleukin-1β-induced hyperresponsiveness to [Sar³, Met(O²)⁵]substance P (4) and nerve growth factor in human bronchi (19). Also, our results suggest that the tachykinin NK₁ and NK₂ receptor antagonists are involved in the mechanisms of sensitiza-
tion of human bronchi by fenoterol. This is in agreement with the recent works reported by Katsunuma and colleagues (35, 36), who found an increase of NK2 receptor expression in bovine tracheal smooth muscle after treatment with fenoterol.

Leukotrienes could amplify neurogenic inflammation by increasing release of the tachykinins from the C-fibers of the nonadrenergic noncholinergic system in asthma (28). We found that the bradykinin B2 receptor antagonist HOE-140 did not significantly enhance the inhibition of the fenoterol-induced sensitization by the tachykinin NK1, NK2, and NK3 receptor antagonists. Ricciardolo and colleagues (49) showed that a combination of the NK1 and NK2 tachykinin receptor antagonists abolished the increased bronchoconstriction produced by NKA and inhibited partially the contractile response induced by bradykinin in ovalbumin-sensitized guinea pigs, whereas HOE-140 had no effect on the increase in bronchoconstriction produced by NKA, suggesting that bradykinin induces the release of tachykinins from sensory nerves in guinea pig airways. Moreover, bradykinin may stimulate the MAPK pathways via the activation of the protein Rho GTPase, PKC, and NF-κB in human lung (29). Also, our study highlights the role of NF-κB, leukotrienes, prostanoids, tachykinin NK1 and NK2 receptor agonists, and bradykinin in the mechanisms of sensitization of human bronchi by fenoterol (Fig. 7).

Our results also show that SB-203580, a p38MAPK inhibitor, at a concentration that did not inhibit COX-1 or COX-2 activity and thromboxane synthesis (Table 3), abolishes the sensitization elicited by fenoterol. Recent studies underline the role of the MAPK in the intracellular processes of airway smooth muscle proliferation and sensitization (2, 30, 32, 46, 58, 61). Three distinct MAPK pathways have been identified in mammals: 1) p42/44MAPK, 2) stress-activated protein kinase (SAPK) or c-Jun NH2-terminal kinase (JNK), and 3) p38MAPK (15). MAPKs are involved in multiple proinflammatory mechanisms, implying humoral and neuronal mediators. Expression of NF-κB, cPLA2, COX-2, tachykinins, bradykinin, ETB, and muscarinic M3 receptors is upregulated by MAPK in airway smooth muscle cells (12, 23, 32, 53). Moreover, MAPK increases the Gs protein activity, which results in a functional uncoupling between Gs protein and β2-adrenoceptor (2, 32, 37). In addition, MAPK enhances the myosin light chain kinase activity and the heavy chain of myosin expression (32) and may increase smooth muscle contraction probably via h-caldesmon phosphorylation and actin-F remodeling (30). Interestingly, prostanoids such as thromboxane A2 stimulate the

![Fig. 7. Proposed mechanisms underlying sensitization of human airway smooth muscle induced by fenoterol. NF-κB, nuclear factor-κB; cPLA2, constitutive phospholipase A2; COX, cyclooxygenase; 5-LPO, 5-lipoxygenase; TxA2, thromboxane A2; MAPK, mitogen-activated protein kinase; PKA, protein kinase A.](http://ajplung.physiology.org/)}
MAPK by coupling the TP receptor with Gαq (activation of PKC) or Gβγ proteins (2, 32, 34), and leukotrienes increase the MAPK expression in humans (48, 49). These data suggest that MAPK pathways could amplify the inflammatory processes induced by NF-κB and could sensitize the airway smooth muscle after prolonged exposure to fenoterol (Fig. 7).

PKC is a cyclic nucleotide-independent protein kinase implicated in regulation of airway smooth muscle tone (61). PKCζ enhances the activity of the protein Raf1 and NF-κB, which activate the p38MAPK and SAPK/JNK pathways (12). Recent publications showed that bradykinin and thromboxane A₂ activate MAPK pathways via PKC-dependent Gαq protein coupling in human cells (20, 29). In addition, PKC may increase the contractility of the airway smooth muscle by inhibiting caldesmon (via the MAPK pathways) and calponin (directly), which are involved in modulation of the actin-myosin interaction (30, 61). We show here that blockage of PKC by calphostin C effectively inhibits fenoterol-induced sensitization. Thus our results suggest that PKC plays a major role in the intracellular mechanisms leading to fenoterol-induced sensitization of human airway smooth muscle (Fig. 7).

We also investigated the mechanisms involved in the increase of the contractility to ET-1 and ACh of human bronchi after sensitization by fenoterol in a protocol where drugs were added after incubation with fenoterol but 45 min before addition of ET-1 or ACh at 37°C for contraction. Our results showed that neither prostanoids nor leukotrienes were involved in this mechanism. In contrast, the NK₁, NK₂, and NK₃ receptors appeared to be implicated in the increase of contractility after sensitization by fenoterol as well as bradykinin, which tended to potentiate, but not significantly, inhibition of the fenoterol-induced sensitization by the NK₁, NK₂, and NK₃ receptor antagonists. In the absence of incubation with fenoterol, we found that tachykinins and bradykinin were not involved in the process of contraction to ET-1 and ACh. Additional studies are needed to clarify the role of tachykinins and bradykinin in the contraction mechanisms after sensitization by fenoterol in human bronchi.

Studies in animal and human airways have shown that the epithelial ETA receptor may mediate NO production (via the constitutive NOS) and prostaglandin E₂ production (via the epithelial COX-2) (3, 8, 31, 44). Epithelial NO and prostaglandin E₂ are relaxant for the airway smooth muscle. In a recent study, Naline and colleagues (44) found that NO is the major determinant of the epithelial regulation of the human airway smooth muscle contraction to ET-1. Our results are in agreement with these authors, because we showed that L-NAME, but not indomethacin and GR-32191, enhanced the contractility to ET-1. The epithelial regulation of the contractility to ACh was also mediated by NO. On the contrary, after sensitization by fenoterol, t-NAME failed to enhance the maximal contractility to ET-1 and ACh. We suggest that chronic exposure to fenoterol induces a disruption of the epithelial regulation of the airway smooth muscle contractility to ET-1 and ACh. Further investigations are needed to confirm and elucidate this mechanism.

Our study has several limitations. First, we studied bronchi obtained from nonhealthy subjects, who were all previous smokers. ΔEₘₐₓ was increased by fenoterol exposure in only 38 of 48 bronchi. β₂-Adrenoceptor polymorphisms inducing variable response to β₂-agonists may constitute a possible explanation of this fickle sensitizing effect (55). For instance, the Gly¹⁶ polymorphism could sensitize the bronchi to ENA, but not ACh. In a randomized study comparing clinical efficacy of nebulized vs. intravenous salbutamol in severe acute asthma, Salmeron and colleagues (51) found plasma concentrations of salbutamol on the order of 10⁻⁵ M. In healthy volunteers, plasma formoterol concentration reached 10⁻⁹ M after inhalation of a single dose of 120 µg of formoterol fumarate (40). Therapeutic plasma concentration of fenoterol of ~10⁻⁸ M was recommended (18), but plasma concentration associated with serious toxicity is not known.

In summary, our study demonstrates the proinflammatory effects of chronic exposure to β₂-agonists in human bronchus. Our results suggest that these proinflammatory effects are mediated by NF-κB and lead to sensitization of airway smooth muscle. MAPK, PKC, and tachykinins seem to play a major role in the sensitization of the human bronchus after chronic exposure to β₂-adrenergic agents.

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