Mechanisms of Impaired β-Adrenoceptor-induced Airway Relaxation by Interleukin-1β In Vivo in the Rat

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Abstract

We studied the in vivo mechanism of β-adrenergic receptor (β-AR) hyporesponsiveness induced by intratracheal instillation of interleukin-1β (IL-1β, 500 U) in Brown–Norway rats. Tracheal and bronchial smooth muscle responses were measured under isometric conditions ex vivo. Contractile responses to electrical field stimulation and to carbachol were not altered, but maximal relaxation induced by isoproterenol (10^{-6}–10^{-5} M) was significantly reduced 24 h after IL-1β treatment in tracheal tissues and to a lesser extent, in the main bronchi. Radioligand binding using [^{125}I]iodocyanopindolol revealed a 32±7% reduction in β-ARs in lung tissues from IL-1β–treated rats, without any significant changes in β_2-AR mRNA level measured by Northern blot analysis. Autoradiographic studies also showed significant reduction in β_2-AR in the airways. Isoproterenol-stimulated cyclic AMP accumulation was reduced by IL-1β at 24 h in trachea and lung tissues. Pertussis toxin reversed this hyporesponsiveness to isoproterenol but not to forskolin in lung tissues. Western blot analysis revealed an IL-1β–induced increase in $G_i\alpha$ protein expression. Thus, IL-1β induces an attenuation of β-AR–induced airway relaxation through mechanisms involving a reduction in β-ARs, an increase in $G_i\alpha$ subunit, and a defect in adenylyl cyclase activity. (*J. Clin. Invest.* 1996. 98:1780–1787.) Key words: β_2-adrenergic receptors • cyclic AMP • adenylyl cyclase • G protein

Introduction

Asthma is a disease characterised by the presence of chronic inflammation of the airways with infiltration of eosinophils, lymphocytes, and mast cells (1, 2), associated with bronchial hyperresponsiveness and bronchoconstriction. There is increasing evidence that a range of pro-inflammatory cytokines play an important role in orchestrating and perpetuating the airway inflammatory response in asthma (3). Among these pro-inflammatory cytokines, interleukin-1β (IL-1β) which is an important mediator controlling inflammatory and immune responses (4, 5) has been implicated in asthma. IL-1 has been measured in increased amounts in bronchoalveolar lavage fluid and in supernatants of alveolar macrophages from asthmatic patients (4, 6). In an allergen-sensitized and challenged guinea-pig model, an IL-1 receptor antagonist has been shown to inhibit allergen-induced bronchial hyperresponsiveness to histamine and substance P, in addition to the accompanying pulmonary infiltration with eosinophils and neutrophils (7–9). IL-1β administered to rats has also been shown to enhance airway responsiveness to bradykinin, in addition to inducing neutrophil infiltration in the airways and lungs (10).

Dysfunction of β-adrenergic receptors has long been postulated as a potential cause of bronchial hyperresponsiveness in asthma (11, 12). Some studies have demonstrated that airways from asthmatic patients fail to relax normally to isoproterenol, supporting a possible defect in β-receptor function in airway smooth muscle (13–15). Whether this is due to a reduction in β-receptors, a defect in receptor coupling, or some abnormality in the biochemical pathways leading to relaxation is not known. In a study of a single asthmatic patient, the density of β-receptors in airway smooth muscle appeared to be normal (16), and in another study, β_2-adrenoceptor mRNA expression in peripheral lung of asthmatics was increased (17). Such studies may be difficult to interpret given that patients with asthma often use β-adrenergic agonists regularly for relieving symptoms of their disease. On the other hand, that the defect in β-receptor function may result from the release of pro-inflammatory cytokines has been recently supported by the observation that incubation of isolated guinea-pig airways with IL-1β or TNF-α led to a reduction in isoproterenol-mediated relaxation in vitro (18). However, the potential mechanisms of any defect in β-receptor function have not been entirely elucidated, particularly in in vivo studies when cytokines such as IL-1β can induce inflammatory changes that may be dependent on an intact circulation, such as the recruitment of inflammatory cells.

In order to address this important issue, we examined the effects of the pro-inflammatory cytokine, IL-1β, administered in vivo on the contribution of β-adrenoceptor expression and of postreceptor coupled transmembrane events involved in β-adrenoceptor relaxation. We found that IL-1β administered directly to the airways of rats resulted in an impaired relaxation of tracheal tissues to isoproterenol in vitro. This impairment in β-adrenoceptor function was associated with a number of abnormalities including uncoupling of β-adrenergic receptors from adenylyl cyclase via increased expression of the inhibitory guanine nucleotide binding protein, G_i, and a reduction in the number of β-receptors and in adenylyl cyclase activity.

Methods

Intratracheal instillation of IL-1β

We used inbred, pathogen-free Brown-Norway rats (Harlan-Olac, Bicester, Oxon, UK) weighing 200–300 g for all studies. Animals were anesthetized with an i.p. injection of 2 mg/kg midazolam (Roche Products Ltd., Welwyn Garden City, UK) and a s.c. injection of 0.4 mg/kg Hypnorm (Janssen Pharmaceuticals Ltd., Wantage, UK), which contains 0.315 mg/ml of fentanyl citrate and 10 mg/ml of fluanisone.

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After adequate anesthesia was achieved, animals were intubated with a nylon cannula (1.02-mm OD), through which recombinant human IL-1β (500 U in 50 μl 0.9% NaCl solution) or 50 μl 0.9% NaCl solution (control group) were instilled intratracheally. We chose this dose of IL-1β because in a previous study we found neutrophil influx into the airways at its most prominent at the dose of 500 U (10).

Measurement of smooth muscle responses in vitro

Airway smooth muscle responses were measured at 4, 12, and 24 h in vitro after instillation of IL-1β or 0.9% NaCl solution. Rats were killed by a lethal dose of pentobarbitone (200 mg/kg i.p.). The lungs were quickly removed and placed in oxygenated modified Krebs-Henseleit (KH) solution of the following composition (mM): 118 NaCl, 5.9 KCl, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 25.5 $NaHCO_3$, and 5.05 glucose. Indomethacin (10^{-5} M) was present throughout experiments. The trachea and the left main bronchus were carefully cleared of adherent connective tissue. The trachea was opened longitudinally and cut into transverse strips ~ 3 mm in length containing three to four cartilaginous rings. The left main bronchus was prepared as rings of 2–3-mm thickness. Tracheal strips and bronchial rings were mounted in 15-ml organ baths containing KH solution, pH 7.4, bubbled with 95% O_2 and 5% CO_2 at 37°C. Tissues were allowed to equilibrate for 60 min under optimal resting tensions of 1.0 g for the tracheal strips and 0.5 g for the bronchial rings. Isometric contractile responses were measured with FT.03 force-displacement transducers (Grass Instruments Co., Quincy, MA) and recorded on a Graphtec Linearcorder polygraph (model Mark VII; Nantwich, Cheshire, UK).

Experimental protocol

Responses to cholinergic agonist. Cumulative concentration–response curves to carbachol (10^{-7} to 10^{-4} M) were determined. Tissues were then washed until tension returned to baseline value and were left for 20 min thereafter before measuring the response to isoproterenol.

Responses to β-adrenergic agonist. After tissues had been precontracted with 10^{-6} M carbachol, which is approximately the ED_{50} carbachol dose, cumulative concentration-response curves to isoproterenol (10^{-8} to 10^{-5} M) were measured. We chose 10^{-6} M of carbachol because in preliminary studies relaxation responses were found to be greatest at this concentration. Relaxation responses were expressed as a percent of maximal contraction induced by carbachol.

Responses to electrical field stimulation. We also evaluated responses of tracheal strips and bronchial rings to electrical field stimulation (EFS)[1] at 24 h after IL-1β administration. EFS was elicited by suspending the tracheal strips and bronchial rings between parallel platinum plate electrodes ~ 1.5 cm apart in 15-ml organ baths. Biphasic square-wave pulses were delivered for 20-s periods from an electrical stimulator with a voltage of 20 V at source and a pulse duration of 0.5 ms. For both tracheal and bronchial tissues pulses of increasing frequency (0.5–50 Hz) were delivered every 4 min. The contractions elicited by EFS were expressed as a percent of maximal response to carbachol.

Radioligand β-adrenergic receptor binding assay

Rat lung was minced coarsely with scissors and suspended in 10 vol of 25 mM Tris HCl buffer, pH 7.4, containing 0.32 M sucrose at 4°C, followed by homogenization with a homogenizer (model Polytron; Kinematica, Basel, Switzerland). The homogenate was centrifuged at 1,000 *g* for 10 min at 4°C to remove unhomogenized debris and the supernatant was then centrifuged at 40,000 *g* for 20 min at 4°C. The resulting pellet was washed and recentrifuged at the same speed. The final membrane was frozen in liquid nitrogen and stored at −80°C without loss of binding characteristics. Lung membranes at a protein concentration of 10 μg per tube were incubated with [^{125}I]iodocyanopindolol (ICYP, specific activity: 2,000 Ci/mmol; 3–100 pM) in the presence or absence of excess (-)-isoproterenol (Iso, 200 μM) in 25 mM Tris HCl buffer, pH 7.4, containing 154 mM NaCl and 1.1 mM ascorbic acid (to prevent oxidation of isoproterenol) in a final volume of 250 μl. Incubation was carried out in triplicate at 37°C for 120 min, which was found to be optimal for specific binding. The incubation was terminated by rapid filtration through Whatman GF/C glass-fiber filters, followed by washing three times with 5 ml ice-cold 25 mM Tris HCl buffer, pH 7.4. The filters were counted in the Auto-Gamma Counting System (model 5550; Packard Instrument Co., Downers Grove, IL) at an efficiency of 80%. Specific binding was calculated by subtracting nonspecific binding from total binding. Protein concentration was determined by the method of Lowry et al. (19), with BSA as a standard.

Receptor autoradiography

Parenchymal tissue was inflated by bronchial instillation of OCT embedding medium diluted 1:4 with PBS. All tissue samples were snap-frozen in isopentane cooled in liquid nitrogen and stored at −80°C until required. Serial frozen sections (10 μm) of parenchymal tissue were cut at −30°C, mounted, and thawed onto gelatinized glass slides. Sections were stored at −80°C for as long as 2 wk before use without loss of binding capacity. Receptor mapping was performed using the method as previously described (20). The slides were warmed to room temperature, washed in incubation buffer (25 mM Tris-HCl, 154 mM NaCl, 0.25% polypeptide, and 1.1 mM ascorbic acid, pH 7.4), and incubated with 25 pM ICYP at 37°C for 120 min. Nonspecific binding was determined by incubating adjacent sections with the same concentration of ICYP and 200 μM (-)-isoproterenol. For mapping of the β_2-receptors, serial sections were incubated with 25 pM ICYP with and without 0.1 μM CGP 20712A. After incubation, slides were washed twice for 15 min in ice-cold buffer (25 mM Tris-HCl, pH 7.4), rinsed in cold distilled water, then rapidly dried in a stream of cold air. Glass coverslips previously coated with Ilford K-5 emulsion were fixed to one end of the slide with cyanoacrylate adhesive and held in contact with the sections with butterfly clips. Slides were exposed to the emulsion for 4 d. The glass coverslip was developed in Kodak D-19 developer and fixed. Sections were stained with cresyl-fast violet and examined under an Axioplan universal microscope (Carl Zeiss, Oberleochem, Germany) equipped with dark- and bright-field illumination. Grain density was measured as optical density with a microscope connected to a computerized image analyzer (Seescan, Cambridge, UK), using a constant magnification. Values of optical density were corrected for background and nonspecific binding. No correction was applied for a possible nonlinearity of emulsion response, as the range of the measurement was small.

RNA extraction and Northern blot analysis

Rat lungs were dissected and total RNAs were isolated according to Chomzynski and Sacchi (21). Poly $(A)^+$ RNA was prepared using PolyTract mRNA system kit (Promega, Southampton, UK) according to the manufacturer's instructions. Samples of mRNA were size-fractioned on a 1% agarose/formaldehyde gel containing 20 mM morpholinosulfonic acid (MOPS), 5 mM sodium acetate and 1 mM EDTA, pH 7.0, and blotted onto Hybond-N filters (Amersham International plc., Buckinghamshire, UK) by capillary action using 20× SSC (standard saline citrate, 1× SSC, 0.15 mM NaCl and 0.015 M sodium citrate, pH 7.0). Random primer labeling was carried out with the 1.8-kb full-length fragment from a rat β_2-receptor cDNA obtained according to Gocayne et al. (22) and the 1.3-kb Pst1 fragment from rat glyceraldehyde-3-phosphate dehydrogenase (GADPH) cDNA using [α-^{32}P]dCTP (3000 Ci/mmol). Prehybridization and hybridization were carried out at 42°C with the labeled probes (~ 1.5×10^6 cpm/ml) in a buffer containing 50% formamide, 50 mM Tris-HCl, pH 7.5, 5× Denhardt's solution, 0.1% SDS, 5 mM EDTA, and 250 μg/ml denatured salmon sperm DNA. After hybridization the blots were washed to a stringency of 0.1× SSC, 0.1% SDS at 60°C before exposure at −80°C for 1–4 d to Kodak X-OMAT-S film with an intensifying screen. The intensity of the signals was then quantified by laser densitometry (New Discovery Series; pdi, Huntingdon Station, NY).

1. *Abbreviations used in this paper:* EFS, electrical field stimulation; GADPH, glyceraldehyde-3-phosphate dehydrogenase; IBMX, isobutylmethylxanthine.

Determination of cyclic AMP accumulation

Tissue blocks ($\sim 2 \times 2 \times 10$ mm) cut out from freshly excised lung tissues of each animal were placed in ice-cold KH solution and were then treated as follows: (*a*) Incubation in 1 ml KH solution with either no drugs (baseline), or 10^{-7}, 10^{-6}, or 10^{-5} M (-)-isoproterenol or 10^{-7}, 10^{-6} , or 10^{-5} M forskolin for 10 min at 37°C in the presence of the phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX; 10^{-4} M). In order to evaluate a possible involvement of a change in phosphodiesterase activity induced by IL-1β, we also measured isoproterenol (10^{-5} M)- or forskolin (10^{-5} M)-stimulated cAMP accumulation in lung tissues in the absence of IBMX. Responses of tracheal tissues to 10^{-5} M isoproterenol in the presence of 10^{-4} M IBMX were also determined. (*b*) Incubation in KH solution with or without cholera toxin (10 μg/ml) for 3 h at 37°C in 15-ml organ baths in the absence or presence of 10^{-4} M IBMX. (*c*) Incubation in KH solution with or without pertussis toxin (2 μg/ml) for 2 h at 37°C in 15-ml organ baths. The baths were aerated continuously with 95% O_2 and 5% CO_2. Tissues were then removed and incubated for 10 min at 37°C in 1 ml KH solution with either no drugs, or 10^{-5} M isoproterenol, or 10^{-5} M forskolin in the presence of 10^{-4} M IBMX. Tissues were removed, blotted, frozen in liquid nitrogen and stored at −80°C until assay for cAMP. cAMP was extracted from tissues by homogenization in 1 M trichloroacetic acid, followed by neutralization with sodium bicarbonate ($NaHCO_3$). The cAMP immunoassay was carried out in 0.05 M sodium acetate buffer, pH 6.2, in duplicate. Samples including standards containing known quantities of cAMP were acetylated by the addition of acetic anhydride and triethylamine and assayed for cAMP by radioimmunoassay using ^{125}I-cAMP as tracer (Amersham). cAMP concentrations were determined by interpolation from a standard curve and expressed as fmol–cAMP/mg wet weight.

Determination of G-protein expression

To determine the expression of the α-subunit of the inhibitory guanine nucleotide binding protein ($G_i\alpha$), Western blot analysis of membrane protein samples isolated from lung tissues from saline-treated (control, $n = 3$) and IL-1β–treated rats ($n = 4$) was performed. Lung tissue was ground in liquid nitrogen, followed by homogenization with the Polytron homogenizer (Kinematica) in 4 vol of 20 mM Tris-HCl, pH 7.4, 5 mM EDTA, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 100 μM leupeptin, and 2 mM benzamidine (Buffer A) with 250 mM sucrose. Nuclei and large particles were removed by centrifugation at 1,000 *g* for 10 min at 4°C. The supernatant was centrifuged at 40,000 *g* for 20 min at 4°C to get the plasma membrane pellet, which was then washed three times in Buffer A. The pellet was resuspended in 75 mM Tris-HCl, pH 7.5, 12.5 mM $MgCl_2$, 1.5 mM EDTA, and 2 mM DTT to give a protein concentration of 2–3 mg/ml. The protein concentration was measured by Bradford assay (23), using bovine serum albumin as a standard. Membrane protein (25 μg) was briefly boiled and fractionated in 12% SDS-polyacrylamide gel, followed by transfer to nitrocellulose membranes. The membranes were placed in 5% dried milk overnight to reduce nonspecific immunoreactivity and then incubated for 1 h with a rabbit polyclonal anti-G_i-common antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA; 1:500 dilution). G_i proteins were detected by enhanced chemiluminescence (Amersham International) after a 1 h-incubation with a 1:6000 dilution of an anti–rabbit horseradish peroxidase-linked secondary antibody (Amersham International) and subsequent exposure to Kodak X-omat-S film. The level of $G_i\alpha$ protein was quantified using laser densitometry (New Discovery Series; pdi). In a separate experiment, Western blot analysis for $G_i\alpha$ was performed using tracheal tissues as described above. Tracheal tissues from six saline-treated and six IL-1β–treated rats were pooled to provide enough membrane protein for Western analysis.

Materials

Unless otherwise stated, all drugs were purchased from Sigma Chemical Co. (Poole, Dorset, UK). Recombinant human IL-1β was a generous gift of Glaxo Laboratories Ltd. (Greenford, UK).

Data analysis

All values are expressed as means±SEM. Statistical differences between two groups were determined by Mann-Whitney U test. A P value less than 0.05 was regarded as significant.

Results

In vitro airway smooth muscle responsiveness. There was no significant difference in tracheal and bronchial responses both to

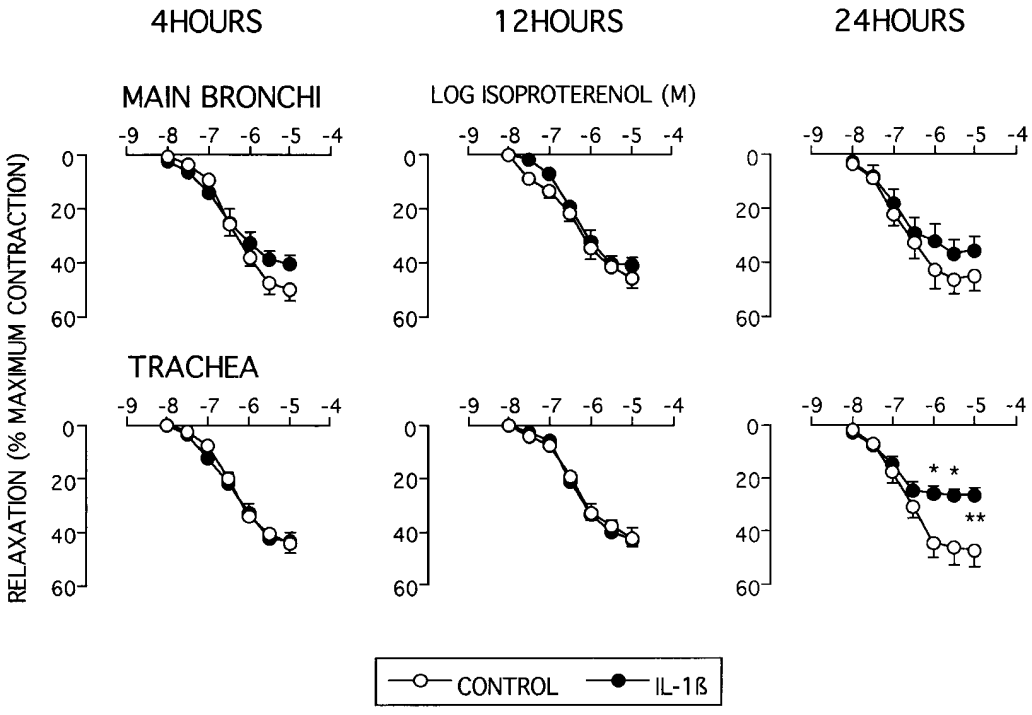

Figure 1. Isometric relaxation expressed as a percentage of maximal contraction induced by carbachol of bronchial ($n = 5$) and tracheal ($n = 7$) tissues in vitro obtained from Brown-Norway rats treated with intratracheal 0.9% NaCl (control) or interleukin-1β (IL-1β). Tissues were precontracted with carbachol (10^{-6} M). There was a significant attenuation of the relaxation response in tracheal tissues obtained from IL-1β–treated rats. $*P < 0.05$ and $**P < 0.01$ compared to control. Data shown as mean±SEM.

Table I. Effect of IL-1β on β-Adrenergic Receptor Binding in Rat Lung Membranes

	K_d (pM)	B_{max} (fmol/mg protein)
Saline-treated	8.32±0.34* ($n = 5$)	275.7±21.9 ($n = 5$)
IL-1–treated	7.34±0.20 ($n = 5$)	186.8±13.1‡ ($n = 5$)

*Results are given as mean ±SEM. The number of animals is denoted in parentheses. ‡$P > 0.05$ vs. saline-treated animals.

EFS at 24 h and to carbachol at 4, 12, and 24 h between saline- and IL-1β–treated animals (data not shown). We standardized relaxation responses to isoproterenol by expressing them as percentage of the corresponding maximal carbachol response. Fig. 1 shows the effect of IL-1β instillation on the responses to isoproterenol. Preincubation of tissues with 10^{-6} M propranolol or 10^{-6}M ICI 118551, a β_2-selective antagonist, completely abolished responses to isoproterenol up to 10^{-6} M, while 10^{-6} M CGP 20172A, a β_1-selective antagonist, had no effect on these responses, confirming that the relaxation induced by isoproterenol was mediated by β_2-adrenoceptors (data not shown). IL-1β caused a significant reduction of tracheal relaxation induced by 10^{-6} to 10^{-5} M of isoproterenol at 24 h after instillation ($P < 0.05$). The concentration of isoproterenol needed to cause 50% relaxation was not significantly different between control and IL-1β–treated animals (0.152±0.043 vs. 0.143±0.045 μM, respectively). Although there was a similar reduction in the responses to isoproterenol in the main bronchi at 24 h after instillation, the difference was not statistically significant. No difference was observed between the two groups at 4 and 12 h after treatment in either trachea or main bronchi.

β-Adrenoceptor binding to lung membranes. Saturation isotherms for specific binding of ICYP to rat lung membranes were best described by assuming the presence of a single class of saturable, high-affinity binding sites. IL-1β caused a 32±7% reduction of the maximal number of β-adrenergic binding sites (B_{max}, $P < 0.05$) in peripheral lung membranes with no significant change in the affinity of binding (K_d, Table I), as assessed by binding of the non-selective β-adrenergic receptor antagonist, ICYP.

Receptor autoradiography. Specific labeling of β-adrenergic binding sites were observed over airway smooth muscle and epithelium and over vascular smooth muscle. Heavy labeling was also localized over the alveoli. Changes in regional β_2-AR subtype due to IL-1β was determined by competition with the selective β_1-antagonist, CGP20712A. In airway and vascular smooth muscle, labeling was reduced by 36.2±5.6% and 40.3±3.5% reductions, respectively after IL-1β–treatment (Figs. 2 and 3). The reduction of β-adrenergic binding sites in lung membranes was reflected by a 65.7±7.1% reduction in labeling over the alveolar walls (Fig. 4). Although there was a low degree of labeling in tracheal sections, a reduction in labeling over tracheal smooth muscle was also observed in IL-1β–treated rats compared to control (data not shown).

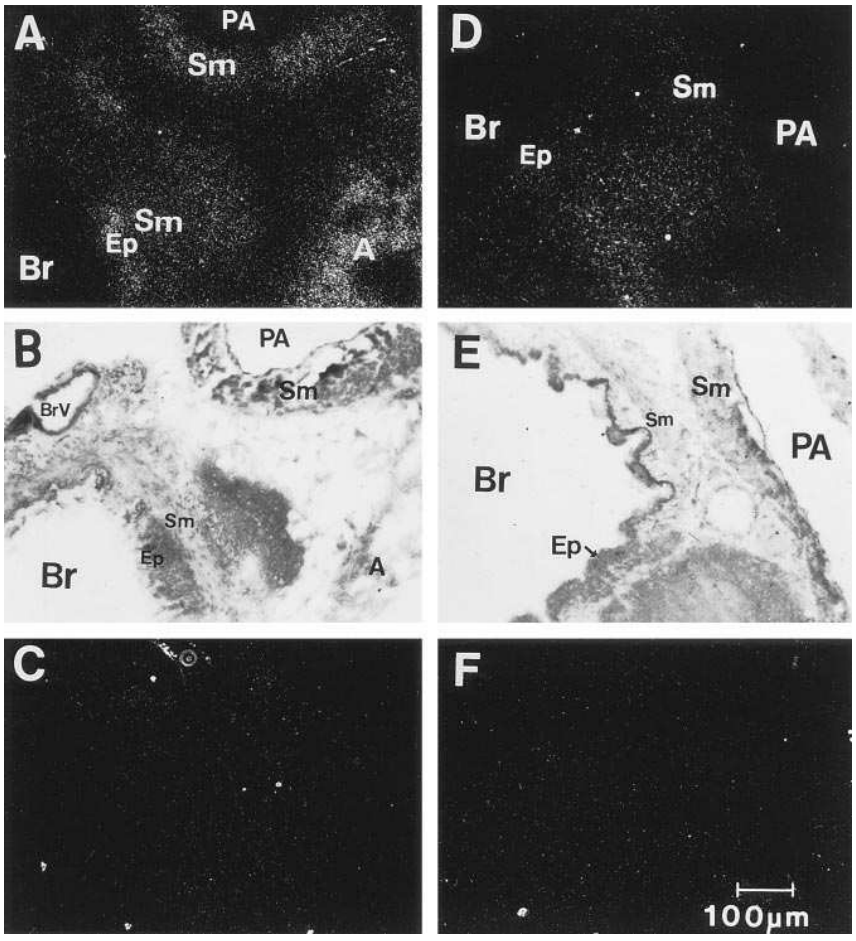

Figure 2. Distribution of β_2-adrenergic binding sites in rat lung. Left-hand panels represent a saline-treated rat, and right-hand panels, an IL-1β-treated rat. (*A*, *D*) Darkfield photomicrographs of autoradiograms demonstrating the distribution of β_2AR to rat lung after incubation sections with ICYP in the presence of 0.1 μM CGP 20712A. (*B*, *E*) Brightfield view of sections (from top panels) stained with 1% cresyl fast violet. (*C*, *F*) Darkfield photomicrographs of adjacent lung sections incubated with ICYP in the presence of 200 μM (-)-isoproterenol, showing nonspecific binding. *Abbreviations*: PA, pulmonary artery; Ep, epithelium; Sm, smooth muscle; A, alveolar walls.

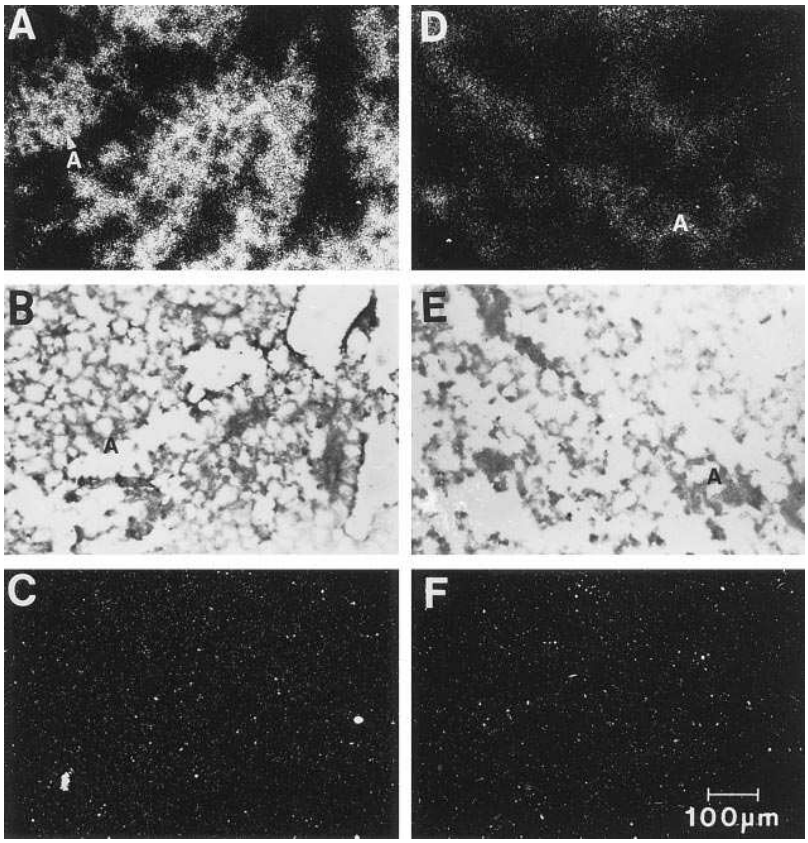

Figure 3. Distribution of β_2-adrenergic binding sites to alveoli (*A*). Left panels represent a saline-treated rat, and right panels, an IL-1β–treated rat. (*A*, *D*) Darkfield photomicrographs of autoradiograms demonstrating the distribution of β_2AR to rat lung after incubating sections with ICYP in the presence of 0.1 μM CGP 20712A. (*B*, *E*) Brightfield view of sections (from top panels) stained with 1% cresyl fast violet. (*C*, *F*) Darkfield photomicrographs of adjacent lung sections incubated with ICYP in the presence of 200 μM (-)-isoproterenol, showing nonspecific binding.

Northern blot analysis for β_2-adrenoceptor mRNA. To address the question of whether IL-1β induced any changes in gene expression of β_2-adrenergic receptor mRNA in rat lung, steady state levels of cellular β_2-receptor mRNA was measured by Northern blot analysis. Using a rat β_2-adrenergic receptor cDNA probe, we detected a single transcript around 2.2 kb from all lungs studied (Fig. 5). To account for differences in loading or transfer of the RNA, the blots were hybridized with a 1,272 bp PstI fragment from rat GAPDH cDNA. Fig. 5 summarizes the mean β_2-adrenergic receptor/GAPDH mRNA ratios over the time investigated and shows that neither saline nor IL-1β treatment affected the β_2-adrenergic receptor mRNA levels.

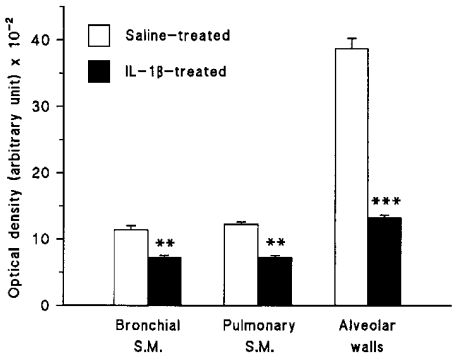

Figure 4. Pulmonary β_2AR distribution in saline-treated ($n = 4$) and IL-1β–treated ($n = 4$) rats. Values are obtained by optical density (OD) measurement of multiple sections from each animal. Each bar is mean±SEM of 36 quantitated fields (9 fields/animal). OD was measured with a microscope connected to a computerised image analyzer (Seescan, Cambridge, UK), using a constant magnification. Values of OD were corrected for background and nonspecific binding. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to saline-treated sham-stimulated rats.

cAMP accumulation to isoproterenol and forskolin. In lung tissues in the presence of the phosphodiesterase inhibitor IBMX, isoproterenol-stimulated cAMP accumulation was significantly less in IL-1β–treated group than in control by ~ 50% at all three doses used while there was no significant difference in the basal cAMP accumulation. Forskolin-stimulated cAMP accumulation was also attenuated at 10^{-6} and 10^{-5} M in the IL-1β group compared with control (Fig. 6, *top*). In the absence of IBMX, the basal cAMP accumulation and the isoproterenol-stimulated cAMP response did not differ significantly between control and IL-1β–treated rats (42.5±3.6 vs. 47.8±4.3 fmol/mg wet weight and 148.5±14.2 vs. 119.2±10.1 fmol/mg wet weight, respectively), but the response to forskolin was reduced by IL-1β treatment (506.6±52.9 to 355.1±42.3, $P < 0.001$). These results indicate that the reduced induction of cAMP accumulation to isoproterenol and forskolin in IL-1β–treated lungs is unlikely to be due to an increase in phosphodiesterase activity. In tracheal tissue, there was also a significant decrease in isoproterenol-stimulated cAMP accumulation, which was less than that found in lung tissue (Fig. 6, *bottom*).

We determined whether ADP-ribosylation of the inhibitory guanine nucleotide binding protein, G_i, with pertussis toxin could modulate the attenuated cAMP response after IL-1β. In tissues incubated at 37°C for 2 h without pertussis toxin, responses to isoproterenol (10^{-5} M) and forskolin (10^{-5} M) were attenuated in IL-1β–treated tissues when compared to sham-treated tissues (Fig. 7). However, in the presence of pertussis toxin (2 μg/ml), IL-

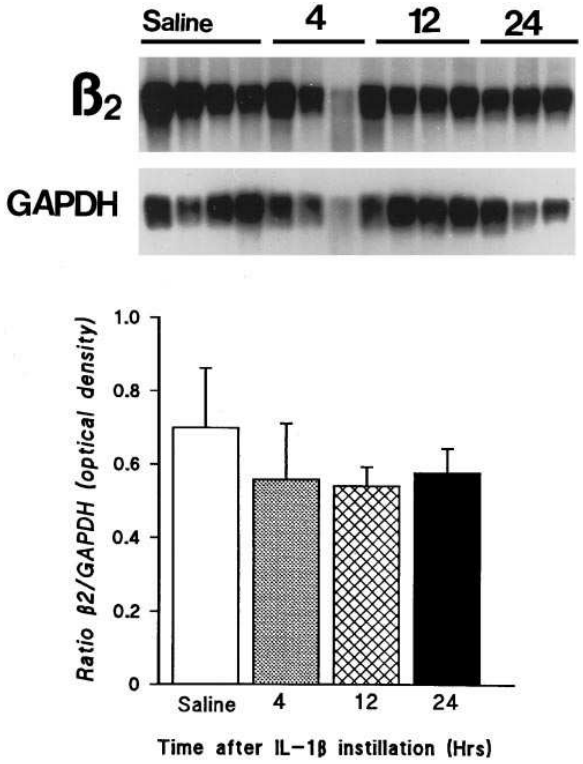

Figure 5. (*Top*) Northern blotting for β_2-adrenergic receptor in rat lungs treated with saline and 4, 12, and 24 h after IL-1β instillation (4, 12, 24, respectively). (*Bottom*) The optical density of the β_2-receptor mRNA signals standardised by GAPDH-optical density. There was no statistical difference between saline- and IL-1β–treated animals at 4, 12, and 24 h. Data shown as mean±SEM. *Abbreviations*: GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

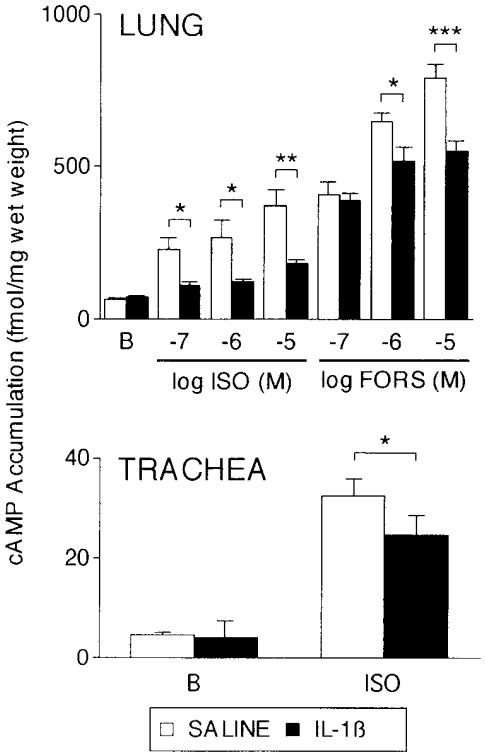

Figure 6. Upper panel. Cyclic adenosine monophosphate (cAMP) accumulation in lung tissue obtained from saline-treated (*solid bars*, $n = 3$) and interleukin-1β (*open bars*, $n = 3$) at 24 h in vitro following baseline conditions (*B*), isoproterenol (ISO, 10^{-7}, 10^{-6}, and 10^{-5} M) and forskolin (FORS, 10^{-7}, 10^{-6}, and 10^{-5} M) in the presence of isobutylmethylxanthine (IBMX). There was a significant attenuation of the responses to all three doses of isoproterenol and to 10^{-6} and 10^{-5} M forskolin in IL-1β–treated rats. (*Lower panel*) cAMP accumulation in tracheal tissue obtained from saline-treated ($n = 3$) and IL-1β–treated ($n = 3$) rats at 24 h following baseline conditions (*B*) and 10^{-5} isoproterenol (ISO). There was a significant attenuation of the isoproterenol-stimulated cAMP accumulation in IL-1β–treated rats. $^*P < 0.01$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared to control. Data shown as mean±SEM.

1β–induced hyporesponsiveness to isoproterenol but not to forskolin was reversed while baseline cAMP levels were unchanged.

We also determined whether cholera toxin stimulation of cAMP production via stimulatory G (G_s) protein ADP-ribosylation was affected by IL-1β treatment. cAMP accumulation in lung tissue were similar between control and IL-1β–treated animals both in the absence or presence of IBMX, indicating that the activity of G_s was not affected by IL-1β (data not shown).

G_i protein expression. Because pertussis toxin reversed the IL-1β–induced cAMP hyporesponsiveness to isoproterenol, we examined the expression of G_i protein in the trachea and lung tissues. $G_i\alpha$-common protein expression was significantly increased 24 h after IL-1β treatment. Fig. 8 shows Western blotting for $G_i\alpha$-common protein, with a significant increase in chemiluminescence from 1.41±0.15 to 2.22±0.20 optical density (arbitrary units) after IL-1β treatment ($P < 0.05$). $G_i\alpha$-common protein expression was also increased in the trachea by approximately 17% (data not shown).

Discussion

To elucidate the mechanisms by which pro-inflammatory cytokines may contribute to impairment in β_2-adrenoceptor-induced airway relaxation, we have examined the effects of IL-1β administered to the lung in vivo on the relative contribution of changes in β-adrenoceptor numbers and affinity, and postreceptor coupled transmembrane events. Our studies show that IL-1β causes a significant reduction in β-adrenergic–induced relaxation of tracheal strips precontracted with carbachol. This was observed at the maximal degree of relaxation without any significant shift of the concentration–relaxation curve. A similar trend was observed in the bronchial preparations, but this did not achieve statistical significance. These changes occurred without any significant effects of IL-1β pretreatment on the contractile responses induced by either carbachol or EFS.

The reduction in β-adrenergic-induced tracheal relaxation was accompanied by a generalized reduction in the number of β_2-adrenergic receptors in airway and vascular smooth muscle and in the alveolar walls, as assessed by autoradiography. In addition, this was accompanied by a significant reduction in the number of β-adrenergic binding sites without any significant changes in the binding affinity of lung membranes. The reduction in β-adrenergic binding sites over tracheal smooth muscle may, at least partly, contribute to the reduction in the maximal relaxation to isoproterenol. Interestingly, the number of β-adrenergic receptors has been reported to remain unchanged when guinea-pig airway smooth muscle was incubated with IL-1β in vitro (24), despite a significant reduction in the maximal relax-

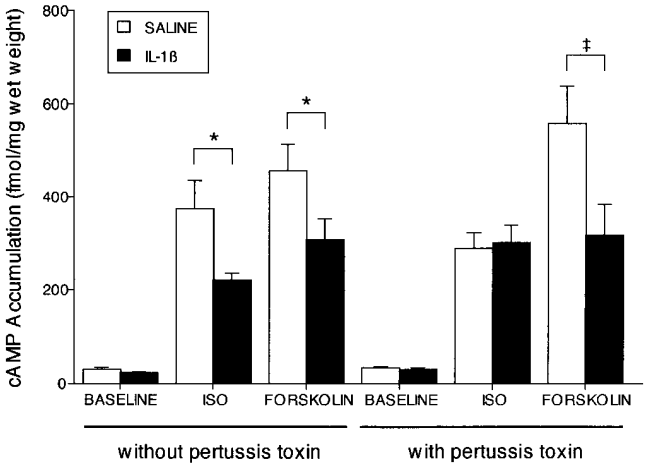

Figure 7. Cyclic adenosine monophosphate (cAMP) accumulation in lung tissue obtained from saline-treated (control, $n = 3$) and interleukin-1β (IL-1β, $n = 3$) at 24 h in vitro following 2 h incubation at 37°C in modified Krebs-Henseleit solution under continuous aeration with 95% oxygen and 5% carbon dioxide. There was a significant attenuation of the isoproterenol (10^{-5} M) and forskolin (10^{-5} M) responses in IL-1β–treated rats (*left*). The IL-1β–induced cAMP hypo-responsiveness to isoproterenol was reversed by ADP-ribosylation by pertussis toxin (2 μg/ml) while hyporesponsiveness to forskolin was not affected (*right*). $^{*}P < 0.05$; $^{\ddagger}P < 0.0005$ compared with control. Data shown as mean±SEM.

ation induced by isoproterenol. Another study reported an increase in the number of β-adrenergic receptors in a lung adenocarcinoma cell line after incubation with IL-1β in vitro (25). Our observation that the reduction in β-adrenergic receptors is not accompanied by significant changes in β_2-adrenergic receptor mRNA indicate that post-translational mechanisms are involved. Taken in the context of the in vitro study of Wills-Karp et al. (24), our results suggest that the inflammatory response evoked by IL-1β may contribute to the reduction in the number of β-adrenergic receptor binding sites observed in the present study. The mechanisms by which the inflammatory process may lead to such an effect remains unknown. Reactive oxygen species released from activated macrophages and hydrogen peroxide inhibit β_2-adrenergic responsiveness in guinea-pig and rat airway smooth muscle, respectively (26, 27). In the Brown-Norway rat, IL-1β–induced increase in bronchial hyperresponsiveness to inhaled bradykinin is partly mediated through the release of reactive oxygen species (28).

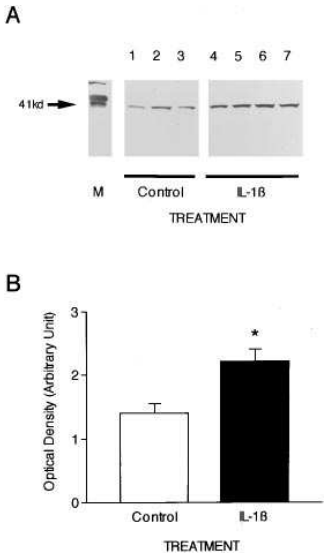

Figure 8. Western blotting with anti-$G_i\alpha$–common antibody in membrane preparations from saline-treated (control) and IL-1β–treated (IL-1β) rats at 24 h after treatment. (*A*) Western blotting showing single band corresponding $G_i\alpha$-protein in each lane. Lanes 1, 2, and 3 represent samples from saline treated rats, and lanes 4–7, from IL-1β–treated rats. (*B*) Mean optical density of $G_i\alpha$-common protein visualized on Western blots. IL-1β treatment caused a significant increase in $G_i\alpha$-common protein expression. $^{*}P < 0.05$ compared to control. Data shown as mean±SEM.

In addition to the reduction in the number of β-adrenergic receptors, we also observed a decrease in isoproterenol-stimulated cAMP accumulation both in tracheal and lung tissues, indicating uncoupling of the β-receptor to adenylyl cyclase. This effect could be directly due to IL-1β because several studies have demonstrated that incubation of IL-1β with various tissues in vitro such as guinea-pig trachea and rat cardiac myocytes leads to an inhibition of β_2-adrenergic responsiveness through changes in the coupling of G-proteins and adenylyl cyclase (24, 29). In our study, incubation of lung tissues with pertussis toxin, which inhibits the action of the G_i-protein by ADP-ribosylation, reversed the IL-1β–induced cAMP hyporesponsiveness to isoproterenol, thus indicating that the IL-1β–induced impairment was due to enhanced G_i-protein–coupled inhibition of adenylyl cyclase. Further support for a role for G_i in IL-1β–induced cAMP hyporesponsiveness to isoproterenol is shown by the increase of 17% and 57% in $G_i\alpha$ protein observed on Western blot analysis in IL-1β–treated trachea and lung tissues, respectively, consistent with a recent in vitro study of rabbit airway smooth muscle (30). Our data indicate that there are regional differences with regard to the effects of IL-1β on G_i protein expression. The topographical differences in the action of IL-1β may result from several factors. Because IL-1β concentration may have been highest in the trachea, it may exert its strongest effect on tracheal tissue, thus contributing to a greater IL-1β–induced impairment of relaxation response as compared to bronchi. In addition, regional differences in β-receptor density may be responsible with tracheal tissues being more susceptible as the receptor density is less in trachea than in more peripheral tissues. The mechanisms by which IL-1β may lead to an increase in G_i expression in the lung are unknown and remain to be elucidated. Some studies have indicated that the $G_i\alpha_2$ subtype is induced by IL-1β at the mRNA level in guinea pig tracheal smooth muscle (31) and in cultured human endothelial cells (32), indicating that IL-1 may increase the transcription of G_i. Our studies are in general agreement with the evidence that β-adrenoceptor stimulation is downregulated by receptor-mediated activation of G_i protein (33, 34). The lack of effect of cholera toxin-catalyzed ADP-ribosylation on IL-1-induced cAMP accumulation indicates that the stimulatory G-protein linked to β-receptors, G_s protein, is not altered in this model.

In addition to uncoupling of the β-adrenergic receptor to adenylyl cyclase, there may also be a defect in adenylyl cyclase as the increase in cAMP accumulation in response to forskolin, a nonselective direct adenylyl cyclase activator (35), was also reduced in IL-1β–treated rats. Pertussis toxin had no effect on this reduced response to forskolin while restoring the attenuated response to isoproterenol, suggesting an abnormality independent of G_i protein, and of β-receptor-linked adenylyl cyclase activity. Indeed, individual adenylyl cyclase species appear to be uniquely regulated by a variety of stimuli (36) and a specific subtype of adenylyl cyclase (Type IV) is inhibited by G_i-linked receptors (37, 38). Our data also indicate that any increase in the activity of phosphodiesterase is not responsible for the impaired β_2-adrenergic receptor response induced by IL-1β instillation, because a phosphodiesterase inhibitor

did not inhibit the IL-1β–induced attenuation of cAMP response to isoproterenol and to forskolin.

In summary, intratracheal instillation of rh-IL-1β caused a reduction in maximal relaxant responses to isoproterenol in the trachea and to a lesser extent in the bronchi. The density of β_2-adrenoceptors was reduced without any changes in β_2-receptor mRNA expression, indicating internalization or degradation of β_2-adrenoceptors rather than inhibition of β_2-adrenoceptor gene transcription. There was a reduction in isoproterenol-induced adenylyl cyclase activation, which could be reversed by pertussis toxin, in association with increased $G_i\alpha$ protein expression in lung tissues. The hyporesponsiveness to isoproterenol and the increase in $G_i\alpha$ protein expression were also noted in IL-1β–treated tracheal tissue. A defect in adenylyl cyclase was also evident in lung tissues with an impairment of forskolin-induced cAMP accumulation, independent of the increase in G_i expression. Thus, the in vivo effect of a pro-inflammatory cytokine, IL-1β, leads to a series of effects that can contribute to impaired airway–smooth muscle relaxation to β-adrenergic agonists. These mechanisms may account for attenuated airway responses to β-adrenergic stimulation in chronic inflammatory airway conditions such as asthma.

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