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β_2-Adrenoceptor Agonists, Like Glucocorticoids, Repress Eotaxin Gene Transcription by Selective Inhibition of Histone H4 Acetylation[1]

Mei Nie,[2] Alan J. Knox, and Linhua Pang[2]

Eotaxin is a potent eosinophil chemoattractant implicated in various allergic inflammatory conditions including asthma, but relatively little is known about its regulation. Human airway smooth muscle cells are an important source of eotaxin in the airway. We have previously demonstrated that β_2-adrenoceptor agonists (β_2-agonists) and glucocorticoids additively inhibit eotaxin production in human airway smooth muscle cells, but the underlying mechanisms are unclear. Here, we studied the molecular mechanisms of their actions and interactions on eotaxin gene transcription. TNF-α-induced eotaxin gene transcription was mediated mainly by the transcription factor NF-κB (p65/p50) as analyzed by luciferase reporter gene assay, Western blotting, EMSA, and electrophoretic mobility supershift assay. Chromatin immunoprecipitation assay demonstrated that TNF-α also induced selective histone H4 acetylation on lysines 5 and 12 at the eotaxin promoter site and p65 binding to the eotaxin promoter, resulting in eotaxin gene transcription. The inhibition of eotaxin production by β_2-agonists and glucocorticoids was transcriptional and not due to altered NF-κB nuclear translocation or in vitro promoter binding capability, but due to their inhibition of TNF-α-induced histone H4 acetylation and p65 in vivo binding to the promoter. Additive inhibition was achieved when the two groups of drugs were combined. Our findings reveal a novel mechanism by which β_2-agonists, like glucocorticoids, regulate NF-κB-mediated inflammatory gene expression through inhibition of histone acetylation. This provides one explanation for the benefits that result when these agents are combined to treat asthma, and may have important implications in a wide range of inflammatory diseases. ***The Journal of Immunology,*** **2005, 175: 478–486.**

Accumulation of eosinophils within local tissues is a prominent feature of a broad range of diseases including allergic conditions such as asthma, rhinitis, and other inflammatory disorders such as inflammatory bowel disease and eosinophilic gastroenteritis (1). Eotaxin, a CC chemokine and potent eosinophil chemoattractant, is believed to be critically involved in the pathogenesis of the above eosinophilic inflammatory diseases (1). β_2-Adrenoceptor agonists (β_2-agonists) are widely used as bronchodilators for asthma treatment. However, recent studies have suggested that long acting β_2-agonists potentiate the effect of glucocorticoids in reducing asthma exacerbations (2, 3), suggesting that they may potentiate the anti-inflammatory effects of glucocorticoids. Studies from our group and others in cell systems have described potential mechanisms that might account for this. We have recently reported that β_2-agonists, like glucocorticoids, partially inhibit TNF-α-induced eotaxin production from human airway smooth muscle (HASM)[3] cells, and that their combined use results in greater inhibition (4). β_2-Agonists also inhibit GM-CSF and RANTES production and enhance the inhibition of TNF-α-induced IL-8 production by glucocorticoids in the same cells (5, 6). Similar effects of β_2-agonists in other cells have also been reported (7). Collectively these studies suggest that β_2-agonists, like glucocorticoids, can inhibit the production of a wide range of chemokines and cytokines in airway smooth muscle and other cells, which would inhibit the trafficking and survival of inflammatory cells in the airway. The underlying molecular mechanisms are largely unknown.

The human eotaxin promoter contains several binding sites for transcription factors including C/EBP, AP-1, STAT6, and NF-κB (8, 9). NF-κB is a key transcription factor regulating the expression of many inflammatory genes and consists of a homodimer or heterodimer of Rel family proteins, including p50 and p65. NF-κB and STAT6 appear to be the main transcription factors that regulate eotaxin gene expression in a cell- and stimulus-dependent manner. For example, NF-κB mediates TNF-α- and IL-1β-induced eotaxin gene transcription (9, 10), whereas STAT6 mediates IL-4-induced eotaxin gene transcription (9) in airway epithelial cells. In contrast, STAT6 is required for eotaxin expression by TNF-α in fibroblasts (11). The transcription factor(s) mediating TNF-α-induced eotaxin transcription in HASM cells have not been identified.

In resting cells, DNA is packaged into a highly organized and dynamic protein-DNA complex known as chromatin. The fundamental subunit of chromatin is the nucleosome, which consists of 146 bp of DNA wrapped twice around an octomer core of four histones (two molecules each of histones H2A, H2B, H3, and H4) (12). This nucleosomic structure prevents the access of transcription factors and RNA polymerase II to their respective recognition sequences and the initiation of transcription (13). Transcriptional regulation involves recruitment of complexes that modify chromatin structure (remodeling) at a gene promoter, transcription factors,

Division of Respiratory Medicine, City Hospital, University of Nottingham, Nottingham, United Kingdom

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[2] Address correspondence and reprint requests to Dr. Mei Nie or Dr. Linhua Pang, Division of Respiratory Medicine, Clinical Sciences Building, City Hospital, University of Nottingham, Hucknall Road, Nottingham NG5 1PB, U.K. E-mail addresses: mszmn1@gwmail.nottingham.ac.uk or linhua.pang@nottingham.ac.uk

[3] Abbreviations used in this paper: HASM, human airway smooth muscle; ChIP, chromatin immunoprecipitation; HAT, histone acetyltransferase; HDAC, histone deacetylase; IP, immunoprecipitate; CBP, CREB-binding protein; β-2M, β_2-microglobulin.

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and the basal transcription machinery, gaining access to their respective binding sites at the promoter, and the altered activity of RNA polymerase at the gene (14–16). One of the chromatin modifications associated with inflammatory gene transcription is the acetylation of histone H4 on the highly conserved lysines at the N-terminal tail (17, 18). Different patterns of histone H4 acetylation are associated with different gene transcription (19), and transcription factor binding to a specific gene promoter is associated with changes in histone acetylation (13). Inhibition of cytokine-induced histone H4 acetylation may be an important mechanism by which glucocorticoids regulate inflammatory gene expression (19). However, so far there is no evidence linking chromatin remodeling to the regulatory effect of β_2-agonists on inflammatory gene expression. How histone H4 acetylation is involved in TNF-α-induced eotaxin expression and whether this process can be modified by β_2-agonists remain to be explored.

In the present study, we studied the transcription factor involvement, histone H4 acetylation and NF-κB subunit p65 promoter binding in TNF-α-induced eotaxin gene transcription in HASM cells and investigated the molecular mechanisms by which β_2-agonists regulate eotaxin gene transcription and compared with those by glucocorticoids. Our results demonstrate a novel chromatin-modifying mechanism by which β_2-agonists, like glucocorticoids, regulate inflammatory gene expression in airway cells and provide an explanation for the benefits that results from their combined use in asthma therapy.

Materials and Methods

Cell culture

Primary cultures of HASM cells were established from explants obtained from four postmortem individuals with no history of respiratory diseases and no evidence of airway abnormalities as previously reported (20). This protocol was approved by the Nottingham City Hospital Research Ethics Committee. Cells at passages 5–6 depict the immunohistochemical and morphological characteristics of typical airway smooth muscle cells and were used throughout this study (20).

DNA constructs

The firefly luciferase reporter constructs in pGL3-Basic vectors containing different human eotaxin promoter fragments (−1363 and −300) and mutations (M1 and M2) (9) were obtained from Prof. R. Schleimer (Johns Hopkins Asthma and Allergy Center, Baltimore, MD). To clarify the involvement of C/EBP in TNF-α-induced eotaxin gene transcription, we generated a 1134-bp DNA fragment containing no C/EBP *cis*-element based on the −1363 fragment by PCR using the primers: forward, 5′-AACGCGTTCGGTACCCGGGGATCCTAGATGGGCACTT-3′ and reverse, 5′-CACGATAGTCATGCCCCGCGC-3′. The PCR product was separated by 2% agarose gel electrophoresis; the 1134-bp fragment was purified with QIAX II kit (Qiagen), digested with *Mlu*I and *Bgl*II (Invitrogen Life Technologies) and then inserted between the *Mlu*I and *Bgl*II sites of the pGL3-Basic vector. The resulted construct was verified by sequencing. The wild-type NF-κB p50 and p65 expression vectors were described before (21). The wild-type STAT6 expression vector (22) was obtained from Tularik. The internal control *Renilla* luciferase reporter construct RL-SV40 was from Promega.

Reporter gene assay

Transient transfections were performed as described previously (23, 24). After transfection, cells were treated with or without the drugs for 0.5 h before incubation with TNF-α for 6 h. The firefly and *Renilla* luciferase activities then were measured, and relative luciferase activity was calculated.

Protein extraction and Western blotting

Cytosolic and nuclear extracts were prepared from cells and transcription factor nuclear translocation was characterized by Western blotting as described previously (23, 24).

EMSA

The in vitro DNA binding of transcription factors was analyzed as described previously (21, 24) using ^{32}P-labeled or unlabeled human eotaxin promoter oligonucleotides containing wild-type STAT6 and NF-κB *cis*-elements (NFS6) (5′-GGCTTCCCTGGAATCTCCCACA-3′), mutated STAT6 *cis*-element (M1) (5′-GGCagCCCTGGAATCTCCCACA-3′), or mutated NF-κB *cis*-element (M2) (5′-GGCTTCCCTGGAATCTgggACA-3′) (9). For competition experiments, nuclear extracts were incubated with excessive unlabeled probes (25-fold) for 15 min and then with labeled probes for another 15 min. For supershift experiments, the reactions with labeled probes were incubated with specific Abs against p50, p65, or STAT6 (Santa Cruz Biotechnology) for another 15 min.

Quantitative real-time RT-PCR

Confluent HASM cells in 24-well plates were serum-starved for 24 h. After treatment, total RNA was extracted and purified using the RNeasy kits with DNase treatment according to the manufacturer's instructions (Qiagen). Up to 2 μg of RNA was used in a reverse transcription reaction with Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies). Quantitative PCR was performed on an ABI prism 7700 (Applied Biosystems) using Excite real-time Mastermix with SYBR green (Biogene). Each reaction consisted of 1× Excite Mastermix, SYBR green (1/60,000 final concentration), 40 nM of both forward and reverse primers, 1.6 μl of cDNA (or H_2O) and H_2O to a final volume of 20 μl. Thermal cycler conditions included an incubation at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. After the 40 cycles, the products were heated from 60°C to 95°C over 20 min to allow melting curve analysis to be performed. This allowed the specificity of the products to be determined (single melting peak) and confirmed the absence of product generated by primer-dimer association.

To enable the levels of transcripts to be quantified, a series of dilutions (2 μg to 0.1 ng) of RNA sample from TNF-α-treated HASM cells (10 ng/ml, 6 h) were included to generate standard curves that were used to obtain relative concentrations of the transcript of interest for each RNA sample. The housekeeping gene β_2-microglobulin (β-2M) was used to confirm the equal loading of total RNA and to calculate the relative expression of eotaxin transcripts by normalizing eotaxin mRNA levels against β-2M mRNA levels. The PCR primers for eotaxin (25) and β-2M (26) have been previously described. Negative controls (no template) were included in each experiment and reactions for standards were run in triplicate and those for samples were run in duplicate.

For RNA stability, confluent and serum-starved cells were treated with or without TNF-α for 2 h and then with or without the drugs for 2 h before the addition of actinomycin D (5 μg/ml) for the times indicated to block new transcript generation. Then total RNA were extracted and real-time PCR was performed as described above.

Chromatin immunoprecipitation (ChIP)

Histone H4 acetylation at the eotaxin gene promoter site and transcription factor in vivo binding to the promoter were analyzed by ChIP as previously described (19, 24). One portion of the soluble chromatin was used as DNA input control, and the remains were subaliquoted and incubated with a nonimmune rabbit IgG (Santa Cruz Biotechnology) or IgG Abs against NF-κB p65 (Upstate Biotechnology), histone H4 AcK5 (acetylated at lysine 5), AcK8, AcK12, AcK16 (Serotec), respectively. The DNA from the immunoprecipitated Ab-protein-DNA complex was purified and amplified by PCR using the primers specific for the eotaxin promoter (−136 to +61) encompassing both STAT6 and NF-κB binding sites: forward, 5′-CTT CATGTTGGAGGCTGAAG-3′ and reverse, 5′-GGATCTGGAATCTG GTCAGC-3′.

Statistical analysis

Data were presented as mean ± SEM of three independent experiments. ANOVA and an unpaired two-tailed Student's t test were used to assess the differences between the means. A value of $p \leq 0.05$ was considered significant.

Results

TNF-α-induced eotaxin gene expression is NF-κB driven

We first determined the transcription factor(s) involved in TNF-α-induced eotaxin gene transcription by transfecting HASM cells with luciferase reporter plasmids containing the wild-type eotaxin

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promoter sequence (−1363) or its deletions or mutations. The constitutive promoter activity of the shortest wild-type eotaxin promoter (−300) plasmid was lower than that of the other two constructs (−1363) and (−1134) (Fig. 1*A*), suggesting a positive regulatory element (possibly AP-1) between positions −300 and −1134. Transfection with the −1363 plasmids containing mutated STAT6 binding site (M1) or mutated NF-κB binding site (M2) revealed that both mutants displayed a lower constitutive promoter activity than the wild-type plasmid (Fig. 1*A*), suggesting that STAT6 and NF-κB binding sites contribute to constitutive eotaxin promoter activity. TNF-α (10 ng/ml, 6 h) markedly increased the promoter activity in cells transfected with the −1363 plasmid containing C/EBP, AP-1, STAT6, and NF-κB binding sites compared with the control (2.90-fold; Fig. 1*A*). TNF-α-induced promoter activity was slightly reduced with transfection of the −1134 plasmid containing no C/EBP binding site (2.25-fold) compared with transfection of the −1363 plasmid. But the activity was not reduced with transfection of the −300 plasmid containing neither C/EBP nor AP-1 binding site (3.34-fold), suggesting that C/EBP binding site may be required to optimize TNF-α stimulation. TNF-α-induced promoter activity was not affected with transfection of the M1 mutant plasmid (3.03-fold) compared with transfection of the wild-type −1363 plasmid, but was completely abolished with transfection of the M2 mutant plasmid (0.63-fold; Fig. 1*A*). Furthermore, the constitutive promoter activity of the −1363 plasmid was increased with overexpression of NF-κB p65 (3.04-fold) and p50 (2.60-fold), to a lesser extent STAT6 (1.59-fold), whereas TNF-α-induced promoter activity of the wild-type −1363 plasmid alone (2.82-fold over control) was markedly enhanced with overexpression of NF-κB p65 (5.75-fold), but not p50 (3.05-fold) or STAT6 (2.45-fold) (Fig. 1*B*). However, the overexpression had no effect on the promoter activity in cells transfected with the M2 plasmid, with or without TNF-α stimulation (Fig. 1*C*). These results strongly suggest that although NF-κB, STAT6, and possibly AP-1, may be involved in constitutive eotaxin transcription, and C/EBP may be required to optimize TNF-α-induced eotaxin transcription, NF-κB is the key transcription factor that mediate TNF-α-induced eotaxin gene transcription in HASM cells.

We then applied Western blotting to assess transcription factor nuclear translocation. Under resting conditions, NF-κB subunit p65, p50, p52, and STAT6 only existed in the cytosol of HASM cells, NF-κB RelB was detected in both cytosol and nucleus, and NF-κB c-Rel was not detected in either cytosol or nucleus. After 0.25 h TNF-α stimulation, a reduction of p65, p50, and STAT 6, but not p52 or RelB, in the cytosol and a corresponding increase in the nucleus were observed (Fig. 2). p65 and p50 nuclear translocation was marked and lasted for up to 2 h after TNF-α stimulation, whereas STAT6 nuclear translocation lasted for only 1 h (Fig. 2).

To determine whether translocated NF-κB or STAT6 could bind to the eotaxin promoter in vitro, nuclear extracts were incubated with DNA probes spanning the binding sites for STAT6 and NF-κB in the eotaxin promoter (Fig. 3*A*). EMSA, with the wild-type NFS6 probe, revealed the formation of two strong protein-DNA complexes (complexes 1 and 2; Fig. 3*B*) in TNF-α-treated cells (10 ng/ml, 1 h; *lanes 2* and *5*) compared with control cells (*lanes 1* and *4*). The complexes were blocked with excessive unlabeled NFS6 probe (Fig. 3*B*, *lane 3*), indicating that they are

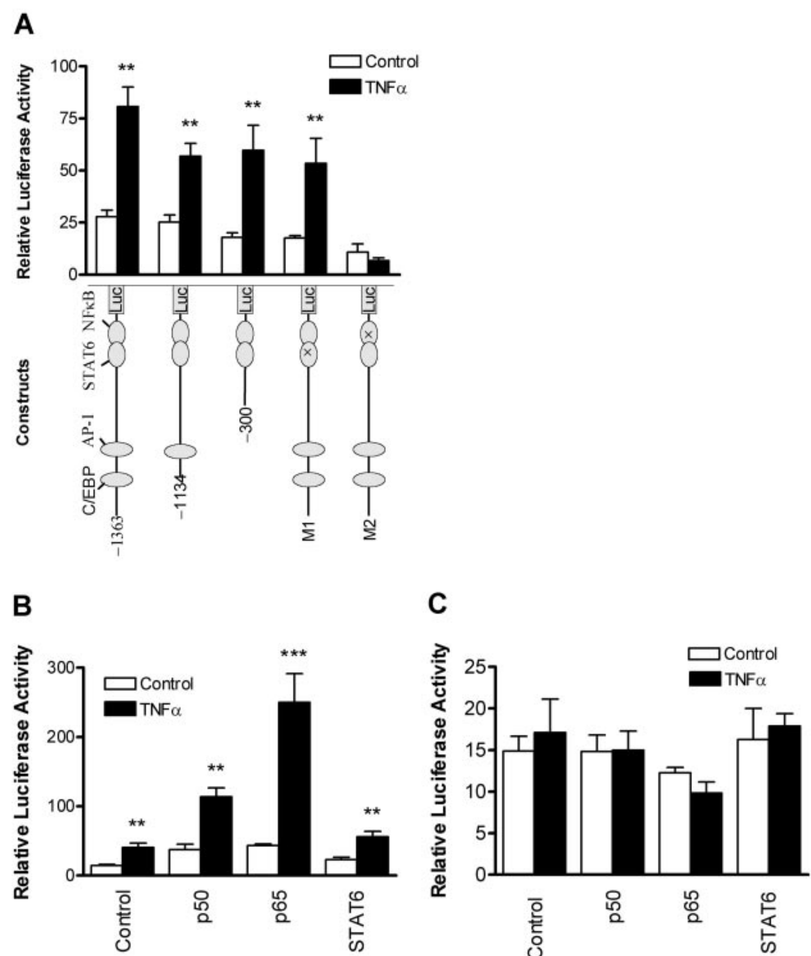

FIGURE 1. Identification of promoter *cis*-elements in TNF-α-induced eotaxin gene transcription. *A*, A total of 50–60% confluent HASM cells in 24-well plates were cotransfected with the internal control *Renilla* luciferase reporter plasmids (4 ng/well) and the eotaxin promoter firefly reporter plasmids (0.4 μg/well) depicted on the *left* for 24 h using FuGene 6 transfection reagent and then treated with or without TNF-α (10 ng/ml) for 6 h. *B*, HASM cells were cotransfected with *Renilla* plasmids, the 1363-bp wild-type eotaxin promoter plasmids, and p50, p65, or STAT6 expression vectors for 24 h, and were treated with or without TNF-α (10 ng/ml) for 6 h. *C*, HASM cells were cotransfected with *Renilla* plasmids, the 1363-bp eotaxin promoter plasmids mutated at the NF-κB site (M2), and p50, p65, or STAT6 expression vectors for 24 h, and then were treated with or without TNF-α (10 ng/ml) for 6 h. The luciferase activities were assayed using the dual-luciferase reporter assay system, and the relative luciferase activity was obtained by normalizing the firefly luciferase activity against the internal control *Renilla* luciferase activity. Each point represents the mean ± SEM of three independent experiments performed in duplicate or triplicate. **, $p < 0.01$; ***, $p < 0.001$ compared with corresponding control.

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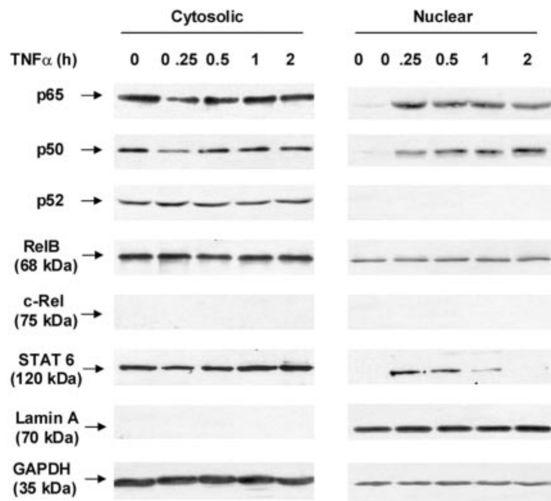

FIGURE 2. TNF-α-induced nuclear translocation of transcription factors. Confluent and serum-starved HASM cells in 90-mm dishes were treated with TNF-α (10 ng/ml) for the times indicated, cytosolic and nuclear extracts were prepared, and the presence of transcription factors in both fractions was analyzed by Western blotting using specific Abs. GAPDH and lamin A were served as protein loading controls. The blots are representative of three independent experiments with similar results.

specific for the probe. When the M1 probe (STAT6 binding site mutated) was used instead of the NFS6 probe, the two complexes remained intact in TNF-α-treated cells (Fig. 3*B*, *lane 7*) compared with control cells (*lane 6*); in contrast, when the M2 probe (NF-κB binding site mutated) was used, the two complexes were abolished (*lane 9*). Furthermore, incubation of the reaction mixtures with anti-p50 Ab resulted in the formation of a strong retarded complex (supershift) with the NFS6 probe, accompanied by the loss of both complexes (Fig. 3*C*, *lane 3*). Incubation with anti-p65 Ab also generated a faint retarded complex (*lane 4*) with a reduction of both complexes, complex 1 in particular; whereas incubation with both Abs abolished the two complexes (*lane 6*). In contrast, anti-STAT6 Ab did not cause any change of the complexes (Fig. 3*C*, *lane 5*). However, EMSA with the wild-type NFS6 probe revealed the formation of one weak protein-DNA complex in IL-4-treated cells (10 ng/ml, 1 h; Fig. 3*C*, *lane 8*) compared with control cells (*lane 7*). Incubation of the reaction mixtures with anti-STAT6 Ab led to complete disappearance of the IL-4-induced complex (Fig. 3*C*, *lane 9*), whereas incubation with anti-p50 and anti-p65 Abs had no effect (*lanes 10* and *11*), indicating that the complex was specifically composed of STAT6-DNA. The results suggest that TNF-α stimulates NF-κB (p50 and p65), but not STAT6, binding to the overlapping STAT6/NF-κB element in the eotaxin promoter in vitro.

β_2-Agonists and glucocorticoids repress TNF-α-induced eotaxin mRNA expression and promoter activity

We evaluated the effect of β_2-agonists and glucocorticoids on TNF-α-induced eotaxin mRNA expression by quantitative real-time RT-PCR. Unstimulated cells expressed low levels of eotaxin mRNA; TNF-α induced eotaxin mRNA expression in a time-dependent manner, with marked expression appearing at 2 h (Fig. 4*A*). The β_2-agonists salmeterol and salbutamol and the glucocorticoids fluticasone and dexamethasone all partially inhibited TNF-α-induced eotaxin mRNA expression (6 h incubation), and greater inhibition was achieved when the two types of drugs were used in combination (Fig. 4*B*). To determine whether posttranscriptional regulation was involved in the reduction of TNF-α-induced

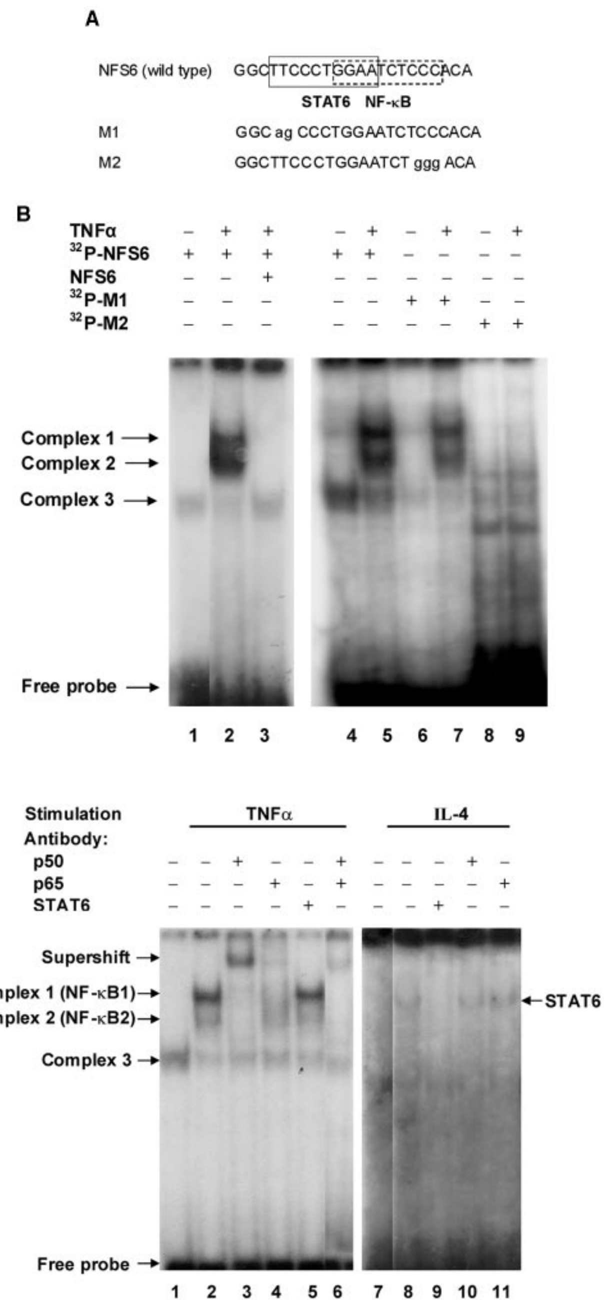

FIGURE 3. TNF-α-induced in vitro promoter binding of transcription factors. Confluent and serum-starved HASM cells in 90-mm dishes were incubated with or without TNF-α (10 ng/ml) or IL-4 (10 ng/ml) for 1 h. Nuclear proteins were extracted and the in vitro nuclear protein-DNA binding was analyzed by EMSA. *A*, Sequences of oligonucleotide probes derived from the eotaxin promoter for EMSA. The binding sites for STAT6 and NF-κB are indicated by a box, and mutated sites are in lowercase. *B*, In vitro binding of nuclear proteins to ^{32}P-labeled probes by EMSA and competition assay with unlabeled probes. *C*, Characterization of TNF-α- and IL-4-induced transcription factor-DNA complexes formed with the ^{32}P-labeled NFS6 probe by supershift assay with specific Abs indicated. Results are representative of three independent experiments with similar results.

eotaxin mRNA by β_2-agonists and glucocorticoids, we performed mRNA stability studies. As shown in Fig. 4*C*, TNF-α-induced eotaxin mRNA was steadily maintained up to 20 h after the addition of actinomycin D although there was a slight trend of mRNA degradation. Treatment with salmeterol, fluticasone, or the combination of both did not alter the stability of TNF-α-induced

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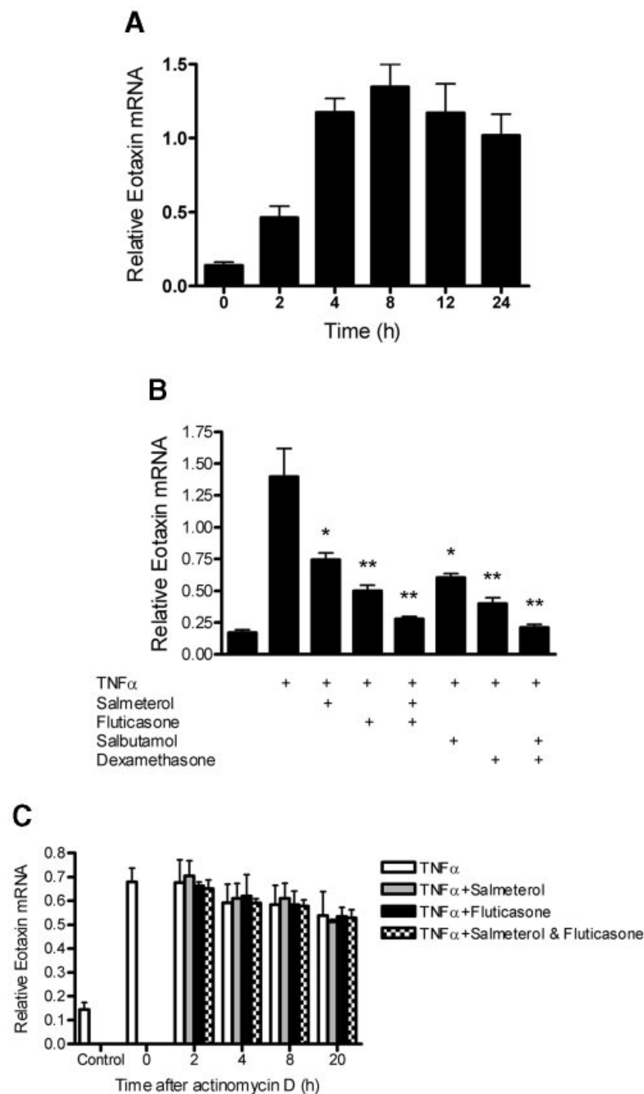

FIGURE 4. Effect of β_2-agonists and glucocorticoids on TNF-α-induced eotaxin mRNA expression and stability. *A,* Confluent and serum-starved HASM cells in 24-well plates were treated with TNF-α for the times indicated. *B*, HASM cells were pretreated with or without salmeterol, fluticasone, salbutamol, and dexamethasone, alone or in combination, for 30 min and then stimulated with or without TNF-α for 6 h. *C*, HASM cells were stimulated with or without TNF-α for 2 h and then treated with or without salmeterol, fluticasone, or their combination for 2 h before the addition of actinomycin D for the times indicated to block the new transcript generation. mRNA levels of eotaxin and the housekeeping gene β-2M were detected by real-time RT-PCR. Relative eotaxin mRNA was obtained by normalizing eotaxin mRNA levels against β-2M mRNA levels. Each point represents the mean $\pm$ SEM of two independent experiments performed in duplicate. *, $p < 0.05$; **, $p < 0.01$ compared with TNF-α alone.

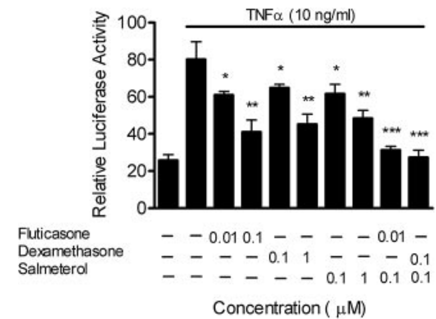

FIGURE 5. Effect of salmeterol and glucocorticoids on TNF-α-induced eotaxin promoter activity. A total of 50–60% confluent HASM cells in 24-well plates were cotransfected with the internal control *Renilla* luciferase reporter plasmids (4 ng/well) and the 1363-bp eotaxin promoter firefly reporter plasmids (0.4 μg/well) for 24 h, pretreated with or without fluticasone, dexamethasone, salmeterol, or their combinations for 30 min, and then stimulated with TNF-α for 6 h. The luciferase activities were assayed using the dual-luciferase reporter assay system and the relative luciferase activity was obtained by normalizing the firefly luciferase activity against the internal control *Renilla* luciferase activity. Each point represents the mean $\pm$ SEM of three independent experiments performed in duplicate or triplicate. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with TNF-α alone.

eotaxin mRNA compared with TNF-α alone. The results are consistent with those obtained by semiquantitative RT-PCR (data not shown). In cells transfected with the -1363 wild-type eotaxin promoter plasmid, fluticasone, dexamethasone, and salmeterol all partially but significantly inhibited TNF-α-induced promoter activity, and greater inhibition was achieved when salmeterol was used together with fluticasone or dexamethasone (Fig. 5). The above results suggest that both β_2-agonists and glucocorticoids had no effect on the stability of TNF-α-induced eotaxin mRNA and that they transcriptionally repress TNF-α-induced eotaxin gene expression in HASM cells through the inhibition of the gene promoter activity.

β_2-Agonists and glucocorticoids do not inhibit TNF-α-induced NF-κB nuclear translocation and in vitro binding to the eotaxin promoter

We next conducted Western blotting to determine whether β_2-agonists and glucocorticoids could affect nuclear translocation and in vitro eotaxin promoter binding of the key transcription factor NF-κB p65. TNF-α induced a reduction of cytosolic p65 and a corresponding increase of nuclear p65 after 0.5 h treatment, the cytosolic levels of p65 recovered after 2 h, whereas the increased nuclear levels of p65 maintained up to 4 h (Fig. 6*A*, *lanes 1–4*). Salmeterol had no effect on TNF-α-induced changes of cytosolic p65 (*lanes 5–7*). Fluticasone did not affect TNF-α-induced p65 reduction in cytosol at 0.5 h but inhibited its recovery at 2 and 4 h (Fig. 6*A*, *lanes 8–10*) and the inhibition was reversed with the addition of salmeterol (*lanes 11–13*). Both drugs, alone or in combination, had no effect on TNF-α-induced increase of nuclear p65 (Fig. 6*A*, *lanes 5–13*). EMSA with the wild-type NFS6 probe revealed that dexamethasone, fluticasone, salbutamol, and salmeterol, either alone or in combination, had no effect on TNF-α-induced NF-κB p50/p65 in vitro binding to the eotaxin promoter (Fig. 6*B*, *lanes 6–13*). These data suggest that the repression of TNF-α-induced eotaxin gene transcription by β_2-agonists and glucocorticoids is not via the inhibition of NF-κB nuclear translocation or in vitro promoter binding.

β_2-Agonists and glucocorticoids inhibit TNF-α-induced histone H4 acetylation at the eotaxin promoter and in vivo promoter binding of NF-κB p65

We then applied the semiquantitative ChIP assay to analyze histone acetylation at the eotaxin promoter site and p65 in vivo binding to the eotaxin promoter in a chromatin context. A fixed amount of immunoprecipitated DNA was amplified by PCR with the primer pairs spanning the eotaxin promoter segment (-136 to $+61$) containing STAT6 and NF-κB binding sites. After TNF-α treatment (10 ng/ml, 1 h), p65 immunoprecipitates showed a marked enrichment of eotaxin promoter DNA segment compared

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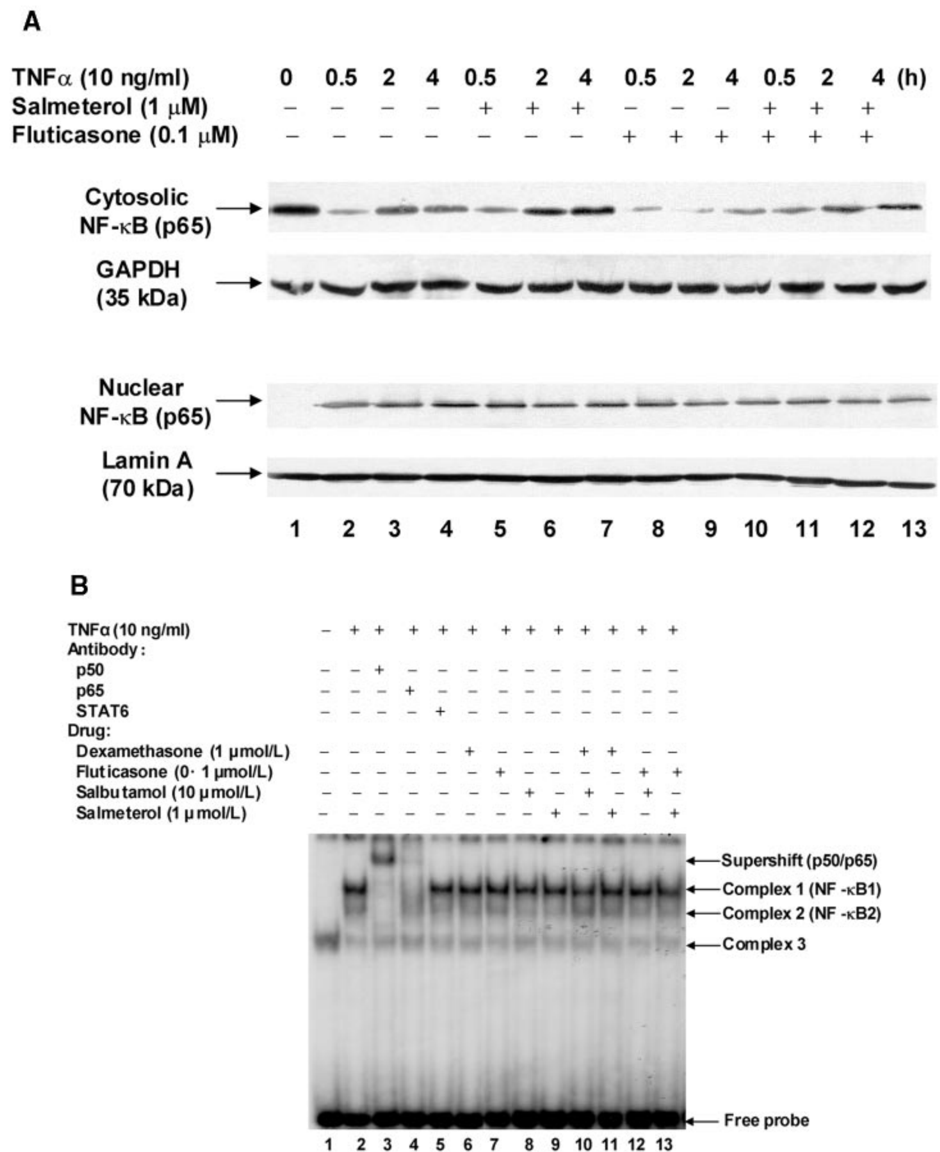

FIGURE 6. Effect of β_2-agonists and glucocorticoids on NF-κB p65 nuclear translocation and in vitro DNA binding. *A*, Confluent and serum-starved HASM cells in 90-mm dishes were pretreated with or without salmeterol, fluticasone, or both for 30 min before stimulation with or without TNF-α for the times indicated. Cytosolic and nuclear extracts were prepared, and the presence of p65 in both fractions was analyzed by Western blotting using specific Ab. GAPDH and lamin A were served as protein loading controls. *B,* Confluent and serum-starved HASM cells in 90-mm dishes were pretreated with or without dexamethasone, fluticasone, salbutamol, salmeterol, or their combinations for 30 min and then stimulated with TNF-α for 1 h. The in vitro binding activity of nuclear proteins to ^{32}P-labeled NFS6 probe was analyzed by EMSA. The transcription factor-DNA complexes were identified by supershift assay with specific Abs indicated. Results are representative of three independent experiments with similar results.

with the nonimmune IgG immunoprecipitate control (Fig. 7*A*), suggesting that p65 binding to the eotaxin promoter DNA is specific. Parallel kinetic analysis showed a time-dependent increase of TNF-α-induced histone H4 acetylation on lysines 5 (AcK5) and 12 (AcK12), but not 8 (AcK8) or 16 (AcK16), at the eotaxin promoter and p65 in vivo binding to the eotaxin promoter DNA (Fig. 7*B*). Compared with the control, marked changes occurred at 5 min for acetylation on lysine 12 and 15 min for acetylation on lysine 5 and p65 promoter binding, and declined after 5 h. TNF-α did not significantly induce histone H3 acetylation at the eotaxin promoter (data not shown). These data suggest that TNF-α-induced selective histone H4 acetylation correlates well with the specific in vivo binding of p65 to the eotaxin promoter, resulting in p65-mediated eotaxin gene transcription.

We finally tested whether the regulation of eotaxin gene transcription by β_2-agonists and glucocorticoids was through chromatin remodeling. As shown in Fig. 7*C*, TNF-α-induced histone H4 acetylation on lysines 5 and 12 and p65 in vivo binding to the eotaxin promoter was markedly reduced by not only dexamethasone and fluticasone but also salbutamol and salmeterol in a concentration-dependent manner, and greater reduction was generally achieved when fluticasone and salmeterol were used in combination. These data strongly suggest that β_2-agonists, like glucocorticoids, repress TNF-α-induced eotaxin gene transcription through inhibition of selective histone H4 acetylation at the eotaxin promoter and p65 in vivo promoter binding.

Discussion

The most important finding of the current study is that β_2-agonists, like glucocorticoids, repress eotaxin gene expression transcriptionally by selective inhibition of histone H4 acetylation and NF-κB p65 binding to the eotaxin promoter. Thus, the present study shows a novel chromatin-modifying mechanism by which β_2-agonists regulate the expression of inflammatory genes. This may be relevant to clinical studies where the addition of long acting β_2-agonists to inhaled glucocorticoids reduced asthma exacerbations.

We studied the eotaxin gene to try to identify important molecular mechanisms of the actions of β_2-agonists and glucocorticoids as eotaxin is an important chemokine relevant to asthma, which we have previously shown is regulated by both groups of drugs. HASM cells provide a useful model system to study such mechanisms as they contain large numbers of β_2-adrenoceptors and are important contributors to asthma pathophysiology through their contractile, synthetic, and remodeling properties. To delineate the

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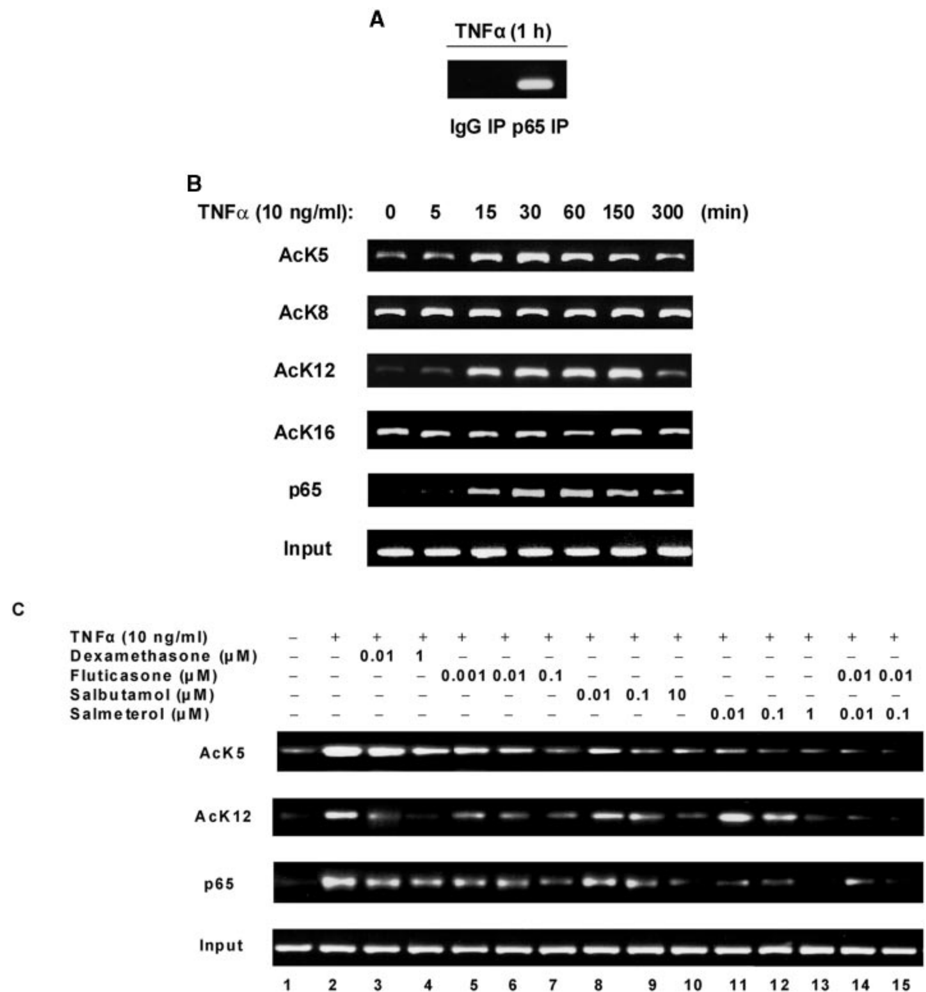

FIGURE 7. Effect of β_2-agonists and glucocorticoids on TNF-α-induced histone H4 acetylation at the eotaxin promoter and in vivo p65 promoter binding. *A*, Confluent and serum-starved HASM cells in 90-mm dishes were treated with TNF-α (10 ng/ml) for 1 h. The in vivo p65 binding to the eotaxin promoter was analyzed by ChIP assay with specific anti-p65 Ab. *B,* HASM cells were treated with TNF-α for the times indicated, and the kinetics of TNF-α-induced histone H4 acetylation on different lysine residues at the eotaxin promoter and the in vivo p65 promoter binding were analyzed by ChIP assay with specific Abs. *C,* HASM cells in 90-mm dishes were pretreated with or without dexamethasone, fluticasone, salbutamol, salmeterol, or the combination of fluticasone and salmeterol for 30 min and then stimulated with or without TNF-α for 1 h. Histone H4 acetylation on lysine residues AcK5 and AcK12 at the eotaxin promoter and the in vivo p65 promoter binding were analyzed by ChIP assay with specific Abs. Results are representative of three independent experiments with similar results.

molecular mechanisms responsible for the effects of β_2-agonists and glucocorticoids on eotaxin production, we first identified with reporter gene assay and EMSA that transcription factor NF-κB, but not STAT6, was critical in TNF-α-induced eotaxin gene transcription. Our in vitro promoter binding results are consistent with previous studies using the same probe in epithelial cells (9) and a different probe in fibroblasts (11), showing that only IL-4, but not TNF-α, induces STAT6 binding to the eotaxin promoter, even though an intact STAT6 binding site and the presence of functional STAT6 protein are required for the activating effect of TNF-α on the eotaxin promoter in fibroblasts (11). The nuclear translocation of STAT6 in our studies suggests that it may be involved in TNF-α-induced transcription of other genes in these cells. By exploring the molecular mechanisms used by β_2-agonists and glucocorticoids to inhibit eotaxin gene transcription, we found that both had no effect on TNF-α-induced p65 nuclear translocation despite the fact that p65 recovery in the cytoplasm after TNF-α treatment was markedly reduced by fluticasone, which was then counteracted by salmeterol. Although the mechanisms of their effects on cytosolic p65 remain to be revealed, our results do suggest that their inhibition on eotaxin transcription is not via inhibition on NF-κB nuclear translocation. This differs from studies showing that β_2-agonists (27) and glucocorticoids (28) decrease NF-κB nuclear translocation in other cell systems. Translocated NF-κB in TNF-α-stimulated HASM cells formed two major NF-κB-DNA complexes with eotaxin promoter in vitro, which were composed mainly of p65 and p50. β_2-Agonists and glucocorticoids, either alone or in combination, had no effect on the formation of these complexes. Although glucocorticoids inhibit NF-κB in vitro DNA binding in other cell systems, this inhibition usually results from reduction of NF-κB nuclear translocation (28). Our results are consistent with the findings in THP-1 cells and endothelial cells that elevated cAMP inhibits NF-κB-mediated transcription of the tissue factor gene but has no effect on p65 nuclear translocation and in vitro promoter binding (29, 30).

The lack of effect on NF-κB nuclear translocation and in vitro promoter binding was surprising and suggested that the drugs might act on chromatin remodeling. Targeted acetylation of histone H4 plays an important role in allowing transcription factors to access their binding sites in the promoter (18, 19). It has been demonstrated that activated transcription factors such as NF-κB p65, form complexes with CREB-binding protein (CBP) and its homologue p300, which have intrinsic histone acetyltransferase (HAT) activity (31, 32), and induce histone acetylation of relevant lysine residues, resulting in local unwinding of DNA, increased transcription factor binding to the promoter, and gene transcription (33). We found in the current study that TNF-α-induced histone acetylation at the eotaxin promoter site was selective to lysines 5 and 12 and preceded p65 in vivo promoter binding. Both β_2-agonists and glucocorticoids inhibited the selective histone H4 acetylation and NF-κB p65 in vivo promoter binding, resulting in repression of eotaxin gene transcription. The disparate drug effects on the in vitro and in vivo p65 promoter binding in our study are very likely due to the chromatin-dependent accessibility of NF-κB binding site in ChIP assay and, therefore, strongly suggest that β_2-agonists, like glucocorticoids, transcriptionally regulate eotaxin

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gene transcription in HASM cells through modification of chromatin structure. There has been one previous report of the chromatin-modifying regulation of the mouse mammary tumor virus promoter by cAMP signaling as a result of inhibition on histone H4 acetylation at specific nucleosomes in the promoter region (34). Our study is the first to demonstrate chromatin-modifying regulation of inflammatory gene transcription by β_2-agonists in any cell system. We have previously shown that the effect of β_2-agonists on eotaxin release from HASM cells is cAMP-mediated (4). There have been reports in other cells of glucocorticoids acting through effects on histone acetylation and chromatin remodeling (19, 35). We also showed an additive inhibition on selective histone H4 acetylation and p65 in vivo promoter binding with the combined use of both classes of drugs, consistent with the additive inhibition on eotaxin protein production (4), mRNA expression and promoter activity (this study). Although histone H4 acetylation, but not histone H3 acetylation, is closely associated with eotaxin gene transcription, our study does not exclude the possible involvement of other histone modifications, e.g., phosphorylation and phosphoacetylation, in this process.

Our present experiments showed that β_2-agonists and glucocorticoids both inhibited TNF-α-induced eotaxin promoter activity in transient transfection. Although the actual chromatin status of the transfected promoter is unclear, it is likely to be a loose and disorganized structure and, therefore, is generally more open and accessible than repressed cellular chromatin structure of endogenous gene promoters (36). In this sense, it is unlikely that histone acetylation is involved in the effects of the drugs. However, there is now good evidence that HAT and histone deacetylase (HDAC) regulate NF-κB-dependent gene expression not only through histone acetylation and deacetylation but also through site specific acetylation and deacetylation of NF-κB p65 (32, 37). Acetylation of p65 lysine 221 enhances DNA binding and impairs IκBα assembly, whereas acetylation of lysine 310 is required for full transcriptional activity of NF-κB in the absence of effects on DNA and IκBα binding (38, 39). HDAC inhibitors such as trichostatin and sodium butyrate have been shown to potentiate TNF-α-induced expression of several natural NF-κB-driven promoters, such as IL-8, IL-6, and ICAM-1, in transient assays (40), strongly suggesting that HAT and HDAC are involved in the transcriptional activity of transiently transfected promoters, probably through the acetylation and deacetylation of NF-κB rather than histones. As we have demonstrated in this study that β_2-agonists and glucocorticoids both inhibit histone H4 acetylation, it is reasonable to speculate that they may also inhibit NF-κB acetylation, leading to the inhibition on TNF-α-induced eotaxin promoter activity in transient assay, whereas the inhibition of acetylation of both NF-κB and histone H4 may account for their inhibition of eotaxin gene transcription in vivo. However, other effects such as inhibition on NF-κB phosphorylation (32) cannot be excluded, and further studies are required to understand the precise mechanisms.

Our results that β_2-agonists, like glucocorticoids, inhibit eotaxin gene transcription through their effects on chromatin remodeling suggest that β_2-agonists and glucocorticoids may share some common pathways in their signaling transduction. Similar findings have been reported from other studies. β_2-Agonists can induce glucocorticoid receptor nuclear translocation in a ligand-independent manner in several cell systems (41, 42) and have complementary effects on C/EBP signaling in HASM cells (42), which inhibits smooth muscle proliferation. Further studies are required to determine how β_2-agonists act on chromatin remodeling, particularly whether they, like those used by glucocorticoids, lead to direct inhibition of CBP/p300-associated HAT activity and/or recruitment of HDAC to the p65-CBP-HAT complex. It will also be important to compare the effects of these drugs on eotaxin expression in HASM cells obtained from healthy subjects with those derived from asthmatics as HDAC levels may be decreased in asthma (43). Although eotaxin is only one of a number of chemokines responsible for inflammatory cell recruitment in asthma, NF-κB signaling is a common feature of the activation of many chemokines, and it would seem likely that the mechanism we have identified with eotaxin may be a more widespread phenomenon among other chemokines.

In summary, we have identified a novel chromatin-modifying mechanism for the anti-inflammatory effect of β_2-agonists, which is shared by glucocorticoids. Histone acetylation status is determined by the balance between HAT activity and HDAC activity. Our findings suggest that selective targeting of members of either of these enzyme families would prove to be useful approaches for the treatment of inflammatory diseases.

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Disclosures

The authors have no financial conflict of interest.

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