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Interaction between Glucocorticoids and β_2-Agonists: α and β Glucocorticoid-Receptor mRNA Expression in Human Bronchial Epithelial Cells

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ABSTRACT. Recent studies have suggested that regular use of β_2-agonists has adverse effects on asthma control, due to the cross-talk between cAMP responsive element binding proteins (CREB) and glucocorticoid receptors (GR). The aim of this study was to investigate the interaction between GR and CREB on cytoplasmic protein level with a gel mobility shift assay and to determine the effect of this interaction on mRNA levels by Northern blot analysis. After exposing human bronchial epithelial cells for 1 hr to either 1 μM terbutaline or budesonide, more binding of CREB and GR, respectively, was observed to their responsive elements in DNA. Simultaneous exposure to terbutaline and budesonide also increased the binding of CREB and GR to DNA. After 4 hr, both α and β GR mRNAs were down-regulated by 1 μM budesonide. Simultaneous addition of 1 μM terbutaline prevented this down-regulation. Adding 100 times more budesonide compared to terbutaline again down-regulated both GR forms, although significantly less compared to the down-regulation induced by 1 μM budesonide alone. Addition of terbutaline to cells already exposed to budesonide did not reverse the GR mRNA expression within 44 hr. Similar results were obtained with metallothionein-2 (MT2) mRNA levels. In conclusion, β_2-agonists interfere with the GR function in human bronchial epithelial cells when given simultaneously, with this being overcome by sequential exposure of the cells to first glucocorticoids and later β_2-agonists. BIOCHEM PHARMACOL **56**;12:1561–1569, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. glucocorticoids; β_2-agonists; glucocorticoid receptor; CREB interactions; *in vitro*

Inhaled β_2-adrenergic agonists are the most effective bronchodilators in current use, and have long been used as the the primary therapy for the treatment of asthmatics. However, since the awareness of asthma as a chronic inflammatory disease of the airways emerged, inhaled glucocorticoids have become the mainstay of treatment. Today, β_2-adrenergic agonists and glucocorticoids are often used in combination for the treatment of asthma. However, despite the their more more widespread use of inhaled β_2-agonists and glucocorticoids, mortality and morbidity from asthma has increased. It is thought that glucocorticoids and β_2-adrenergic agonists function antagonistically in their control of asthma [1]. Although glucocorticoids sometimes appear able to diminish or reverse the negative effects of β_2-agonists on asthma control, reports to this effect are limited [2, 3]. Therefore, the underlying mechanisms remain to be elucidated.

Exposure of cells to glucocorticoids or β_2-adrenergic agonists results in the activation of two separate signal transduction pathways in these cells. The GR§ plays a central role in the function of glucocorticoids [4]. It exists in an α and β form, although Oakley and coworkers *et al.* [5] recently reported, using Northern blot analysis, the existence of 2 α GR isoforms, α1 and α2 with Northern blot analysis. The α1, α2, and β bands were demonstrated at 7 kb, 5.5 kb, and 4.3 kb, respectively. Translation of both α forms results in the same GR protein. After entering the cell by passive diffusion, glucocorticoids bind to an inactive GR, which then becomes activated [6]. Gene transcription is regulated by the binding of a GR dimer to GRE in the DNA [7]. The β_2-adrenergic agonists, on the other hand, bind to a cell-membrane-bound receptor, which, after binding of the ligand, activates the receptor-associated stimulating G protein [8]. Subsequent production of cAMP leads to the phosphorylation of the CREB [9], which can regulate gene transcription by binding to CRE in the DNA [10]. The modulation of gene transcription not only depends on the presence or absence of responsive elements in

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§ Abbreviations: AP-1, activator protein 1; CREB, cAMP responsive element binding protein; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GR, glucocorticoid receptor; MT2, metallothionein-2; NFκB, nuclear factor κB; Stat5, and signal transducers and activators of transcription 5.

the genes, but also on the direct interaction between transcription factors [11]. Direct protein–protein interaction of the GR has been is described with CREB [12–14]. In addition, the activated GR is also capable of binding with other transactivation proteins, such as AP-1 [15, 16], NFκB [17, 18], and Stat5 [19]. In particular, cross-talk of the GR with AP-1 or NFκB is now believed to play an essential role in the reduction of cytokine production, an important aspect of the antiinflammatory action of glucocorticoids [20, 21].

Both α1 and β GR mRNAs are shown to be present in all tissues investigated [5, 22], but because of its ligand binding capacity, most previous articles have been mainly concerned with the α1 GR isoform [5, 23]. The levels of GR expression vary between cell types and individuals, but in all cases higher levels of α1 GR mRNA expression have been observed compared to β GR mRNAs [5]. The fact that the β form is widely expressed in many cell types indicates that it may play a role in the cellular response to glucocorticoids. However, because the antibodies available were only able to recognize the common part of both isoforms, earlier studies could not distinguish between the 2 GR proteins. In the last 2 years, several reports have discussed the role of the β GR [5, 22, 24]. These studies, however, are contradictory regarding a negative, inhibitory effect of the β GR on the activity of the α GR. Therefore, more research needs to be performed to solve the questions concerning the functional activities of the β form.

Scavenging of the GR by CREB before binding to DNA may be one explanation for a possible antiglucocorticoid activity of β_2-adrenergic agonists. Cross-talk between CREB and GR has been demonstrated previously in rat and human lung [12, 25], rat hepatoma cells [26], placental cells [13, 14], and human pulmonary and bronchial epithelial cells [27]. In three studies [12, 25, 27], β_2-adrenergic agonists were used to demonstrate interactions between GR and CREB. No studies have been performed so far on alterations in mRNA levels after simultaneous exposure of cells to glucocorticoids and β_2-agonists.

The purpose of this present study was to investigate a possible effect of β_2-adrenergic agonists on the action of glucocorticoids. To this end, we exposed a human bronchial epithelial cell line to terbutaline and budesonide separately and simultaneously. Gel mobility shift assays were performed to determine the binding of GR and CREB to their responsive elements in the DNA. The effect of this interaction was studied by measuring the mRNA levels of the α and β GR and MT2 genes.

MATERIALS AND METHODS

In Vitro *Experiments*

Bet1A, a human bronchial epithelial cell line transformed by the SV40 virus [28], was cultured in LHC-8 medium containing 2×10^{-7} M hydrocortisone (LHC-8-, Biofluids) and an addition of 33 μM retinoic acid and 546 (M epinephrine [29]. During the experiments, LHC-8 medium without hydrocortisone (LHC-8-, Biofluids) was used without the additives. Budesonide (Astra) was dissolved in 10 mL of 100% ethanol to a concentration of 10^{-2} M. Terbutaline (Astra) was dissolved in 10 mL of DMSO to 10^{-2} M. Before the addition of the β_2-adrenergic agonist terbutaline or the glucocorticoid budesonide, cells were pre-incubated for 24 hr with the LHC-8- medium to create a balanced starting point. Separate experiments were performed to investigate the mRNA levels and transcription factor DNA binding. The experiments were planned with harvesting of the cells at approximately 70–85% confluency and were repeated 2- to 4-fold. Controls for terbutaline contained equivalent amounts of DMSO. Equal amounts of 100% alcohol were added to the controls for budesonide.

mRNA Expression

HIT AND RUN. To mimic the *in vivo* situation, a short-term exposure to terbutaline was performed. With this hit and run phenomenon [30], cells were incubated with 1 μM terbutaline for 1 hr in fresh LHC-8- medium and subsequently cultured in LHC-8- medium alone for different incubation times, 1,2,3,4,5,6 and 12 hr. Incubation of the control samples was performed at every time point sampled.

CONTINUOUS EXPOSURE. During the continuous time experiment, incubation times of 1, 2, 3, 4, 5, 6 and 12 hr with a terbutaline concentration of 1 μM were used. Control samples consisted of only DMSO and were taken at equal time points.

INTERVENTION. To study the interaction of terbutaline and budesonide, both hormones were simultaneously added to Bet1A cells for 4 hr. The compounds were added to the cells in an equimolar concentration of 1 μM or with a 100-fold lower concentration of terbutaline. In a separate experiment, the reversibility of the known GR mRNA down-regulation by budesonide [30] was studied by adding, after 4 hr, terbutaline to the cells for different lengths of time. Thus, after 4 hr, budesonide and terbutaline were simultaneously present in the culture medium. In the controls, DMSO was added without removing the budesonide.

RNA Isolation, Northern Blotting and Hybridization

RNA was isolated from the cells as described previously by Korn *et al.* [30]. In short, after the removal of the culture medium, Bet1A cells were harvested by adding 8 mL of a 4 M GTC solution directly into the tissue culture plates. Total RNA was isolated by the GTC/CsCl method and 20 μg was run on Northern blot. The GR, GAPDH, and MT2 probes were subsequently hybridized. The GR probe was obtained using a polymerase chain reaction on the cDNA

of Bet1A cells with primers specific for a *Bam*H1 and *Xho*1 restriction site. After the digestion of the polymerase chain product with *Bam*H1 (Boehringer Mannheim) and *Xho*1 (Boehringer Mannheim), the DNA was ligated into Bluescript 13+. The 2.67-kb probe was sequenced using the sequenase method. This probe detects both the α- and β-forms [23, 31]. The MT probe was also obtained through polymerase chain reaction from cDNA of Bet1A cells with primers specific for an *Eco*R1 and *Xho*1 restriction site and cloned in Bluescript 13+ (240 bp). The GAPDH probe (1007 bp) was kindly provided by Dr. R. G. Crystal (formerly NHLBI), National Institutes of Health. All probes were labeled with [^{32}P]dCTP (Amersham) using the random primed labeling method. Sample signals were analyzed visually as well as semiquantitatively with a phosphorimaging system (Molecular Dynamics). GR and MT2 mRNA levels were expressed relative to GAPDH levels of the same sample and were then compared to control samples at each identical time point.

Electrophoretic Mobility Shift Assays (EMSA)

INTERVENTION. To study the binding of GR and CREB to DNA, cells were exposed for 1 hr to budesonide and terbutaline, respectively. As in the previous experiments, concentrations of 1 μM terbutaline and budesonide were used.

Protein Isolation and Mobility Shift Assays

Gel mobility shift studies were performed as described previously by Adcock *et al.* [32], with these slight modifications: Cytoplasmic proteins were isolated as described and 5 μg was used for the EMSA. Double-stranded GREs (5′-TCGACTGTACAGGATGTTCT AGCTACT-) and CREs (5′-AGAGATTGCCTG ACG-TCAGAGAGCTAG) were end-labeled with [γ-^{32}P]ATP, and 50 ng of GRE and 80 ng of CRE were added per reaction. Proteins and labeled responsive elements were incubated for 20 min at room temperature in 30 μL of buffer containing 4% glycerol, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10 mM Tris-HCL (pH 7.5), and 0.8 mg/mL sonicated salmon sperm DNA. As a negative control, a sample was added without proteins. To check the specificity of the binding, 100-fold excess, unlabeled, cold GRE or CRE was used, respectively. As a control, double-stranded oligonucleotides of the MT2 gene (5′-GGGGCGTCCTCACAAT GGTGTA-) were also added unlabeled. The samples were separated on a 6% polyacrylamide gel (29:1) for 4 hr at 200 V in 0.25× Tris-borate EDTA running buffer. After vacuum drying the samples for 2 hr at 80°, the gel was analyzed semi-quantitatively with a phosphorimaging system (Molecular Dynamics).

Statistics

For all studies, means ± SD were calculated, and the Mann–Whitney *U*-test was performed to determine possible differences. Differences of $P < 0.05$ were considered statistically significant.

RESULTS

mRNA Expression

To observe the effect of terbutaline on the GR mRNA levels, two different time experiments were performed with 1 μM terbutaline. With the hit and run experiments, except for a slight down-regulation of the β GR gene expression after 4 hr (Fig. 1), no difference in either α or β GR mRNA expression was obtained. Because a 1-hr exposure might be too short to obtain transcriptional modification of the GR by terbutaline, a continuous time experiment was performed with 1 μM terbutaline for up to 12 hr. GR mRNA levels varied slightly along baseline values (Fig. 2), with a small but significant upregulation after 3 hr for β GR mRNA. Thus, terbutaline alone appears to have no essential effect on GR mRNA levels.

Exposure of the cells to budesonide alone for 4 hr down-regulated both α and β GR mRNAs ($P < 0.05$). Simultaneously exposing cells to terbutaline and budesonide, in equimolar concentrations, for 4 hr did not change GR mRNA levels (Fig. 3). Addition of 100 times more budesonide to terbutaline, again, significantly down-regulated both GR mRNAs ($P < 0.05$), but to a lesser degree than budesonide alone. The inhibition of the budesonide-induced down-regulation by terbutaline was thus dose related.

To demonstrate that this inhibition of GR function by budesonide was not limited to the GR gene, MT2 mRNA levels, which are usually up-regulated by glucocorticoids, were determined. Similar results were obtained (Fig. 4): terbutaline alone had no effect on MT2 mRNA levels after a 4-hr exposure of the cells. However, the budesonide-induced up-regulation of the MT2 mRNA was inhibited by terbutaline when added in equimolar concentrations. Exposure of the cells to 100-fold more budesonide, again, up-regulated MT2 mRNA levels ($P < 0.05$), but to a lesser extent than with budesonide alone.

To test whether this interaction would also occur after the budesonid-induced GR mRNA down-regulation had already taken place, terbutaline was added to the bronchial epithelial cells after 4 hr pre-incubation with budesonide alone. The GR mRNA downregulation induced by budesonide, present after 4 hr, was not reversed to pre-exposure levels by subsequent simultaneous incubation of the cells to budesonide and terbutaline up to 44 hr (Fig. 5).

Similar results were obtained for the MT2 mRNA levels (Fig. 6). The budesonide-induced up-regulation of the MT2 mRNA was not reversed by terbutaline within 44 hr, although it appeared that the MT2 mRNA levels returned to the 100% pre-exposure level after 44 hr.

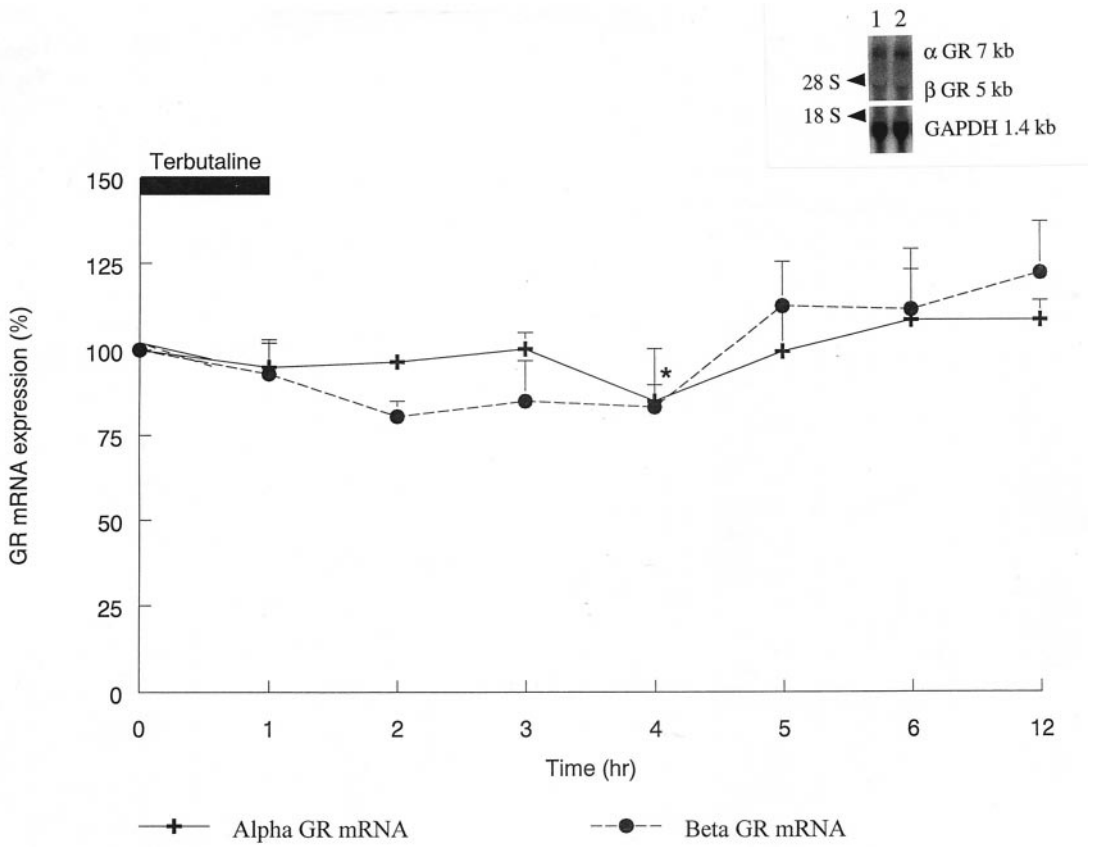

FIG. 1. GR mRNA expression is shown after exposure of bronchial epithelial cells for 1 hr to 1 μM terbutaline. Circles represent the α GR mRNA, and triangles represent the β-form. No change in either α or β GR mRNAs is observed in time. Represented are the means ± SDs of two to four experiments (*$P < 0.05$). mRNA expression levels were corrected for GAPDH and then compared to the control samples, which were set to 100%. Insertion is a Northern blot hybridized with both GR and GAPDH probes. A strong band is seen at 7 kb, representing the α GR. The β GR mRNA is expressed to a lower extent and is demonstrated at 5 kb. The control GAPDH is 1.4 kb long. Both 28S and 18S bands are shown.

EMSA

To test whether the disturbance of the GR and MT2 mRNA levels that were observed after the simultaneous addition of terbutaline and budesonide to epithelial cells was due to an interaction of the transcription factors GR and CREB, an electrophoretic mobility shift assay was performed. To this end, cells were exposed to budesonide, to terbutaline, or to budesonide and terbutaline simultaneously for 1 hr, and cytoplasmic proteins were isolated. Specificity of the signal was tested by the addition of unlabeled GRE, CRE, or control DNA not representing any responsive element (MT2 oligonucleotides). Cells exposed to budesonide alone showed an increased binding of GR to GRE. Exposure to terbutaline alone resulted in an increase of CREB binding to CRE. And the simultaneous exposure to equimolar concentrations of budesonide and terbutaline increased the binding of both transcription factors to GRE and CRE equally (Fig. 7), indicating protein interaction. Protein-GRE bindings were strongly inhibited by 100-fold excess, unlabeled GRE and CRE, but not by the unspecific control DNA. Similar results were obtained for the protein-CRE binding, which was also inhibited by both responsive elements and not by MT2 oligonucleotides.

DISCUSSION

In this study, the interaction between glucocorticoids and β_2-agonists was investigated at the mRNA level. As expected [30], exposure of a bronchial epithelial cell line to 1 μM budesonide significantly down-regulated both α and β GR mRNAs. No change was observed in α and β GR mRNA after exposing the cells to 1 μM terbutaline. The simultaneous incubation of bronchial epithelial cells with equimolar concentrations of budesonide and terbutaline prevented the budesonide-induced dose-dependent down-regulation, indicating an interaction between the two signal transduction pathways. This interaction between CREB and GR was further supported by the EMSA experiments. However, initial down-regulation of the α and β GR mRNA by budesonide alone was not reversed by subsequent treatment with a combination of budesonide and terbutaline. Similar results were obtained with the control gene, MT2, which is up-regulated by glucocorticoids.

The down-regulation of both GR mRNAs triggered by budesonide is a well-known phenomenon and has been demonstrated previously in this cell type [30]. However, the physiologic significance of this down-regulation is still not known and has to be further evaluated. Because five CRE

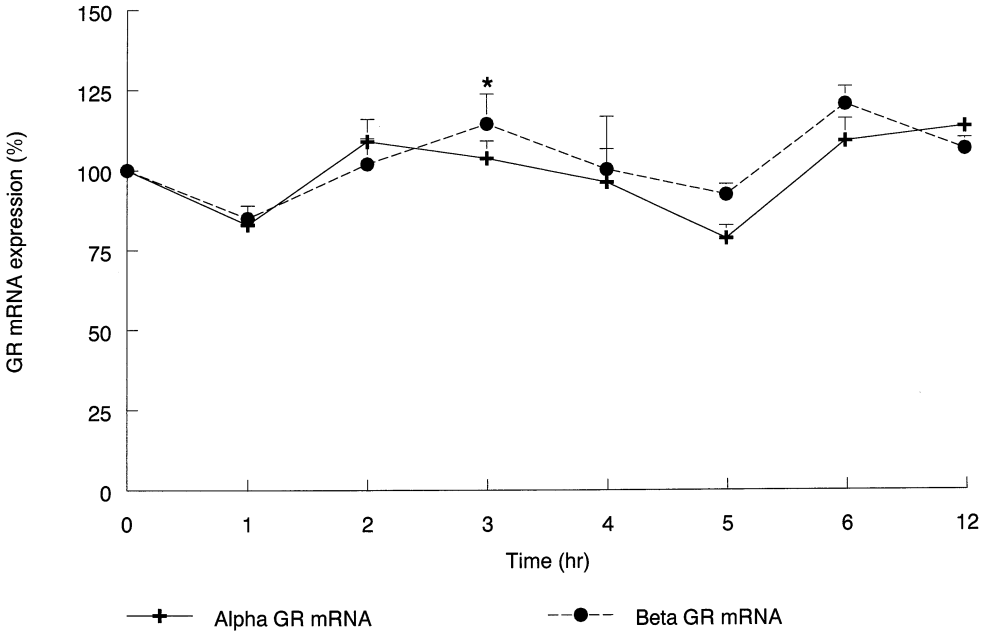

FIG. 2. Continuous exposure of bronchial epithelial cells to 1 μM terbutaline did not change either α or β GR mRNAs in time. Given are means and SDs of two to four experiments (*$P < 0.05$). mRNA expression levels were corrected for GAPDH and then compared to the control samples, which were set to 100%.

sides in the promotor region of the GR have been demonstrated [34], the activation of cAMP may also lead to the modulation of GR mRNA levels [33]. In rat hepatoma cells, exposure to 8-bromo-cAMP for 5 hr resulted in an increase in GR mRNA, due to an increased GR mRNA stability [33]. Because β_2-agonists increase intracellular cAMP levels, the modulation of GR mRNA expression might be expected after exposing cells to β_2-adrenergic agonists. In this study, no direct effect of terbutaline on the GR mRNA levels was observed. However, we demonstrated that incubation with equimolar concentrations of terbutaline and bunesonide, simultaneously, resulted in an inhibition of the budesonide-induced GR mRNA down-regulation. This inhibition was dose related; the addition of 100-fold more budesonide again down-regulated both GR mRNAs, but to a lesser extent than with budesonide alone. The inhibition of GR-mediated gene transcription due to interactions with other transcription factors has been shown for CREB [13, 14, 26], AP-1 [15, 16], NFκB [17, 18] and Stat5 [19]. Interestingly, addition of terbutaline to cells that were already exposed to budesonide for 4 hr did not reverse GR mRNA down-regulation. Similar results were obtained with MT2 mRNA. The fact that GR mRNA down-regulation and MT2 mRNA upregulation continued for a longer period of time, despite the addition of

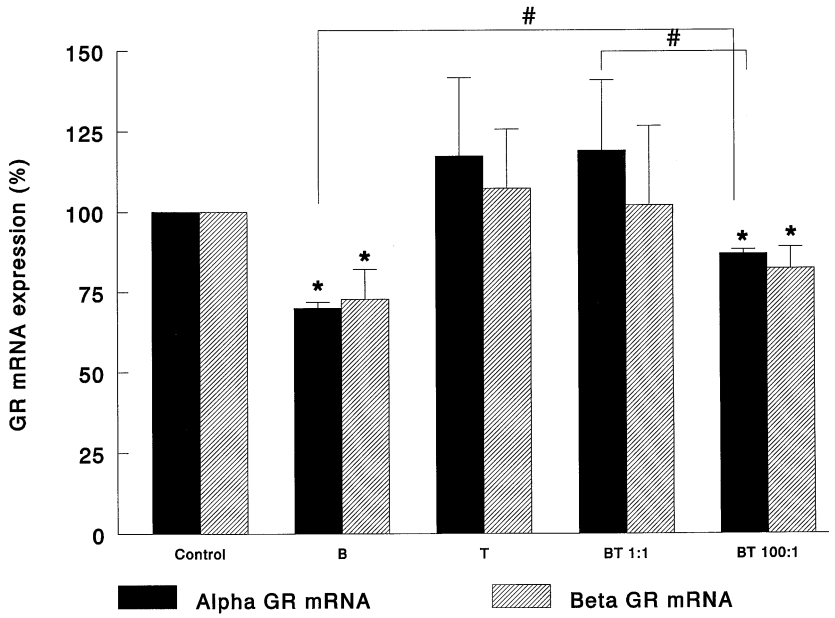

FIG. 3. Intervention between budesonide (B) and terbutaline (T). After exposing cells to 1 μM budesonide for 4 hr, a downregulation of both α and β GR mRNA levels was observed. Simultaneously adding equal amounts of terbutaline to the cells prevented this GR mRNA down-regulation. Addition of 100 times more budesonide restored the down-regulatory capacity of budesonide, although to a lower level than obtained with budesonide alone (*$P < 0.05$ compared to control, #$P < 0.05$ compared to budesonide alone or budesonide and terbutaline 1:1). mRNA expression levels were corrected for GAPDH and then compared to the control samples, which were set to 100%.

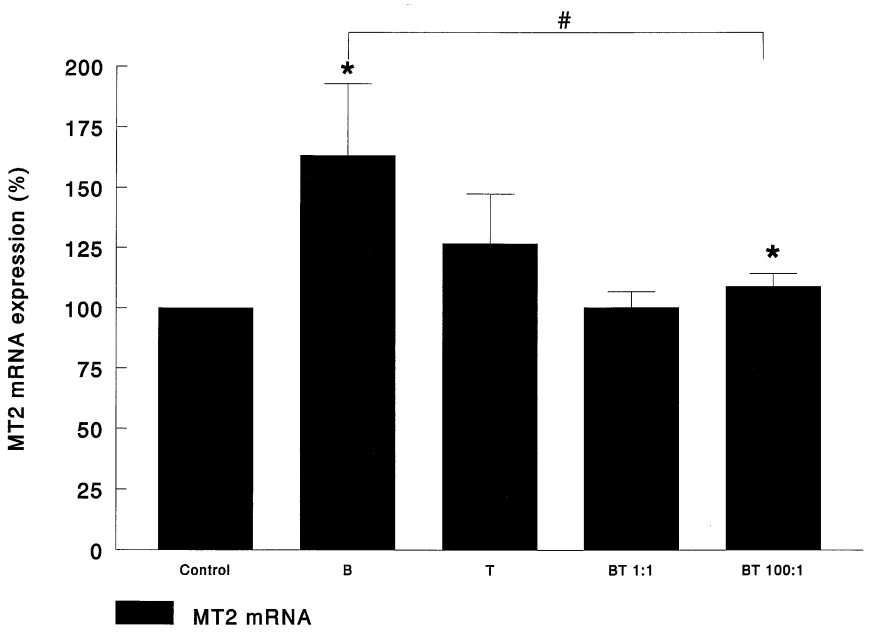

FIG. 4. MT2 mRNA levels in cells exposed to budesonide and terbutaline separately and simultaneously for 4 hr. A dose-dependent inhibition of the budesonide-induced up-regulated MT2 mRNA level was demonstrated by terbutaline. mRNA expression levels were corrected for GAPDH and then compared to the control samples, which were set to 100%. For an explanation of the symbols, see Fig. 3.

terbutaline, suggests that the activated DNA-bound transcription factors present are not inhibited by binding to other transcription factors.

Because, despite the use of inhaled β_2-agonists and glucocorticoids, morbidity and mortality of asthma has increased worldwide, there is, at present, a controversy as to whether regular treatment with β_2-agonists reduces overall asthma control [1]. An explanation for the detrimental effects of β_2-adrenergic agonists on asthma control might be found in the interaction between CREB and GR, which has also been demonstrated by Barnes *et al.* [12, 25, 27] where rat and human lung were exposed to albuterol and dexamethasone, which resulted in a reduced binding of both transcription factors to their responsive elements. In the present study, we also demonstrated an interaction between CREB and GR in bronchial epithelial cells,

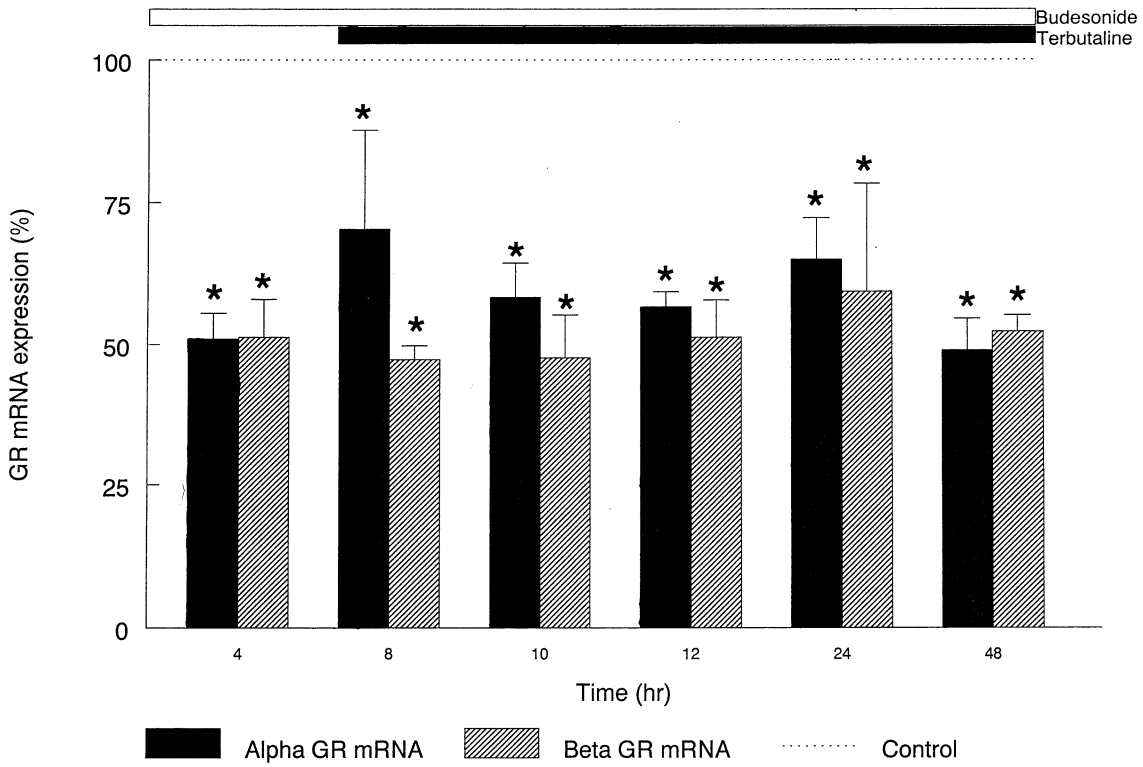

FIG. 5. After establishing a down-regulation of α and β GR mRNA by exposing bronchial epithelial cells to budesonide for 4 hr, incubation of terbutaline combined with budesonide did not reverse the down-regulation up to 44 hr (*$P < 0.05$). mRNA expression levels were corrected for GAPDH and then compared to the control samples, which were set to 100%.

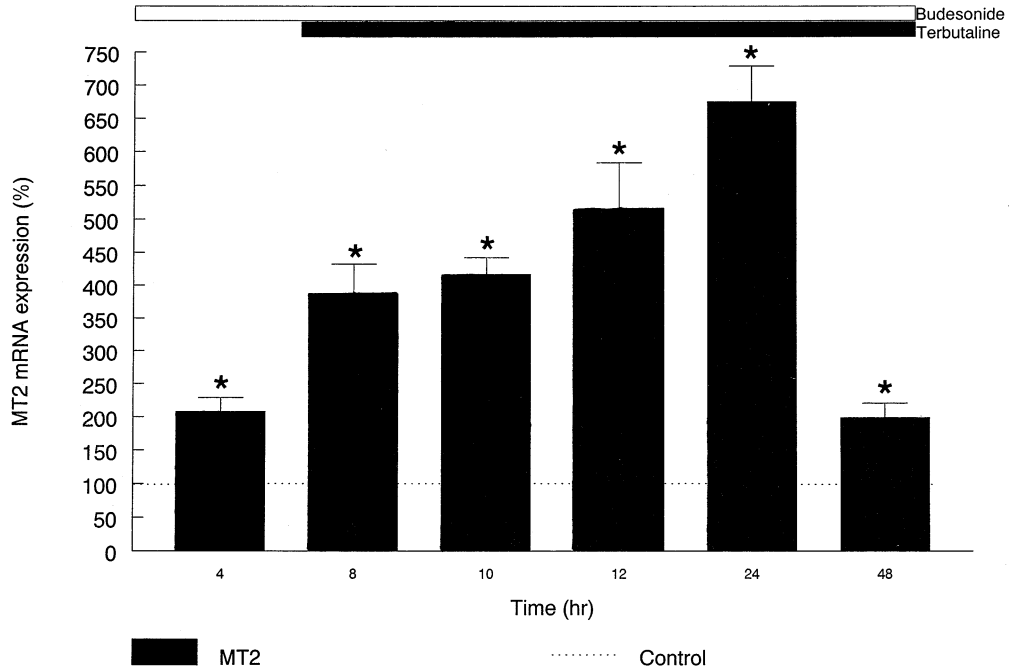

FIG. 6. The budesonide-induced up-regulated MT2 mRNA levels after 4 hr were not reversed by combined terbutaline and budesonide exposure up to 44 hr (*$P < 0.05$). mRNA expression levels were corrected for GAPDH and then compared to the control samples, which were set to 100%. The rise in MT2 mRNA levels was solely due to budesonide and was not changed by the addition of terbutaline.

because the proteins arising after exposure to terbutaline and glucocorticoids were able to bind both labeled GRE and CRE. Another support for this interaction is that the GRE and CRE signals not only disappeared with their own unlabeled responsive elements, but also with 100 times more of the other responsive element. However, contrary to the studies by Barnes *et al.*, in the present study an increased binding of this complex to DNA was demonstrated after simultaneous exposure to terbutaline and budesonide, in comparison to cells exposed to the dilutant only. These paradoxical results may be explained by the fact that in the above-mentioned studies [12, 25, 27] proteins were isolated from the nucleus, whereas in the present study the proteins were of cytoplasmic origin. Combining these data strengthens the notion of a cross-talk between GR and CREB transcription factors in the cytoplasm and not in the nucleus, suggesting that cytoplasmic transcription factor complexes do not enter into the nucleus. This is supported

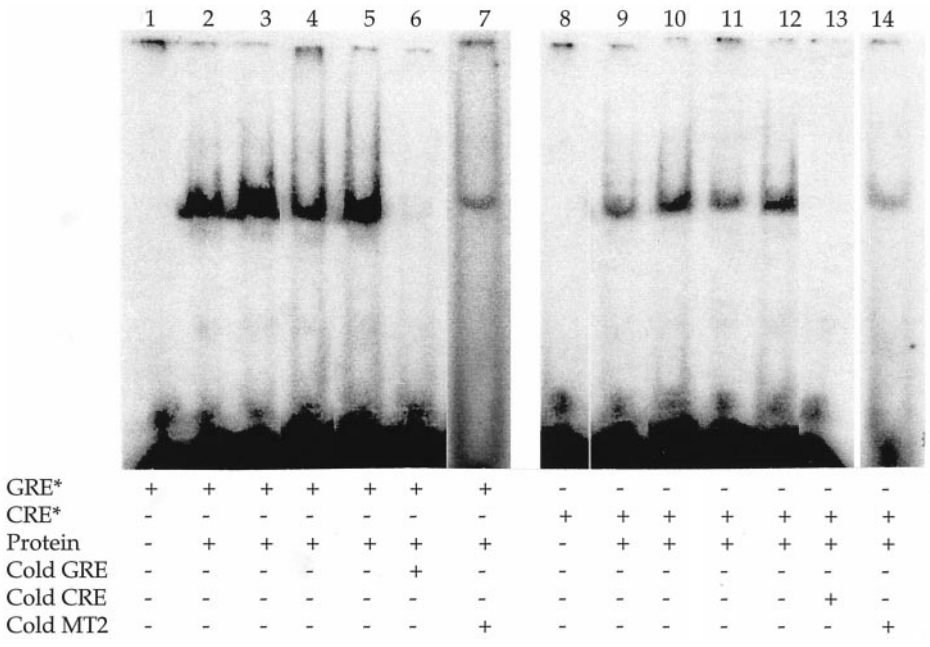

FIG. 7. Gel mobility shift assays demonstrated a cross-talk between GR and CREB. In lanes 1–7, binding to GREs is shown. Lanes 8–14 represent binding of transcription factors to CREs. No proteins were added to lanes 1 and 8. Lane 2 represents the control sample of the in-lane 3 run budesonide-exposed cells. Lanes 4 and 11 represent the controls of the cells exposed to budesonide and terbutaline simultaneously, shown in lanes 5 and 12. Lanes 6 and 13 are similar to lanes 3 and 10, except for the addition of 100-fold unlabeled GRE or CRE, respectively. Lane 9 represents the control of the terbutaline-exposed cells, presented in lane 10. In lanes 7 and 14 100-fold excess unspecific responsive element was added.

by a study of Adcock *et al.* [35, 36], in which simultaneous exposure of cells to dexamethasone and 4β-phorbol 12-myristate 13-acetate (PMA)/tumor necrosis factor β/Interleukin 1α results in cross-talk between the GR and NFκB and/or AP-1 in the cytoplasm. The surprising findings that: 1) the GRE and CRE bands are on the same level in the EMSA assays; and 2) the GRE and CRE are mutual inhibitors of GR and CREB DNA binding suggest that these interacting proteins are part of a larger protein complex, perhaps consisting of AP-1, NFκB, Stat5, or other transcription factors. An explanation for the cytoplasmic localization may be that the nuclear localization signals from the individual transcription factors, necessary for nuclear influx [37, 38], are blocked due to the protein-protein interactions. Alternatively, the protein complex may be too large to enter the nuclear pores [39]. In Fig. 8a–c, schematic presentations of the hypothesis of the interaction between CREB and GR are provided.

Extrapolation of the results to the in vivo situation depends on the time span. In the long term, the effect of higher GR mRNA levels is as yet unclear and is dependent on the presence of several transcription factors playing a pathophysiologic role in diseases such as asthma. In the short term, simultaneous inhalation of glucocorticoids and β_2-adrenergic agonists induces an interaction of transcription factors, thereby disturbing the regulation of mRNA levels by glucocorticoid treatment alone. In this respect, the modulation of GR and MT2 mRNA can be looked upon as one of the effects of glucocorticoid treatment. In asthma, several cytokines whose expression is inhibited by glucocorticoids play a role (for review, see Brattsand and Linden [40]). This inhibition occurs by binding the activated GR to transcription factors AP-1 and NFκB, which induces cytokine mRNA expression (for review, see Barnes [20]). If the cross-talk with AP-1 and NFκB is blocked by the scavenging of activated GR by CREB, the antiinflammatory effects of glucocorticoids may be inhibited. To avoid the inhibition of GR function by β_2-agonists, it can be hypothesized from the present study that, to allow the beneficial effects of both bronchodilator and antiinflammatory agonists, glucocorticoids should be inhaled first, followed after a few hours by inhalation of β_2-agonists.

This hypothesis is based on *in vitro* experiments where the dose of the medication is supposed to be uniformly distributed over all cells. However, in the human lung, the drug deposition after inhalation will be nonhomogeneous, and it is not likely that all airway cells will be exposed to similar doses of β_2-agonists and glucocorticoids. Thus, a mixture of effects may occur *in vivo*. However, this hypothesis remains to be investigated in a clinical study.

In conclusion, β_2-agonists interfere with the GR function in human bronchial epithelial cells *in vitro*, when given simultaneously, with this being overcome by sequential exposure of the cells to first glucocorticoids and then β_2-agonists.

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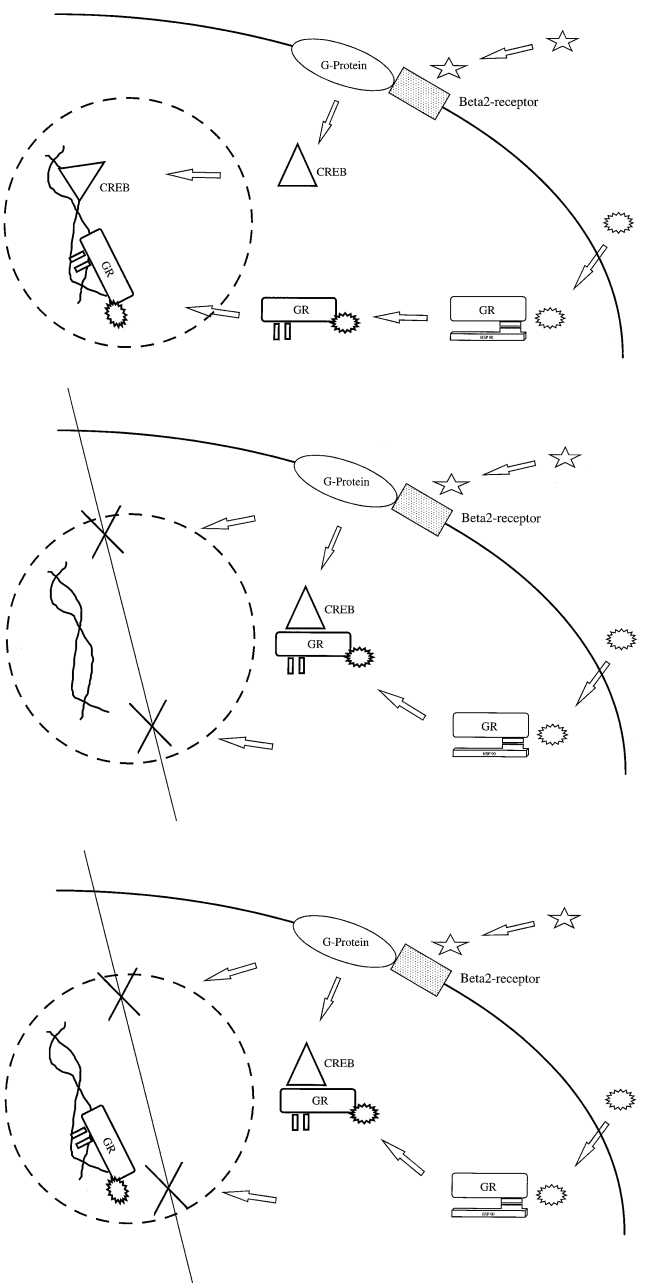

FIG. 8. Proposed mechanism of cross-talk between CREB and GR: (a) Signal transduction pathways after separate exposure of cells to budesonide or terbutaline to genes containing GREs or CREs, respectively; (b) interaction between CREB and GR after simultaneous exposure to budesonide and terbutaline; and (c) exposure of cells to budesonide, followed by exposure to budesonide and terbutaline simultaneously.

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