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Effect of short- and long-acting β_2-adrenoceptor agonists on pulmonary β_2-adrenoceptor expression in human lung

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Abstract

β-Adrenoceptor agonists induce the down-regulation of β_2-adrenoceptors and mRNA expression in animal lung. The down-regulation of β_2-adrenoceptors may limit the therapeutic efficacy of β_2-adrenoceptor-mediated bronchodilators in obstructive airways disease. We examined the effects of three selective β_2-adrenoceptor agonists, salbutamol, salmeterol and formoterol on β_2-adrenoceptor binding and mRNA levels in human lung in vitro. Human lung was obtained from cardiac transplantation donors. Peripheral lung was chopped and incubated with three selective β_2-adrenoceptor agonist for 3 h or 24 h at three different concentrations (0.1, 1 and 10 μM). The affinity and density of β_2-adrenoceptors was determined by [^{125}I]iodocyanopindolol equilibrium binding in a lung membrane preparation in the presence of 0.1 μM CGP 20712 A (1-{2-[(3-carbamoyl-4-hydroxy)phenoxy]ethylamino}-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)phenoxy]-propan-2-ol), a selective β_1-adrenoceptor antagonist. Although treatment with salbutamol for 3 h did not change β_2-adrenoceptor density, both salmeterol and formoterol reduced β_2-adrenoceptor density, and exposure to each agonist for 24 h reduced β_2-adrenoceptor density at all concentrations. Treatment with 10 μM salmeterol increased the eqilibrium dissociation constant (K_d), and also shifted the competition curves of (−)-isoprenaline to the left. β_2-Adrenoceptor mRNA, measured by Northern blot analysis using a human β_2-adrenoceptor cDNA probe, was reduced after exposure to all β_2-adrenoceptor agonists at 3 h. Our data provide evidence for down-regulation of β_2-adrenoceptor protein and mRNA after selective β_2-adrenoceptor agonist treatment in human lung.

Keywords: Down-regulation; β_2-Adrenoceptor; Salbutamol; Salmeterol; Formoterol; Lung, human

1. Introduction

Selective β_2-adrenoceptor agonists are far the most effective bronchodilators in current use for the treatment of asthma. When taken by inhalation they rapidly relieve symptoms and protect against all known bronchoconstrictor mechanisms, since they are functional antagonists and are effective on large and small airways (Nelson, 1995). It is common clinical practice for β_2-adrenoceptor agonists to be given as a regular medication, but this may mask the underlying inflammatory process and the need for anti-inflammatory drugs (Barnes and Chung, 1992; Barnes, 1995). There is some evidence that regular treatment with β_2-adrenoceptor agonists makes asthma more difficult to control (Sears et al., 1990; Taylor et al., 1993) and may even increase airway inflammation (Manolitsas et al., 1995).

Whether desensitization or tolerance to inhaled β_2-adrenoceptor agonists is a clinical problem is still not clear. Although there is no loss of bronchodilator response to selective β_2-adrenoceptor agonists after regular treatment (Tattersfield, 1985; O'Connor et al., 1992), several studies demonstrated loss of protection against bronchoconstrictor challenges with both short-acting β_2-adrenoceptor agonists (O'Connor et al., 1992; Cockcroft et al., 1993), and the long-acting β_2-adrenoceptor agonists, salmeterol and formoterol (Cheung et al., 1992; Yates et al., 1995). Down-regulation after selective β_2-adrenoceptor agonist use could limit the therapeutic efficacy of β_2-adrenoceptor-mediated bronchodilation therapy in bronchial asthma (Barnes and Chung, 1992). Prolonged administration of β_2-adrenoceptor agonists induces the down-regu-

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lation of β_2-adrenoceptor protein and mRNA expression in cultured cells in vitro (Hadcock and Malbon, 1988), and in animal lungs in vivo (Nishikawa et al., 1993, 1994). Chronic β_2-adrenoceptor agonist therapy in asthmatic patients reduces β_2-adrenoceptor density in circulating polymorphonuclear leukocytes and lymphocytes (Galant et al., 1978; Aarons et al., 1983; Brodde et al., 1985; Hataoka et al., 1993), but it is not clear to which extent down-regulation can occur in human lung. The predominant β-adrenoceptor subtype in human lung are the β_2-adrenoceptors (70%), but β_1-adrenoceptors (30%) are also present, mostly in the alveolar walls (Carstairs et al., 1985; Hamid et al., 1991). In the present study, we examined the effects of the short-acting β_2-adrenoceptor agonist salbutamol, and the long-acting β_2-adrenoceptor agonists salmeterol and formoterol, on β_2-adrenoceptor binding sites and mRNA levels in human lung in vitro.

2. Materials and methods

2.1. Experimental procedure

Fresh human lungs were obtained from 7 normal donors (4 male, age 18–48 years, mean age 27.8 years), in which the hearts were used for cardiac transplantation. The donors were maintained on ventilation prior to organ donation and were treated with inhaled β_2-adrenoceptor agonists. Macroscopically normal areas of the lung were dissected free of pleura, large vessels, large airways and connective tissue. The peripheral lung tissues containing mainly bronchioles and small pulmonary blood vessels were chopped into small pieces. Incubation was performed in Ham's F-12 medium (ICN, Thame, UK) supplemented with 2 mM glutamate, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B at 37°C in an incubator containing 95% air/5% CO_2. The chopped lung was placed in 100 mm dishes and incubated in the absence and presence of each selective β_2-adrenoceptor agonist, salbutamol, salmeterol or formoterol for 3 h or 24 h at three concentrations (0.1, 1 and 10 μM) (Kerrebijin, 1991). After washing, the tissue was frozen for the measurement of β_2-adrenoceptor affinity and density, and the affinity of β-adrenoceptors to (−)-isoprenaline. Furthermore, to investigate the change in mRNA levels, the tissue treated for 3 h was also stored for the assessment of steady-state β_2-adrenoceptor mRNA level.

2.2. Radioligand receptor binding assay

The minced lung, suspended in 10 volumes of 25 mM Tris-HCl buffer (pH 7.4, Sigma, Poole, UK) containing 0.32 M sucrose (Sigma) at 4°C, was then homogenized with a Polytron homogenizer (Kinematica, Littau-Lucerne, Switzerland) at setting 6 in 30 s bursts. The homogenate was centrifuged at $1000 \times g$ for 10 min at 4°C to remove unhomogenized debris and the supernatant was then centrifuged at $40\,000 \times g$ for 20 min at 4°C, the resulting pellet being washed and recentrifuged at the same speed. The final membranes were resuspended in incubation buffer, frozen as aliquots in liquid nitrogen and stored at −80°C, without loss of binding characteristics (Engel, 1981; Carstairs et al., 1985).

Portions of lung membrane at a protein concentration of 7.5 μg per tube were incubated with a range of [^{125}I]iodocyanopindolol (specific activity: 2000 Ci/mM, Amersham International, Amersham, UK) concentrations from 3 to 250 pM in the presence or absence of excess (−)-isoprenaline (200 μM, Sigma) in 25 mM Tris-HCl buffer (pH 7.4) containing 154 mM NaCl and 1.1 mM ascorbic acid (to prevent oxidation of isoprenaline) in a final volume of 250 μl. Incubation was carried out at 37°C for 120 min, which was found to be optimal for specific binding. Incubations were performed in triplicate. The incubation was terminated by rapid filtration through Whatman GF/C glass-fibre filters. Each filter was rapidly washed with 3×5 ml ice-cold 25 mM Tris-HCl buffer (pH 7.4). The filters were counted in an Auto Gamma Counting System (model 5550, Packard Instrument, Pangbourne, UK) at an efficiency of 80%. Specific binding was calculated by subtracting non-specific binding from total binding. Protein concentration was determined by the method of Lowry et al. (1951), with bovine serum albumin (Sigma) as standard.

The density of β_2-adrenoceptors was analyzed by [^{125}I]iodocyanopindolol equilibrium binding in the presence of 0.1 μM CGP 20712 A (1-{2-[(3-carbamoyl-4-hydroxy)phenoxy]ethylamino}-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)phenoxy]-propan-2-ol), a selective β_1-adrenoceptor antagonist, a concentration at which practically all β_1-adrenoceptors are occupied (Dooley et al., 1986). The affinity of β-adrenoceptors to (−)-isoprenaline was measured by competition curves of [^{125}I]iodocyanopindolol in the presence of (−)-isoprenaline (10^{-10} to 10^{-4} M).

Equilibrium dissociation constant (K_d), maximal binding capacity (B_{max}), Hill coefficient (n_H) and IC_{50} (concentration of drug producing 50% inhibition) were obtained from individual experiments using the computer program GraphPad InPlot (ISI Software, San Diego, CA, USA).

2.3. Northern blot analysis

Random primer labeling was carried out with the 439 bp *Sma*I fragment from human β_2-adrenoceptor cDNA and the 1272 bp *Pst*I fragment from rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA using [α-^{32}P]dCTP (3000 Ci/mmol; Amersham).

Total RNA from human lung was isolated according to Chromczynski and Sacci (1987). Total cellular RNA (20 μg per lane) was subjected to electrophoresis on a 1% w/v agarose, 18% formaldehyde gel and blotted onto

Table 1
Effects of selective β_2-adrenoceptor agonists (10 μM) on equilibrium dissociation constant (K_d) and maximal binding capacity (B_{max}) of [^{125}I]iodocyanopindolol binding in the presence of CGP 20712 A in human lung membranes

		K_d (pM)		B_{max} (fmol/mg protein)	
		Control	Treatment	Control	Treatment
SALB	3 h	20.09 ± 3.97	18.29 ± 3.50	96.86 ± 11.55	70.51 ± 6.81
	24 h	16.09 ± 3.15	12.54 ± 1.61	79.06 ± 12.33	47.14 ± 10.96 [a]
SALM	3 h	20.09 ± 3.97	55.02 ± 14.80 [a]	96.86 ± 11.55	33.67 ± 10.49 [b]
	24 h	16.09 ± 3.15	113.92 ± 35.11 [b]	79.06 ± 12.33	35.31 ± 6.18 [b]
FORM	3 h	20.09 ± 3.97	20.56 ± 3.822	96.86 ± 11.55	48.53 ± 7.60 [b]
	24 h	16.09 ± 3.15	20.89 ± 5.56	79.06 ± 12.33	43.60 ± 5.35 [b]

SALB, salbutamol; SALM, salmeterol; FORM, formoterol. [a] $P < 0.05$; [b] $P < 0.01$ compared to corresponding value.

Hybond-N membranes (Amersham) by capillary blotting. After prehybridization for 4 h at 42°C in buffer containing 5 × Denhardt's solution, 5 × standard saline citrate (SSC), 10 mM NaH_2PO_4, 0.1% sodium dodecyl sulfate (SDS), 250 μg/ml sonicated denatured salmon sperm DNA and 50% formamide, the blot was hybridized for 20 h at 42°C with ^{32}P-labeled human β_2-adrenoceptor DNA probe (1×10^6 cpm/ml) in prehybridization buffer. Each blot was washed twice with 2 × SSC/0.1% SDS at room temperature and twice with 2 × SSC/0.1% SDS for 30 min, once with 1 × SSC/0.1% SDS for 30 min at 42°C and finally with 0.1 × SSC/0.1% SDS for 20 min at 55°C, and exposed at −80°C for 7 days to Kodak X-OMAT S film with an intensifying screen. After autoradiography, blots were stripped for reprobing with GAPDH cDNA probe.

The autoradiograms were scanned with a laser densitometer (Protein and DNA ImageWare System, Discovery Series, New York, NY, USA). The amount of β_2-adrenoceptor mRNA was quantified relative to the amount of GAPDH mRNA on the same filter.

2.4. Analysis of results

The experimental data are expressed as mean ± standard error of the mean (S.E.M.). Groups of the data were evaluated by analysis of variance (ANOVA) following Dunnet's test for comparing the means of multiple groups using the computer program GraphPad InStat. Values of $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. Effects of selective β_2-adrenoceptor agonists on β_2-adrenoceptor affinity and density

The β_2-adrenoceptor affinity and density after incubation with selective β_2-adrenoceptor agonists, salbutamol (10 μM), salmeterol (10 μM) and formoterol (10 μM), are summarized in Table 1. Incubation with salbutamol for 3 h caused no change in β_2-adrenoceptor density, whereas incubations with salbutamol for 24 h or with salmeterol and formoterol for 3 h and 24 h caused 40–65% reduction in B_{max} values compared with control incubation. There was a significant reduction in β_2-adrenoceptor density after incubation with salbutamol for 24 h and with salmeterol and formoterol for 3 h and 24 h (Figs. 1 and 2). The K_d values after treatment with 10 μM salmeterol for 3 h and 24 h were significantly increased compared to the corresponding control values, whereas K_d was unaffected after incubation with salbutamol and formoterol.

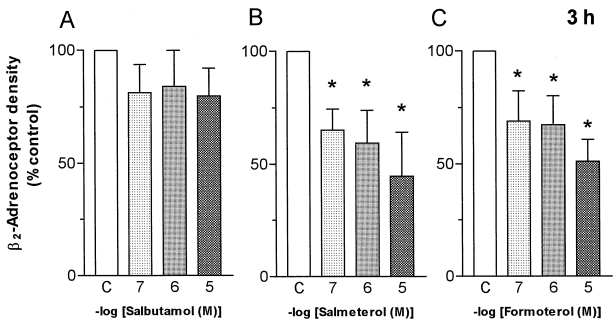

Fig. 1. Effects of salbutamol (panel A), salmeterol (panel B) and formoterol (panel C) treatment for 3 h on β_2-adrenoceptor density in human lung. Mean values (±S.E.M.) are shown, expressed as percent change from the control value; significance of difference from the control value: * $P < 0.05$.

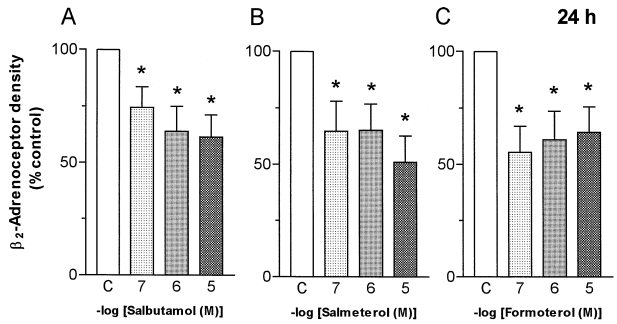

Fig. 2. Effects of salbutamol (panel A), salmeterol (panel B) and formoterol (panel C) treatment for 24 h on β_2-adrenoceptor density in human lung. Mean values (±S.E.M.) are shown, expressed as percent change from the control value; significance of difference from the control value: * $P < 0.05$.

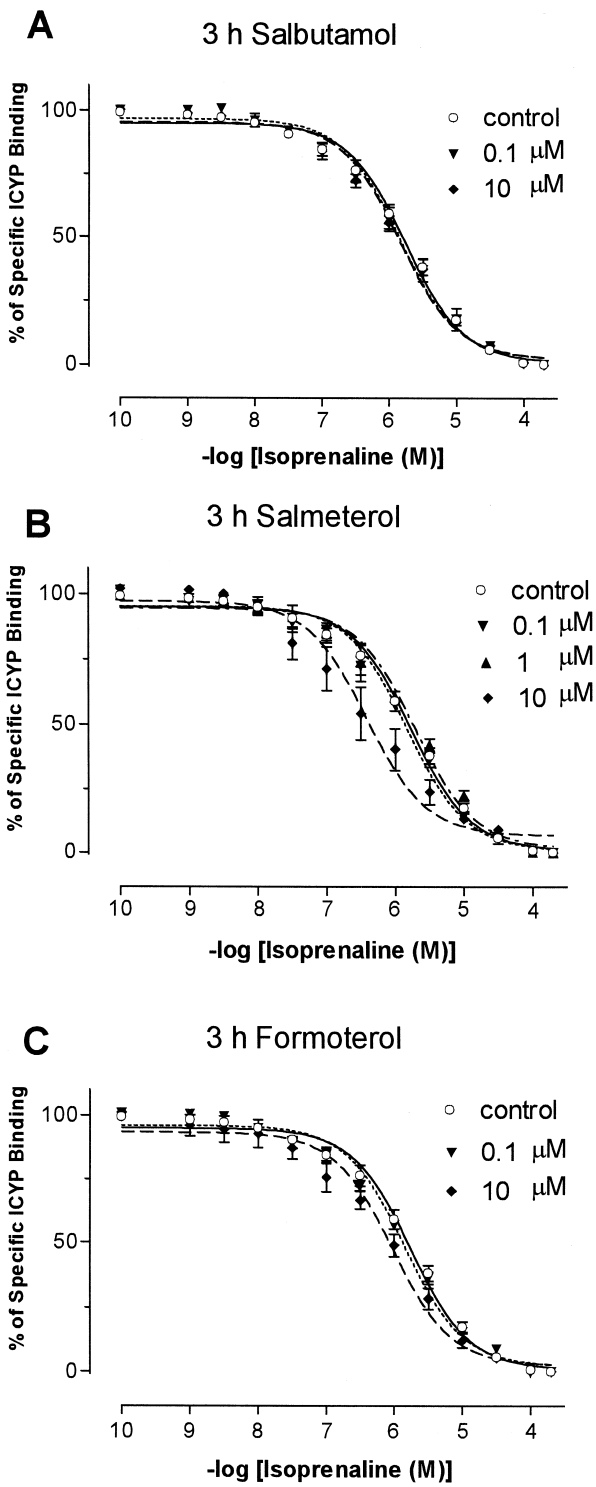

Fig. 3. Competition curves of (−)-isoprenaline for [^{125}I]iodocyanopindolol binding to human lung, treated for 3 h with salbutamol (panel A), salmeterol (panel B) and formoterol (panel C). Each data point represents the mean of seven lung membrane preparations. Only the curve obtained from human lung tissue incubated with 10^{-5} M salmeterol showed a significant leftward shift.

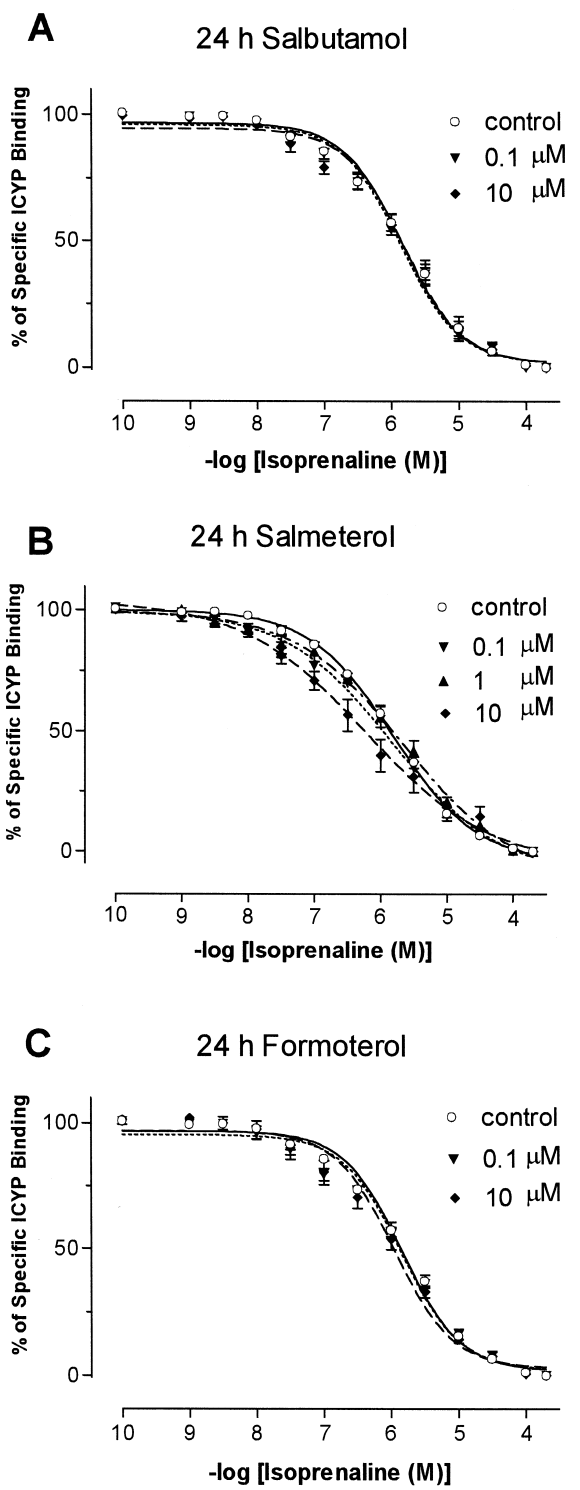

Fig. 4. Competition curves of (−)-isoprenaline for [^{125}I]iodocyanopindolol binding to human lung, treated for 24 h with salbutamol (panel A), salmeterol (panel B) and formoterol (panel C). Each data point represents the mean of seven lung membrane preparations. Only the curve obtained from human lung tissue incubated with 10^{-5} M salmeterol showed a significant leftward shift.

Table 2
Effects of selective β_2-adrenoceptor agonists (10 μM) on Hill coefficient (n_H) and pIC_{50} ($-\log IC_{50}$) values of (−)-isoprenaline competition curves for [^{125}I]iodocyanopindolol binding sites in human lung membranes

		n_H		pIC_{50} ($-\log IC_{50}$)	
		Control	Treatment	Control	Treatment
SALB	3 h	0.78 ± 0.05	0.71 ± 0.03	5.85 ± 0.07	5.93 ± 0.05
	24 h	0.76 ± 0.03	0.70 ± 0.03	5.91 ± 0.04	5.92 ± 0.10
SALM	3 h	0.78 ± 0.05	0.57 ± 0.05 [a]	5.85 ± 0.07	6.57 ± 0.12 [b]
	24 h	0.76 ± 0.03	0.58 ± 0.05 [a]	5.91 ± 0.04	6.28 ± 0.14 [a]
FORM	3 h	0.78 ± 0.05	0.65 ± 0.06	5.85 ± 0.07	6.00 ± 0.05
	24 h	0.76 ± 0.03	0.69 ± 0.04	5.91 ± 0.04	5.99 ± 0.09

SALB, salbutamol; SALM, salmeterol; FORM, formoterol. [a] $P < 0.05$; [b] $P < 0.01$ compared to corresponding control value.

3.2. Effects of selective β_2-adrenoceptor agonists on the affinity of β-adrenoceptors for (−)-isoprenaline

The Hill coefficient (n_H) and pIC_{50} values of (−)-isoprenaline for the sites labelled by [^{125}I]iodocyanopindolol binding in human lung after incubation with selective β_2-adrenoceptor agonists are shown in Table 2. Competition curves for (−)-isoprenaline on [^{125}I]iodocyanopindolol binding exhibited a steep monophasic shape characteristic of a homologous class of receptor in control, salbutamol- and formoterol-treated lung membranes (Figs. 3 and 4). Treatment with 10 μM salmeterol for 3 h and 24 h shifted the competition curves for (−)-isoprenaline to the left and significantly reduced n_H and IC_{50} values, indicating the existence of high and low affinity states of the receptor (Figs. 3 and 4).

3.3. Effects of selective β_2-adrenoceptor agonists on β_2-adrenoceptor mRNA level

Fig. 5 illustrates the representative autoradiograms of the Northern blot analysis of human lung tissues treated without or with various concentrations of salbutamol, salmeterol and formoterol using human β_2-adrenoceptor cDNA probe. There were single transcripts of 2.2 kb, in agreement with our previous studies (Hamid et al., 1991; Mak et al., 1995a). The small variation between lanes was corrected by the hybridization signal with GAPDH cDNA probe. Although treatment with each of the three selective β_2-adrenoceptor agonists at 0.1 μM for 3 h did not affect the β_2-adrenoceptor mRNA expression, treatment at 1 and

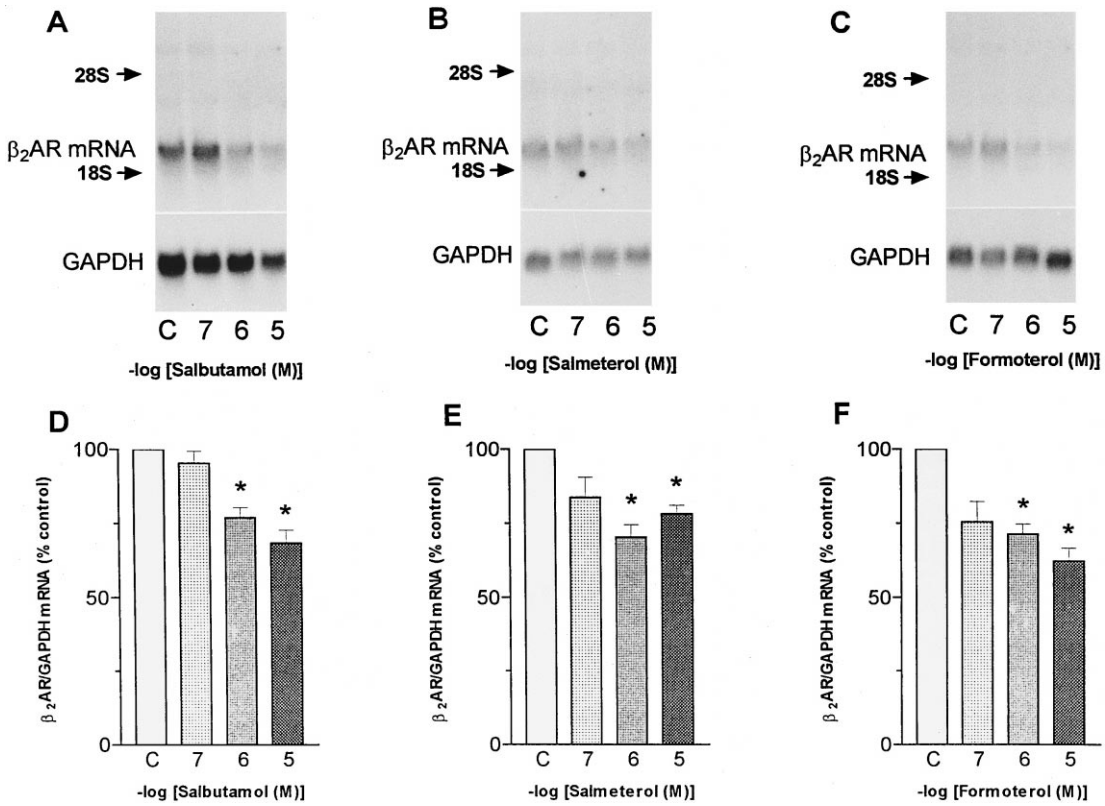

Fig. 5. Effects of salbutamol, salmeterol and formoterol treatment on β_2-adrenoceptor mRNA expression in human lung. Panels A–C: Representative Northern blot analysis of human β_2-adrenoceptor mRNA (upper panels) and GAPDH mRNA (lower panels) after incubation with salbutamol (panel A), salmeterol (panel B) and formoterol (panel C) at indicated concentrations for 3 h. The size of the mRNA was estimated from rRNA markers as 2.2 kb. Panels D–F: Densitometric measurement of β_2-adrenoceptor mRNA from control and after treatment for 3 h with salbutamol (panel D), salmeterol (panel E) and formoterol (panel F). Relative β_2-adrenoceptor mRNA abundance was expressed as percent change from the control value; significance of difference from the control value: * $P < 0.05$.

10 μM significantly reduced it to 62–78% (mean range) of the control ($P < 0.05$, respectively; Fig. 5D, E, F).

4. Discussion

In this study, we investigated the effects of three selective β_2-adrenoceptor agonists, salbutamol, salmeterol and formoterol, on β_2-adrenoceptor protein and steady state mRNA level in human lung in vitro. Our receptor binding study using the ligand [^{125}I]iodocyanopindolol in the presence of CGP 20712 A, a selective β_1-adrenoceptor antagonist, showed a reduction in β_2-adrenoceptor density after treatment with salmeterol and formoterol for 3 h, and to all three selective β_2-adrenoceptor agonists after 24 h. Furthermore, Northern blot analysis showed a reduced β_2-adrenoceptor mRNA expression after treatment with all three selective β_2-adrenoceptor agonists at 1 and 10 μM for 3 h.

Our findings are in agreement with previous studies demonstrating down-regulation of β_2-adrenoceptors in cell lines, animal lung and human leukocytes. Hadcock and Malbon (1988) revealed the down-regulation of β_2-adrenoceptor protein and mRNA expression after chronic stimulation of a hamster vas deferens cell line (DDT_1MF-2) with a β-adrenoceptor agonist. We have also reported the down-regulation of β_2-adrenoceptors and steady state mRNA level in guinea pig and rat lung after prolonged treatment of β-adrenoceptor agonists (Nishikawa et al., 1993, 1994). Furthermore, several radioligand binding experiments with polymorphonuclear leukocytes and lymphocytes from normal humans and asthmatics have demonstrated down-regulation of β_2-adrenoceptors after in vitro and in vivo treatment with β_2-adrenoceptor agonists (Galant et al., 1978; Aarons et al., 1983; Brodde et al., 1985; Hataoka et al., 1993). In contrast, Hauk et al. (1990) have failed to demonstrate the down-regulation of pulmonary β_2-adrenoceptors in lung from patients treated with the β_2-adrenoceptor agonist terbutaline before undergoing lobectomy for lung cancer. The difference between these results and our own may relate to their patients in which some of them have been on bronchodilators, such as theophylline, before preoperative treatment with subcutaneous injection of terbutaline. This route of administration may be less susceptible for pulmonary β_2-adrenoceptor down-regulation.

Competition curves for (−)-isoprenaline on [^{125}I]iodocyanopindolol binding exhibited a steep monophasic shape characteristic of a homologous receptor state, even in control lung membranes. This could reflect prior β_2-adrenoceptor agonist therapy in the donors, who were routinely treated in intensive care units. High and low affinity states of β-adrenoceptors are well demonstrated by the (−)-isoprenaline competition curves for radioligand binding. Treatment with a β-adrenoceptor agonist results in a significant shift of the (−)-isoprenaline competition curve to the right, indicating a change from high to low affinity state of the receptors in frog erythrocytes (Kent et al., 1980) and human lymphocytes (Meurs et al., 1987). In contrast, we found no shift of the (−)-isoprenaline competition curves after treatment with either salbuatamol or formoterol, but there was a significant shift from the right to the left after salmeterol treatment. We also found an apparent decrease in β_2-adrenoceptor density after incubation with salmeterol and formoterol for 3 h, but no significant decrease with salbutamol. This may reflect occupation of the β_2-adrenoceptors with the long-acting β_2-agonists rather than a loss of receptors. However, a reduction in β_2-adrenoceptor density after 24 h with all three β_2-adrenoceptor agonists is more likely to reflect a loss of receptors and is consistent with the reduction in mRNA. With salmeterol treatment, there was an apparent reduction in binding affinity (K_d) and a leftward shift of the competition curves for (−)-isoprenaline, which was not observed with either salbutamol or formoterol treatments. This may reflect the high lipophilicity and prolonged binding of this compound.

Several mechanisms of desensitization have been described. Short-term exposure (a few minutes) to β_2-adrenoceptor agonists results in receptor phosphorylation by both cAMP-dependent protein kinase (protein kinase A) and specific β_2-adrenergic receptor kinase, whereas more prolonged exposure results in changes in transcription or mRNA stability (Barnes, 1995). The reduction in β_2-adrenoceptor mRNA after incubation with all three β_2-adrenoceptor agonists is consistent with decreased synthesis of β_2-adrenoceptors after prolonged agonist exposure, and this measurement is independent of continued occupation of β_2-adrenoceptors on the cell surface.

The effects of selective β_2-adrenoceptor agonists on the expression of β_2-adrenoceptor protein and mRNA in human lung indicates that selective β_2-adrenoceptor agonists have the ability to down-regulate β_2-adrenoceptors, but the clinical relevance of this is not yet clear. We have previously demonstrated that glucocorticoids increase transcription of β_2-adrenoceptors in human lung in vitro (Mak et al., 1995a) and in animal lungs in vivo (Mak et al., 1995b). This suggests that glucocorticoid treatment in asthma may counteract the down-regulation of β-adrenoceptors and this may be particularly relevant with long-acting β_2-adrenoceptor agonists. Current recommendations are that patients should not be treated with long-acting inhaled β_2-adrenoceptor agonists unless concurrently treated with inhaled steroids (Guidelines British Thoracic Society, 1993).

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