Protective Effects of a Glucocorticoid on Downregulation of Pulmonary β_2-Adrenergic Receptors In Vivo

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Abstract

We investigated the in vivo effects of a glucocorticoid on β-agonist–induced downregulation of β_1- and β_2-adrenergic receptors (determined by [^{125}I]iodocyanopindolol binding), mRNA expression (assessed by Northern blotting), and gene transcription (using nuclear run-on assays) in rat lung. Dexamethasone (Dex) (0.2 mg/kg/d, days 1–8) increased β_1- and β_2-receptor numbers by 70 and 69% above control, respectively, but did not change their mRNA expression. Isoproterenol (Iso) (0.96 mg/kg/d, days 2–8) decreased β_1- and β_2-receptor numbers by 48 and 51%, respectively, and also reduced mRNA expression by 69 and 57%, respectively. The combination of Dex and Iso resulted in no net change in β_2-receptor number and its mRNA expression, although there was a significant reduction in β_1-receptor number and mRNA expression. The mapping of β_1- and β_2-receptors by receptor autoradiography confirmed these findings over alveoli, epithelium, endothelium, and airway and vascular smooth muscle. We also measured the activation of the transcription factor, cyclic AMP response element binding protein (CREB) using an electrophoretic mobility shift assay. CREB-like DNA-binding activity was decreased after Iso treatment but this decrease was prevented after treatment with Dex. Nuclear run-on assays revealed that the transcription rate of the β_1-receptor gene did not alter after Dex treatment, but was reduced after Iso treatment. The transcription rate of the β_2-receptor gene was increased after Dex treatment by approximately twofold, but there was no change after Iso treatment. We conclude that glucocorticoids can prevent homologous downregulation of β_2-receptor number and mRNA expression at the transcriptional level without affecting β_1-receptors and that the transcription factor CREB may be involved in this phenomenon. Such an effect may have clinical implications for preventing the development of tolerance to β_2-agonists in asthmatic patients treated with β-agonist bronchodilators. (*J. Clin. Invest.* 1995. 96:99–106.) Key words: glucocorticosteroid • upregulation • downregulation • mRNA expression • β-adrenergic receptors • CREB

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Introduction

Glucocorticosteroids and β_2-adrenergic agonists are the mainstay of asthma treatment and are usually given together (1). β-Adrenergic agonists cause bronchodilation predominantly in the activation of β_2-adrenergic receptors (β_2-receptors) on airway smooth muscle cells (2). There has been concern that regular use of inhaled β_2-agonists may result in tolerance to their beneficial effects in asthma. Although there is no loss of bronchodilator response to β_2-agonists, several studies have demonstrated loss of protection against various bronchoconstrictor challenges (3, 4) and this may be relevant to the reduced asthma control seen with the regular use of inhaled β_2-agonists (5, 6). There is a downregulation of β-adrenergic receptors (β-receptors) in lung after chronic administration of β-agonists in animals in vivo, although this is less marked in airway smooth muscle than in lung parenchyma (7, 8). Agonist-promoted downregulation of β_2-receptors may be reversed by treatment with glucocorticoids in vitro (9). Glucocorticoids induce an increase in the synthesis of β-receptors in human and rat lung (10) and restoration of desensitization of β-receptors in human neutrophils and lymphocytes (11, 12). The reversal of agonist-induced downregulation of the β_2-receptor by glucocorticoids has been reported in cultured vas deferens smooth muscle cells (DDT_1-MF2) at the levels of radioligand binding and of mRNA (13).

Cyclic AMP and glucocorticoid response elements (CREs and GREs)[1] have been identified in the promoter region of the β_2-receptor gene (14, 15), suggesting the involvement of these transcription factors in the regulation of β_2-receptors. CRE binding protein (CREB) appears to maintain the basal transcription of the β_2-receptor gene (16). On the other hand, glucocorticoids appears to increase β_2-receptor mRNA expression, exerted at GREs in the 5′-noncoding region (15, 17, 18).

Chronic β-agonist therapy in asthmatic subjects results in reduction in β-receptor density in circulating polymorphonuclear leukocytes and lymphocytes (19) and the downregulated receptor number is restored with oral prednisone. However, a difference in susceptibility to downregulation between lung and lymphoid tissue has been reported (20). To investigate whether the in vivo treatment of glucocorticoids prevent the β-agonist–promoted downregulation of pulmonary β_2-receptors, we studied the effects of dexamethasone (Dex) and isoproterenol (Iso) on β_2-receptor number, and mRNA expression in rat lung in vivo. In addition, we examined effects on the transcription factor CREB. We also used autoradiographic mapping of β_2-receptors to study the effects of Iso and Dex on different cell types in lung. Direct receptor binding techniques using selective β-antagonists have shown the coexistence of β_1- and β_2-receptor

1. *Abbreviations used in this paper:* CRE, cAMP response element; CREB, CRE binding protein; Dex, dexamethasone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GRE, glucocorticoid response element; ICYP, [^{125}I]iodocyanopindolol; Iso, isoproterenol.

subtypes in rat and human lung (7, 21). Both Dex and Iso may also affect β_1-receptors as well as β_2-receptors, so we also examined the expression of β_1-receptors under the same conditions.

Methods

Experimental procedures. Male Wistar rats weighing 300–320 g were used. One group ($n = 7$) was injected with Dex (0.2 mg/kg/d) subcutaneously for 1 d, with another group ($n = 7$) once a day for 8 d (days 1–8). A third group ($n = 7$) was treated with Iso (0.96 mg/kg/d) for 7 d (days 2–8) delivered by an osmotic minipump (Alzet model 2001; Alza Corp., Palo Alto, CA), which was implanted subcutaneously under anesthesia and sterile conditions. A fourth group ($n = 7$) was treated with both Dex and Iso simultaneously in the same manner described above. A control group ($n = 7$) was treated with vehicle both by subcutaneous injection and by a minipump simultaneously. Dex was dissolved in sterilized isotonic saline only; Iso was dissolved in sterilized isotonic saline containing 1.1 mM ascorbic acid to prevent its oxidation. On the ninth day the animals were killed by 100% CO_2 exposure and the lungs were quickly removed.

Radioligand receptor binding assay. 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, and was then homogenized with a Polytron homogenizer (Kinematica, Basel, Switzerland) at setting 6 in 30-s bursts. The homogenate was centrifuged at 1,000 g for 10 min at 4°C to remove unhomogenized debris, the supernatant was then centrifuged at 40,000 g for 20 min at 4°C, and the resulting pellet was washed and recentrifuged at the same speed. The final homogenate was frozen in liquid nitrogen and stored at −80°C without loss of binding characteristics. Portions of lung membrane at a protein concentration of 10 μg/tube were incubated with [^{125}I]iodocyanopindolol (ICYP) (sp act: 2,000 Ci/mmol; 3–100 pM) in the presence or absence of excess 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 Iso) in a final volume of 250 μl. The density of β_1-receptors was analyzed by ICYP saturation binding in the presence of 0.1 μM ICI 118551, a β_2-selective antagonist, a concentration at which practically all β_2-receptors are occupied. The density of β_2-receptors by ICYP saturation binding in the presence of 0.1 μM CGP 20712 A, a β_1-selective antagonist, a concentration at which practically all β_1-receptors are occupied. 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 GF/C glass-fiber filters (Whatman Inc., Clifton, NJ). Each filter was rapidly washed with 3 × 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 Instruments, 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. (22), with bovine serum albumin 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 as long as 2 wk before use without loss of binding capacity.

Receptor mapping was performed using the method as described previously (8). 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 Iso. For mapping of the β_1-receptors, serial sections were incubated with 25 pM ICYP with and without 0.1 μM ICI 118551, and for β_2-receptors with and without 0.1 μM CGP 20712 A. 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, and 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 a Zeiss microscope equipped with dark- and bright-field illumination. Grain density was measured as optical density with a microscope connected to Image Analysis (Seescan, Cambridge, United Kingdom), 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 measurements was small.

Northern blot analysis. Random primer labeling was carried out with the 1.8-kb full-length fragment from rat β_2-receptor cDNA (23), the 851-bp SmaI/PvuII fragment from human β_1-receptor cDNA, and the 1.3-kb PstI fragment from rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA using [α^{32}P]dCTP (3,000 Ci/mmol). GAPDH is a constitutive (housekeeping) gene that is expressed at constant levels in all cells (24) and used as an internal control for the quantity of the RNA loaded into each lane.

Total RNA from rat lung was isolated according to the method of Chromczynski and Sacchi (25). Total cellular RNA (20 μg/lane) was subjected to electrophoresis on a 1% wt/vol agarose, 6.6% formaldehyde gel and blotted onto Hybond-N membranes (Amersham International, Amersham, United Kingdom). After prehybridization for 5 h at 42°C in buffer containing 5 × Denhardt's solution, 5 × standard saline citrate (SSC), 50 mM Na_2HPO_4, 0.1% sodium dodecyl sulfate (SDS), 250 μg/ml sonicated denatured salmon sperm DNA, and 50% formamide, the blot was incubated for 20 h at 42°C with either first to a ^{32}P-labeled rat β_2- or a human β_1-receptor cDNA 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, once with 0.5 × SSC/0.1% SDS for 30 min at 50°C, and finally with 0.1 × SSC/0.1% SDS for 30 min at 55°C, and exposed at −80°C for 10–14 d to Kodak OMAT XS film with an intensifying screen. After autoradiography, blots were stripped for reprobing with a ^{32}P-labeled rat GAPDH cDNA probe.

Electrophoretic mobility shift assay. The CREB consensus oligonucleotide was labeled at the 5′-ends using T4 polynucleotide kinase and [γ^{32}P]ATP (> 5,000 Ci/mmol). Nuclear protein from rat lung was isolated according to a method described previously (7). Binding reactions between ^{32}P-labeled CREB consensus oligonucleotide and nuclear protein were performed in a final volume of 10 μl in 4% glycerol, 1 mM EDTA, 1 mM DTT, 100 mM NaCl, 10 mM Tris (pH 7.5), and 0.08 mg/ml sonicated salmon sperm DNA. Binding was allowed to proceed for 20 min at room temperature. To resolve the complexes, the reactions were applied to 6% nondenaturing polyacrylamide gels in 0.25 × TBE buffer (10 × TBE is 0.89 M Tris base [pH 8.0], 0.89 M boric acid, and 20 mM EDTA) containing 0.1% ammonium persulfate. The gels were run in 0.25 × TBE buffer at 100 V/cm for 1 h at room temperature with buffer recirculation, then dried and autoradiographed. The specificity of binding was studied by incubation of ^{32}P-labeled CREB consensus oligonucleotide with nuclear protein in the presence of excess amount of unlabeled oligonucleotide. We have demonstrated previously that CREB-like DNA-binding activity is increased in a concentration-related manner by β-agonists in rat lung (26).

Nuclear run-on transcription assay. To determine whether Dex or Iso changed the transcription rate of β_1- and β_2-receptor gene, nuclear run-on transcription assays were performed as described previously (27). Nuclei from frozen rat lung tissues treated with vehicle, Dex, Iso, or both were isolated and stored at −80°C in Keller storage buffer at 25×10^6 nuclei/100 μl. Each reaction (final volume, 400 μl) was carried out in the presence of 5×10^7 isolated nuclei, 40 mM Tris-HCl (pH 8.3), 150 mM NH_4Cl, 7.5 mM $MgCl_2$, 0.625 mM ATP, 0.313 mM GTP, 0.313 mM CTP, 0.5 mCi [α^{32}P]UTP (800 Ci/mmol), and 120

U/ml recombinant RNasin. Transcription reactions were allowed to proceed for 30 min at 27°C before termination by the addition of 40 U of recombinant RNasin and 75 U of RQ-1 DNase. After DNase and proteinase K treatments, the radiolabeled RNA formed was purified by phenol/chloroform extraction and precipitated with ethanol three times in the presence of 1.33 M ammonium acetate. An equal number of counts from each sample (2×10^6 cpm) was added to slot blots, 4 slots on the same blot of which 10 μg of either pGEM-3Z plasmid (as control), plasmid containing inserts of full-length human β_1-receptor cDNA, rat β_2-receptor cDNA, or rat GAPDH cDNA has been immobilized to a nylon filter (Hybond-N). After hybridization for 72 h at 42°C, the filters were washed at a final stringency of 0.1 × SSC and 0.1% SDS at 55°C, including a 30-min digestion with 1 μg/ml RNase A and 10 U/ml RNase T_1 at 37°C to digest any single-stranded RNA not hybridized to DNA. The filters were exposed to Kodak OMAT XS film with an intensifying screen at −80°C for 1–3 d.

Analysis of results. The experimental data are given as means±SEM. The significance of difference was tested by an ANOVA; $P < 0.05$ was considered to be statistically significant. Parameters (dissociation constant, K_d; maximal binding capacity, B_{max}) of ICYP binding were obtained from individual experiments using the program GraphPAD (ISI Software, San Diego, CA). Data from the autoradiograms of Northern blot analysis, gel shift assays, and nuclear run-on assays were assessed using laser densitometry (Howtek, Hudson, NH) linked to a computer analysis system (PDI, Huntington Station, NY).

Materials. [^{125}I]Iodocyanopindolol, [α^{32}P]dCTP, [γ^{32}P]ATP, Hybond-N membranes, and random primer labeling kit were purchased from Amersham International. [α^{32}P]UTP was obtained from DuPont/New England Nuclear (Stevenage, United Kingdom). Rat β_2-receptor cDNA was obtained from American Type Culture Collection (Rockville, MD). CREB consensus oligonucleotide, recombinant RNasin, RQ-1 DNase, and restriction endonucleases, such as SmaI and PvuII, were purchased from Promega (Southampton, United Kingdom). Dex, Iso, and other substances were obtained from Sigma Chemical Co. (Poole, United Kingdom). CGP-20712A was a gift from Ciba-Geigy (Basel, Switzerland) and ICI-118,551 was from Zeneca Pharmaceuticals (Macclesfield, United Kingdom).

Results

Radioligand receptor binding assay. Chronic injection of Dex (8 d) significantly increased the number of β_1- and β_2-receptor density by 70±15% ($P = 0.001$) and 69±10% ($P < 0.001$), respectively (Fig. 1), but there was no increase after treatment for 1 d. Continuous infusion of Iso significantly reduced β_1- and β_2-receptor density by 48±5% ($P < 0.001$) and 51±6% ($P = 0.001$), respectively. Combined treatment of Dex and Iso did not significantly change β_2-receptor density (decrease by 15±6%, not significant), but significantly reduced β_1-receptor density by 23±5% ($P = 0.01$). These treatments did not change the affinity of binding, nor the ratio of the β_2- and β_1-receptors (~ 3:1).

Receptor autoradiography. The distribution of β_1- and β_2-receptors in rat lung (Fig. 2) is in good agreement with previous findings in human and guinea pig lung (8, 28) and confirms and extends the results of the receptor binding study. The increase in β_1- and β_2-receptors after Dex, the decrease in both receptor subtypes after Iso, the decrease in β_1- but no change in β_2-receptor after combined treatment of Dex and Iso were found over alveoli, airway epithelium, vascular endothelium, and airway and vascular smooth muscle (Table I).

Northern blot analysis. A single band of 3.2 and 2.2 kb for β_1- and β_2-receptor mRNA was observed in rat lung, respectively, in agreement with previous reports (29). Chronic injection of Dex (8 d) did not change β_1- or β_2-receptor mRNA expression (Figs. 3 and 4, respectively). Similarly, 1-d treatment with Dex did not change β_1- or β_2-receptor mRNA expression. Continuous infusion of Iso significantly decreased the ratio of β_1- and β_2-receptor mRNA to GAPDH mRNA by 69±7 ($P < 0.01$) and 57±8% ($P = 0.01$), respectively. Combined treatment of Dex and Iso did not affect β_2-receptor mRNA expression, but significantly decreased β_1-receptor mRNA expression by 66±8% ($P < 0.01$).

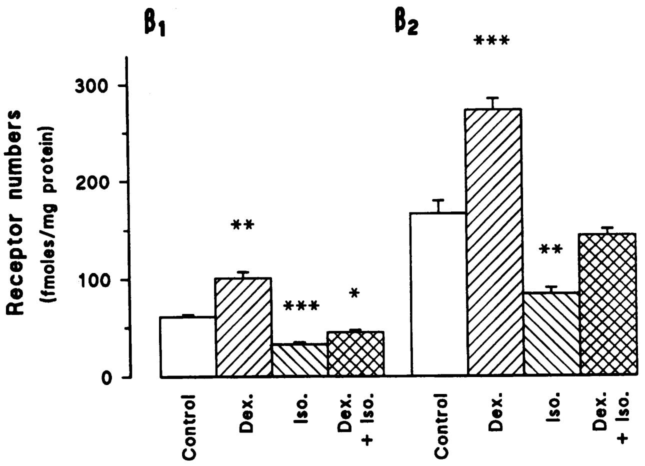

Figure 1. Effects of treatment with Dex and/or Iso on β-adrenergic receptor subtypes in rat lung. Control, Dex, Iso, and combination of Dex and Iso groups are shown. The density of β_1-receptor subtype was analyzed by ICYP saturation binding in the presence of 0.1 μM ICI 118,551 and the density of β_2-receptor subtype by ICYP saturation binding in the presence of 0.1 μM CGP 20712 A. Significance of difference from the control value, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ ($n = 7$).

Electrophoretic mobility shift assay. The specificity of CREB-like DNA-binding activity was demonstrated by the abolition of the single complex formed by excess amount of unlabeled CREB consensus oligonucleotide and the absence of the complex in the control incubation lacking nuclear protein (Fig. 5 *A*). Chronic injection of Dex did not significantly affect the CREB-like DNA-binding activity, whereas continuous infusion of Iso significantly reduced the CREB-like DNA-binding activity ($P < 0.05$). Combined Dex and Iso treatment showed no change in CREB-like DNA-binding activity (Fig. 5, *B* and *C*).

Nuclear run-on transcription assay. The transcription rates of β_1- and β_2-receptor genes were measured by nuclear run-on assays in lung tissues from control, Dex-, Iso-, or both treated groups. Chronic injection of Dex showed an increase in transcription rate of β_2-receptor gene without affecting β_1-receptor gene. The transcription rate of β_2-receptor gene, calculated from the ratio of transcription rate of β_2-receptor gene to that of the GAPDH gene, was increased by approximately twofold of control in nuclei from Dex group, whereas continuous infusion of Iso showed a decrease in transcription rate of β_1-receptor gene without affecting the transcription rate of β_2-receptor gene. Combined Dex and Iso treatment did not affect the transcription rate of either β_1- or β_2-receptor genes (Fig. 6, *A* and *B*).

Discussion

In this study we investigated the effect of treatment of a glucocorticoid and a β-agonist, alone and in combination, on the

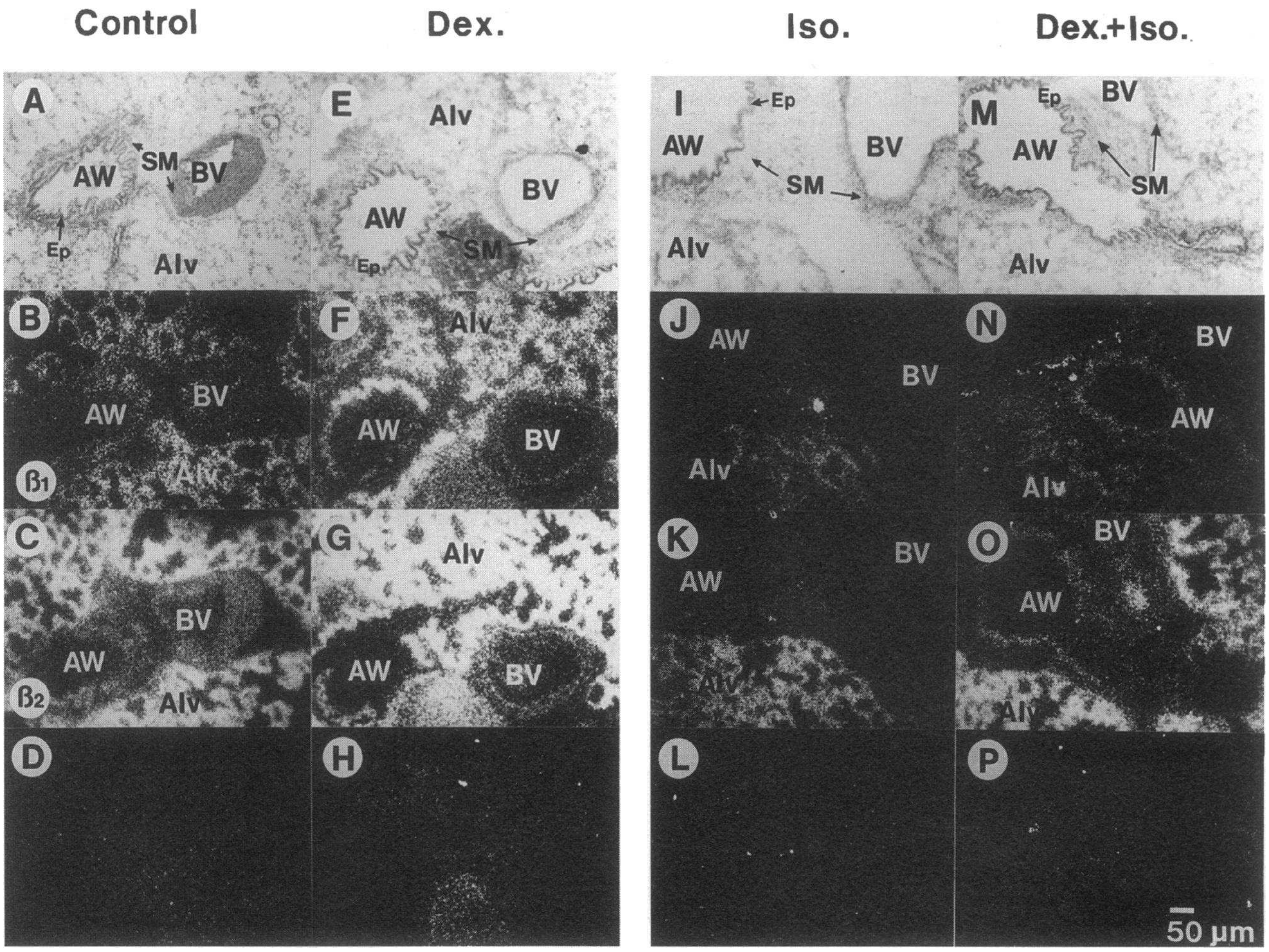

Figure 2. Distribution of β-adrenergic receptor subtypes in rat lung using in vitro receptor autoradiography in control (*A–D*), Dex-treated (*E–H*), Iso-treated (*I–L*), and Dex and Iso–treated (*M–P*) animals. *A, E, I,* and *M* show sections stained with 1% cresyl fast violet. *B, F, J,* and *N* show the distribution of β_1-receptor subtype in the presence of 0.1 μM ICI 118,551. *C, G, K,* and *O* show the distribution of β_2-receptor subtype in the presence of 0.1 μM CGP 20712 A. *D, H, L,* and *P* show the nonspecific binding of ICYP to lung sections. *Alv,* alveoli; *AW,* airway; *BV,* blood vessel; *SM,* airway smooth muscle; and *Ep,* airway epithelium. Bar, 50 μm.

density and distribution of β_1- and β_2-receptors, their mRNA expression, and CREB-like DNA-binding activity in rat lung in vivo. Chronic treatment with Dex resulted in an increased density of both β_1- and β_2-receptors and an increase in the transcription rate of β_2-receptor gene, but no change in their mRNA expression, in the transcription rate of β_1-receptor gene, and in CREB-like DNA-binding activity. Chronic treatment with Iso resulted in a reduced density of both β_1- and β_2-receptors, decreases in their mRNA expression, in the transcription rate of β_1-receptor gene, and in CREB-like DNA-binding activity, but no change in the transcription rate of β_2-receptor gene. Combined treatment of Dex and Iso resulted in no change in β_2-receptor number nor its mRNA expression, while there was a reduction in β_1-receptor number and mRNA expression (Table II). Autoradiographic mapping showed that the changes in β-receptor expression after Dex, Iso, and combined treatment were present on all cell types identified, including airway smooth muscle, epithelium, and alveolar walls.

As observed previously (10), the present study also provides evidence that an increase in pulmonary β_1- and β_2-receptor levels after chronic in vivo dexamethasone treatment can be detected. In vitro, glucocorticoids have been shown to induce the upregulation of β-receptors in DDT$_1$-MF2 (17, 18) and 3T3-F442A (30) cell lines in culture. The downregulation of β_1- and β_2-receptors in rat lung after chronic stimulation by β-agonist is also in good agreement with previous in vivo and in vitro studies (7, 8, 19, 31). When rats were exposed to both Dex and Iso simultaneously, β_1-receptors were reduced, but β_2-receptors were unchanged. This protection of dexamethasone on β-agonist–promoted downregulation of β_2-receptors is consistent with the in vitro study using DDT$_1$-MF2 cells (13). However, Dex had no protective effect on β-agonist–promoted downregulation of β_1-receptors.

In agreement with previous reports (7, 8, 31), the present study also provides evidence for a reduction in β_1- and β_2-receptor mRNA after chronic in vivo β-agonist treatment. The fall in β_1-receptor mRNA started at 2 h, maximum at 1 d, and persisted for 7 d while β_2-receptor mRNA did not change at 2 h but was significantly reduced at 1 and 7 d after continuous infusion with Iso (7). Furthermore, our data showed an apparent association between these reductions in mRNA and the reduction in CREB-like DNA-binding activity, suggesting the possible involvement of this transcription factor(s). CREB, a transcription factor, recognizes CRE and stimulates target gene tran-

Table I. Influence of Treatment on β-Adrenergic Receptors by Receptor Autoradiography on Different Structures in Rat Peripheral Lung, as Determined by Optical Density

Structure	Treatment	β_2-Subtype	β_1-Subtype
Alveoli	Control	102.4±3.2	38.0±1.3
	Dex	184.0±3.7*	60.0±0.9*
	Iso	47.3±1.7*	15.8±0.4*
	Dex + Iso	103.1±2.3	26.6±0.9*
Vascular smooth muscle	Control	26.4±1.8	14.8±0.7
	Dex	62.9±2.1*	26.7±1.4*
	Iso	7.0±0.5*	1.4±0.2*
	Dex + Iso	24.5±1.1	6.4±0.6*
Endothelium	Control	18.4±1.2	9.6±0.5
	Dex	45.8±1.3*	22.8±1.0*
	Iso	4.2±0.4*	0.6±0.1*
	Dex + Iso	15.0±0.7‡	2.0±0.4*
Airway smooth muscle	Control	18.3±1.3	8.6±0.7
	Dex	67.9±3.1*	19.6±1.4§
	Iso	5.4±0.6*	2.4±0.4§
	Dex + Iso	20.8±1.3	7.0±0.5‡
Epithelium	Control	31.2±1.8	19.4±1.7
	Dex	92.9±4.4*	37.6±2.1*
	Iso	8.0±0.9*	2.7±0.4*
	Dex + Iso	27.1±1.2	9.4±0.7*

Values are the means±SEM from 40 separate optical density measurements ($\times 10^{-3}$) of multiple sections from three control and three treated animals. β_2-Receptor subtype was determined in the presence of 0.1 μM CGP 20712 A and β_1-receptor subtype in the presence of 0.1 μM ICI 118,551. ‡ $P < 0.05$, § $P < 0.01$, * $P < 0.001$, as compared with the control values.

scription (14). The presence of CRE in the β_2-receptor gene and the ability of β-agonists to increase intracellular cAMP prompted the suggestion that these elements may be involved in the negative control of transcription (30). However, this is unlikely since activation of CRE enhances rather than suppresses transcription rates of target genes (16). Thus, it is possible that the reduced CREB-like DNA-binding activity contributes, in part, to the downregulation of β_2-receptor mRNA after prolonged exposure to a β-agonist, in good agreement with our previous findings in rat and guinea pig lung (7, 8). Recently, the presence of a CRE has also been identified in the 5′-flanking promoter region of the β_1-receptor gene (32) and may be involved in the negative control of transcription of this gene.

Although both β_1- and β_2-receptor densities were increased, neither β_1- nor β_2-receptor mRNA was increased after chronic in vivo glucocorticoid treatment, in agreement with an in vivo study using Sprague-Dawley rats (33). In contrast, induction of β_2-receptor mRNA by glucocorticoids has been demonstrated in human lung and several cell lines in vitro (17, 18, 34). The lack of change in the levels of β_1- and β_2-receptor mRNAs after dexamethasone in this study may be due to the fact that the accumulation of mRNA is transient. Indeed in our previous study in human lung in vitro (34), we observed that the increase in β_2-receptor mRNA was maximal 2 h after exposure to glucocorticoid and returned to baseline by 24 h. Glucocorticoids have the ability to downregulate their own receptors, which act as transcription factors (35). Several putative GREs are identified in the promoter region of the β_2-receptor gene and are obligatory for glucocorticoid regulation of receptor mRNA levels (15).

After combined treatment with both glucocorticoid and β-agonist, β_1-receptor mRNA and the density of β_1-adrenoceptors were reduced, but β_2-receptor mRNA and β_2-receptors remained unchanged, compared with control animals. The differential regulation of β_1- and β_2-receptor gene expression after

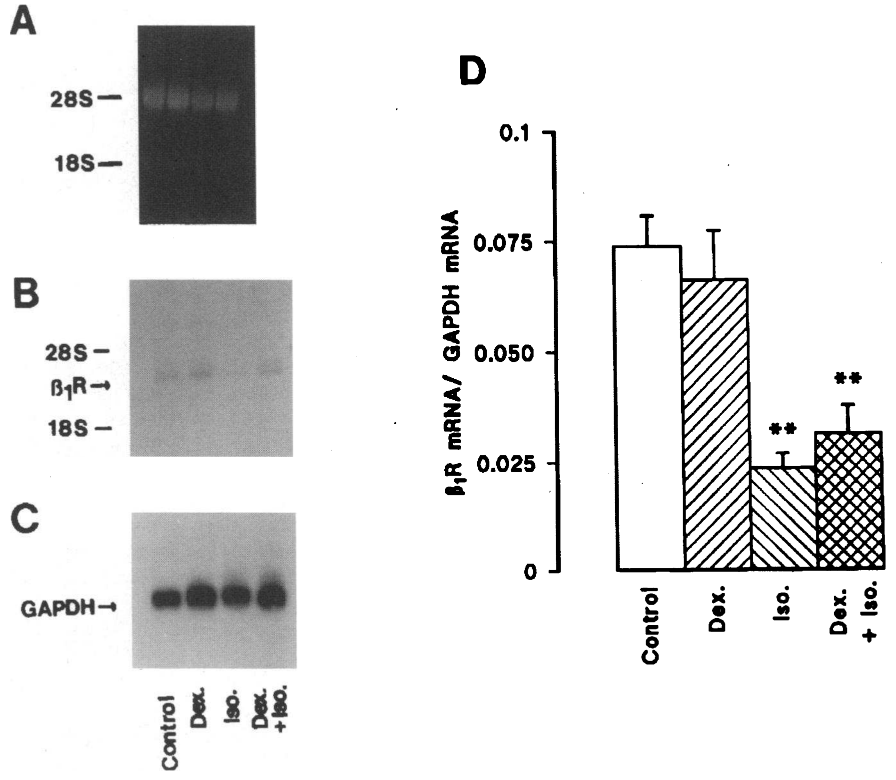

Figure 3. Effects of treatment with Dex and/or Iso on β_1-adrenergic receptor mRNA in rat lung. (*A*) A photograph of the ultraviolet light–illuminated, ethidium bromide staining of the RNA on the membrane used for Northern blot in *B* and *C*. The signal intensity of the 28S and 18S rRNA bands demonstrated the quantity of the RNA loaded into each lane. (*B*) Representative autoradiogram from Northern blot of rat β_1-receptor mRNA. Total RNA from one control, one Dex, one Iso, and one Dex and Iso–treated rat lung was hybridized with a ^{32}P-labeled SmaI/PvuII fragment human β_1-receptor cDNA probe. The size of the mRNA (in kilobases) was estimated from rRNA markers as 3.2 kb. (*C*) Representative autoradiogram from Northern blot of rat GAPDH mRNA. The same membrane as in *A* was later probed with a ^{32}P-labeled rat GAPDH cDNA probe to control for the quantity of the RNA loaded into each lane. (*D*) Densitometric measurement of β_1-receptor mRNA from control, Dex-treated, Iso-treated, and Dex and Iso–treated rat lungs (n = 7 in each group). β_1-receptor mRNA was normalized to that for GAPDH mRNA. Significance of difference from the control value, ** $P < 0.01$.

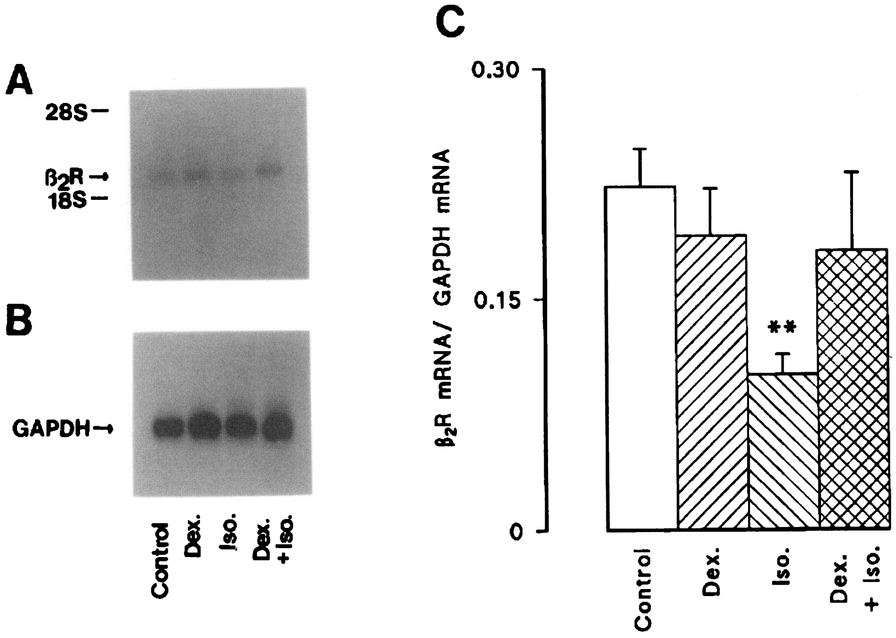

Figure 4. Effects of treatment with Dex and/or Iso on β_2-adrenergic receptor mRNA in rat lung. (*A*) Representative autoradiogram from Northern blot of rat β_2-receptor mRNA using rat full-length β_2-receptor cDNA. The same membrane as used in Fig. 3. was hybridized with a ^{32}P-labeled full-length rat β_2-receptor cDNA probe. The size of the mRNA (in kilobases) was estimated from rRNA markers as 2.2 kb. (*B*) Representative autoradiogram from Northern blot of rat GAPDH mRNA. (*C*) Densitometric measurement of β_2-receptor mRNA from control, Dex-treated, Iso-treated, and Dex and Iso–treated rat lungs ($n = 7$ in each group). β_2-receptor mRNA was normalized to that for GAPDH mRNA. Significance of difference from the control value, ** $P < 0.01$.

combined treatment of Dex and Iso may be complex. Glucocorticoids alone have been shown to selectively upregulate β_2-receptors in brown fat, 3T3-L1 preadipocytes, and 3T3-F442 adipocytes, with no effect or an actual decrease in the expression of β_1-receptors (29, 36, 37).

The molecular explanation for regulation of receptor

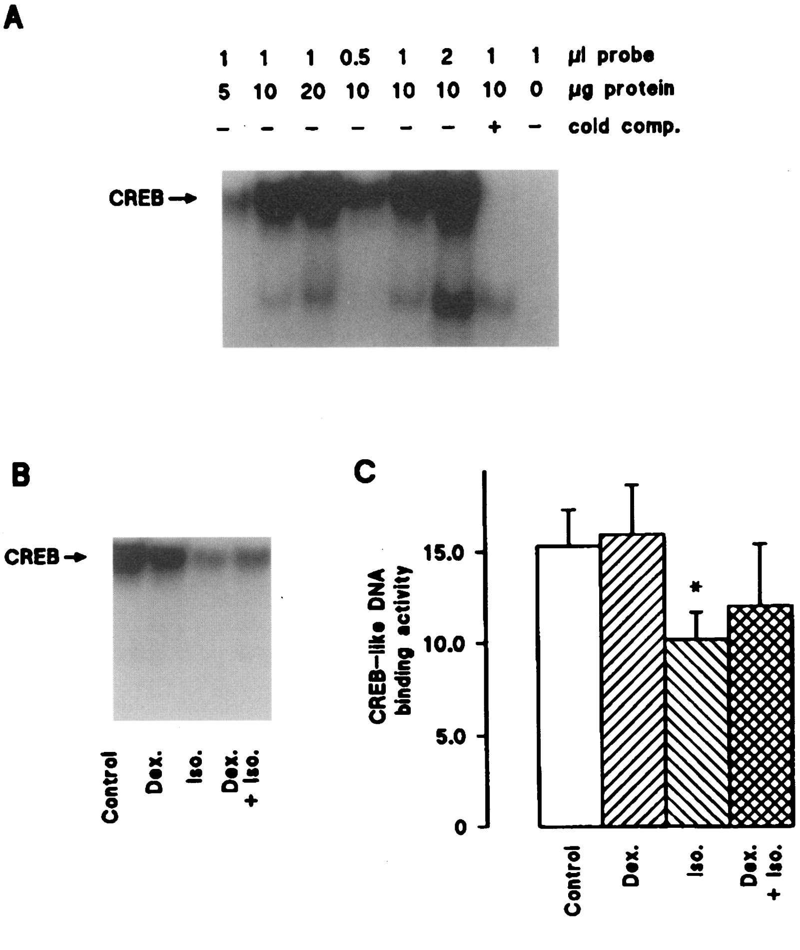

Figure 5. Effects of treatment with Dex and/or Iso on CREB-like DNA-binding activity in rat lung. (*A*) Specificity of the nuclear protein–CREB consensus oligonucleotide complex. The indicated amounts of nuclear protein from one control animal were incubated with the indicated amounts of ^{32}P-labeled CREB consensus oligonucleotide in the presence or absence of unlabeled competitor oligonucleotide. Arrow indicates the specific complex. (*B*) Representative autoradiogram from gel shift assay of CREB in rat lung. Nuclear protein from one control, one Dex-treated, one Iso-treated, and one Dex and Iso–treated rat lung was hybridized with a ^{32}P-labeled CREB consensus oligonucleotide. Arrow indicates the specific complex. (*C*) Densitometric measurement of CREB-like DNA-binding activity from control, Dex-treated, Iso-treated, and Dex and Iso–treated rat lungs ($n = 5$ in each group). Significant different from the control value. * $P < 0.05$.

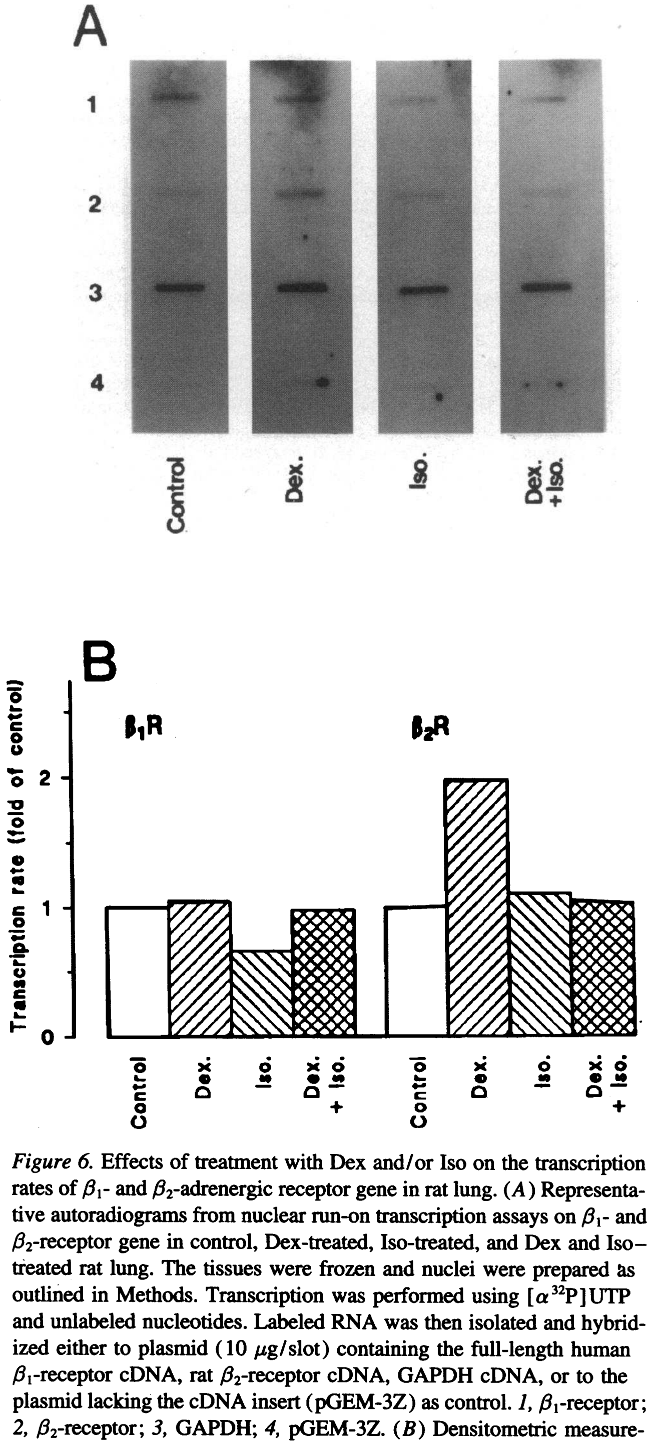

Figure 6. Effects of treatment with Dex and/or Iso on the transcription rates of β_1- and β_2-adrenergic receptor gene in rat lung. (*A*) Representative autoradiograms from nuclear run-on transcription assays on β_1- and β_2-receptor gene in control, Dex-treated, Iso-treated, and Dex and Iso–treated rat lung. The tissues were frozen and nuclei were prepared as outlined in Methods. Transcription was performed using [α^{32}P]UTP and unlabeled nucleotides. Labeled RNA was then isolated and hybridized either to plasmid (10 μg/slot) containing the full-length human β_1-receptor cDNA, rat β_2-receptor cDNA, GAPDH cDNA, or to the plasmid lacking the cDNA insert (pGEM-3Z) as control. *1*, β_1-receptor; *2*, β_2-receptor; *3*, GAPDH; *4*, pGEM-3Z. (*B*) Densitometric measurement of β_1- and β_2-receptor transcription rate from control, Dex-treated, Iso-treated, and Dex and Iso–treated rat lungs. The transcription rate was calculated as the ratio of β_1- or β_2-receptor cDNA signal relative to the GAPDH cDNA signal. Average values from two separate experiments are shown.

mRNA, the balance and interplay between glucocorticoid induced upregulation and agonist-promoted downregulation, has been demonstrated in cultured cell lines (13). Glucocorticoids enhance the rate of transcription at a GRE in the 5′-noncoding portion of the gene which is responsible for the steroid-induced upregulation of receptor mRNA, and the stability of mRNA appears to play no major role in the glucocorticoid effect. On the other hand, β-agonists reduce the half-life of mRNA and this is responsible for the short-term agonist-induced downregulation of receptor mRNA, without any affect on the rate of transcription. An intriguing finding in the present study was the observation that there was no change on the steady state level of β_1- and β_2-receptor mRNA after chronic in vivo glucocorticoid treatment, indicating that the observed increase in β_1- and β_2-receptor density might reflect the involvement of different molecular mechanisms. For β_1-receptor, the synthesis of receptor protein may be unchanged but the stability of receptor protein may be increased. On the other hand, both an increase in the rate of transcription of the β_2-receptor gene as well as an consequent increase in the synthesis of the receptor protein may occur. After chronic in vivo β-agonist, the observed decrease in β_1-receptor mRNA and rate of transcription, followed by a decrease in β_1-receptor density, suggests that it is primarily the reduced rate of transcription which is responsible for the β-agonist–induced downregulation of β_1-receptor mRNA. On the other hand, the decrease in β_2-receptor mRNA and density without any detectable decline in the rate of transcription indicates that β-agonist might destabilize β_2-receptor mRNA. In these studies with chronic in vivo β-agonist and/or glucocorticoid treatment, it is not possible to measure whether mRNA stability is changed, as it is not possible to perform the transcriptional blocking studies that can be carried out in vitro. Recently, a regulatory factor, known as β-adrenergic receptor-binding protein, has been identified that selectively modulates the stability of β_2-receptor mRNA in DDT$_1$-MF2 cells (38). The abundance of this factor varies inversely with the level of receptor mRNA, being induced by β-agonists that downregulate receptor mRNA, and being reduced by glucocorticoids that upregulate receptor mRNA.

It is now increasingly recognized that chronic treatment with β-agonist bronchodilators may result in densitization to the protective effects of β-agonists on the airways (3–6). Our results suggest that concomitant treatment with glucocorticoids should prevent changes in β_2-receptors which mediate most of the antiasthma effects of β-agonists, whereas the desensitization of β_1-receptors would remain. Taken together, our findings suggest that different molecular mechanisms may be involved in the regulation of β_1- and β_2-receptor expression after chronic in vivo β-agonist and/or glucocorticoid treatment. This may be

Table II. Summary of Results

	Treatment		
	Dex	Iso	Dex + Iso
β_1-Receptors			
Number	↑	↓	↓
mRNA	No change	↓	↓
Rate of transcription	No change	↓	No change
β_2-Receptors			
Number	↑	↓	No change
mRNA	No change	↓	No change
Rate of transcription	↑	No change	No change
CREB-like DNA-binding activity	No change	↓	No change

of clinical benefit, since some of the cardiovascular side effects of β-agonists may be mediated via stimulation of cardiac β_1-receptors.

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