Chemokine-mediated inflammatory cell infiltration is a hallmark of asthma. We recently demonstrated that glucocorticoids and \( \beta_2 \)-agonists additively or synergistically suppress tumor necrosis factor-\( \alpha \) (TNF\( \alpha \))-induced production of chemokines eotaxin and interleukin-8 (IL-8), respectively, in human airway smooth muscle (HASM) cells, which may partly explain their combined benefits in asthma. Peroxisome proliferator-activated receptors (PPARs) also modulate inflammatory gene expression. We reported here that the PPAR\( \gamma \) agonists 15-deoxy-\( \Delta^{12,14} \)-PGJ\(_2\), WY-14643, and troglitazone, but not PPAR\( \alpha \) agonist inhibited TNF\( \alpha \)-induced production of eotaxin and monocyte chemotactic protein-1 (MCP-1) but not IL-8. Eotaxin inhibition was transcriptional and additively enhanced by the glucocorticoid fluticasone and the \( \beta_2 \)-agonist salmeterol, whereas MCP-1 inhibition was post-transcriptional and additively and synergistically enhanced by fluticasone and salmeterol, respectively. Coimmunoprecipitation revealed the PPAR\( \gamma \) agonist 15d-PGJ\(_2\) induced a protein-protein interaction between PPAR\( \gamma \) and the glucocorticoid receptor (GR) in TNF\( \alpha \)-treated HASM cells, which was enhanced by fluticasone and salmeterol. 15d-PGJ\(_2\), fluticasone, and salmeterol all inhibited TNF\( \alpha \)-induced histone H4 acetylation at the eotaxin promoter and NF-kB p65 binding to the eotaxin promoter and induced PPAR\( \gamma \) and GR association with the eotaxin promoter, as analyzed by chromatin immunoprecipitation assay. Our data suggest that chemokine expression in HASM cells is differentially regulated by PPAR\( \gamma \) agonists and that the interaction between PPAR\( \gamma \) and GR may be responsible for the additive and synergistic inhibition of chemokine expression by PPAR\( \gamma \) agonists, glucocorticoids, and \( \beta_2 \)-agonists, particularly the chromatin-dependent suppression of eotaxin gene transcription. The interaction may have wide applications and may provide a potential target for pharmacological and molecular intervention.

Asthma is a common chronic disease featured by airway inflammation. Inflammatory cells are key players in the inflammatory process of asthma and are attracted to the airways by a network of chemokines (1). Mast cells and eosinophils are the most important inflammatory cells in asthmatic airways (2), and neutrophil infiltration is associated with asthma exacerbations (3). Eosinophils are attracted by chemokines such as eotaxin, IL-5, and MCP-1 (monocyte chemotactic protein-1), and neutrophils are recruited by chemokines such as IL-8 (4). Eotaxin and IL-8 are also mast cell chemotactic factors (5), and MCP-1 also attracts T-lymphocytes and monocytes (4). We and others have demonstrated that human airway smooth muscle (HASM) cells produce a number of important chemokines such as IL-8 (6, 7), eotaxin (8), MCP-1 (9), RANTES, and GM-CSF (10) and is an important source of chemokines in the airways, consequently contributing to the orchestration and perpetuation of airway inflammation. Thus, understanding the molecular mechanisms of chemokine gene expression and regulation in HASM cells will have important implications for the regulation of airway inflammation in asthma.

Peroxisome proliferator-activated receptors (PPARs) belong to a subfamily of the nuclear receptor superfamily and are potentially important transcription factors that modulate inflammatory responses (11). PPARs are comprised of three isoforms, \( \alpha \), \( \beta \)\( \delta \), and \( \gamma \), and are activated by a heterogeneous group of structurally dissimilar endogenous and synthetic agonists. Of these PPAR agonists, the prostaglandin D\(_2\) metabolite 15-deoxy-\( \Delta^{12,14} \)-PGJ\(_2\) (15d-PGJ\(_2\)) is a direct-binding natural ligand for PPAR\( \gamma \) (12). It has been documented that PPAR\( \gamma \) agonists may play an important role in the regulation of inflammatory process and may become a new class of anti-inflammatory compounds (11, 13, 14). Studies have shown that regulation of chemokine expression by PPAR\( \gamma \) activation is cell- and stimulus- and gene-specific. For instance, 15d-PGJ\(_2\) induces IL-8 expression, reduces MCP-1 expression, and has no effect on RANTES expression in human monocytes/macrophages (15), whereas it reduces both IL-8 and MCP-1 gene expression in colon epithelial cells (14). We have shown that PPAR\( \alpha \)s and \( \gamma \) are constitutively expressed in HASM cells and that activation of PPAR\( \gamma \), but not PPAR\( \alpha \), induces the expression of cyclooxygenase-2 (16). PPAR\( \gamma \) activation also inhibits...
GM-CSF and G-CSF production, whereas glucocorticoid dexamethasone inhibits only GM-CSF in HASM cells (17). However, the effects of PPAR agonists on other chemokine expression in HASM cells have not been investigated so far.

Glucocorticoid receptors (GR), like PPARs, are ligand-activated nuclear receptors, and glucocorticoids (GCs) are commonly applied in the treatment of inflammatory diseases. β3-Agonists promote bronchodilation by increasing intracellular cAMP and stimulating cAMP-dependent protein kinases. We have recently reported that β3-agonists synergistically and additively enhance the inhibitory effect of GCs on TNF-α- and eotaxin production, respectively (7, 8), from HASM cells, which may partially explain their combined benefits in asthma therapy (18). However, many patients have persistent symptoms despite these treatments. Approaches seeking new therapeutic targets for asthma treatment are therefore required.

The study of crystal structures has revealed that nuclear receptors share highly conserved ligand-binding domain, denominated activation function-2, which is necessary for transcriptional activation (19). Because steroids estrogen, progesterone, and GCs interact with each other (20) and GCs also interact with nuclear receptors and glucocorticoids (GCs) are commonly applied in the treatment of inflammatory diseases.

The local hyper-acetylation of histone H4 at a specific gene promoter plays a key role in the regulation of gene transcription (24). The local hyper-acetylation of histone H4 at a specific gene promoter plays a key role in the regulation of gene transcription (24). GR activation has been shown to suppress inflammatory gene transcription by inhibiting histone acetylation associated with specific gene promoters (24, 26); whether PPARγ has a similar effect is not yet known. The purpose of this study was to investigate the regulation of TNFα-induced eotaxin, MCP-1, and IL-8 expression by PPAR agonists, either alone or in combination with GCs and β3-agonists on chemokine expression.

In unstimulated 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 octamer core of four histones (two molecules each of histones H2A, H2B, H3, and H4) (21). This nucleosome structure prevents the access of transcription factors and RNA polymerase II to their respective recognition sequences and the initiation of transcription (22). When the cells are stimulated with inflammatory mediators, histones undergo an array of post-transcriptional modifications on N-terminal domains, including acetylation, phosphorylation, and methylation (23), which result in chromatin remodeling and transcription factors binding to specific gene promoters, leading to the initiation of gene transcription (24). The local hyper-acetylation of histone H4 at a specific gene promoter plays a key role in the regulation of gene transcription (24, 25). GR activation has been shown to suppress inflammatory gene transcription by inhibiting histone acetylation associated with specific gene promoters (24, 26); whether PPARγ has a similar effect is not yet known. The purpose of this study was to investigate the regulation of TNFα-induced eotaxin, MCP-1, and IL-8 expression by PPAR agonists, either alone or in combination with GCs and β3-agonists and to explore the underlying molecular mechanisms.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dubelco’s modified Eagle’s medium, penicillin and streptomycin, 1-glutamine, amphotericin B, recombinant human TNFα, Me6SO, actinomycin D (Act D), and other unspecified chemicals were all purchased from Sigma; fetal calf serum was from Seralab (Loughborough, UK); W31463, 15d-PGJ2, and troglitazone (TRO) were from Calbiochem; salmeterol (Salme) and fluticasone propionate (Flut) were kindly supplied by Professor Malcolm Johnson (Glaxo-SmithKline, Middlesex, UK); ELISA kits for human eotaxin, MCP-1, and IL-8 were from R&D Systems (Abingdon, Oxon, UK); RNasey mini kit was from Qiagen (West Sussex, UK); Moloney murine leukemia virus-reverse transcriptase was from Invitrogen; and FuGENE 6 transfection reagent was from Roche Diagnostics. Anti-acetylated histone H4 antibody was from Upstate Biotechnology, Inc. (Milton Keynes, UK); anti-p65, -PPARγ, and -GRα antibodies and normal rabbit IgG were from Santa Cruz Biotechnology (Santa Cruz, CA).

**Cell Culture**—Primary HASM cells were prepared from explants of HASM as reported previously (27) and cultured in Dubelco’s modified Eagle’s medium containing 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), amphotericin B (2.5 μg/ml), and 1-glutamine (4 mM) in humidified 5% CO2, 95% air at 37 °C. This protocol was approved by the Nottingham City Hospital Research Ethics Committee. Cells at passage 6 were used for all experiments. We have shown previously that the cells grown in this manner depict the immunohistochemical and morphological features of typical HASM cells (27). HASM cells were grown to confluence in 24-well plates and growth-arrested in serum-free medium for 24 h prior to experiments in fresh serum-free medium unless otherwise stated. Because we have reported previously that HASM cells produce ample amounts of eotaxin (8) and IL-8 (7) after 8 h of stimulation with TNFα (10 ng/ml), this time point was chosen to examine the drug effects. In the concentration-response for drug combination experiments, the cells were incubated with increasing concentrations or a single concentration of drug(s) or the drug vehicle Me6SO (maximum concentration 0.4%) for 0.5 h and then stimulated with TNFα (10 ng/ml) for 8 h. The supernatants were collected and stored at −20 °C until ELISA for chemokines, whereas in some experiments total RNA was extracted from the cells and stored at −20 °C until further detection of mRNA by RT-PCR.

**Chemosine Assays**—The concentrations of eotaxin, MCP-1, and IL-8 in the culture media were measured by ELISA according to the manufacturer’s instructions as reported previously (7, 8). The sensitivity of the ELISA kits at our hands was at least 5 pg/ml, which was consistent with the manufacturer’s specifications. According to the kit insert, there is no significant cross-reactivity or interference with other human cytokines and chemokines.

**RT-PCR**—After treatment, total RNA was extracted from cells using the RNasey mini kit following the manufacturer’s protocol and transcribed into cDNA using Moloney murine leukemia virus-reverse transcriptase. cDNA from one sample was simultaneously applied to detect levels of eotaxin, MCP-1, and IL-8 mRNA and the internal control GAPDH mRNA. The primer sequences were as follows: GAPDH forward, 5′-GATGTTTGGGCATGAGTAC-3′, and reverse, 5′-CTCTGAGACGCCGATGTGTTAC-3′; eotaxin forward, 5′-GAGTCAAGCTGCTTTTC-3′, and reverse, 5′-GAATCCTAGCTGCTTTTCC-3′; MCP-1 forward, 5′-GATCTCAGTGAGCAAGGCTCG-3′, and reverse, 5′-TGCTTGTCAGTGGTCATCT-3′; IL-8 forward, 5′-AGCGTCAAGCTGCTTTTC-3′, and reverse, 5′-CTCTAGCTTCTGCTTTTCC-3′; cDNA samples were annealed at 95 °C for 5 min (25 s for eotaxin, 95 °C for 10 s, 72 °C for 10 s; IL-8 and MCP-1, 95 °C for 1 min, 60 °C for 10 s, 72 °C × 10 s) followed by extension at 72 °C for 10 min. The PCR products were separated on 3% agarose gel and visualized by ethidium bromide staining.

**RNA Stability**—Confluent and growth-arrested HASM cells were incubated with or without TNFα for 2 h and then treated with the drugs over 4 h before adding Act D (5 μg/ml) for the times indicated to block new transcription generation. Total RNA was then extracted from the cells, and eotaxin mRNA level was analyzed by RT-PCR using the full-length eotaxin coding primers described above.

**Plasmids and Transient Transfection**—The firefly luciferase reporter gene constructs in pGL3-basic vectors containing the full-length eotaxin promoter fragment (−1363) were obtained from Professor Robert Schleimer (Johns Hopkins University) and were described previously (28). IL-8 promoter fragment (−1481 to +44) was digested with XhoI and HindIII from the IL-8 PUX-CAT (chloramphenicol acetyltransferase) reporter plasmids (a gift from Professor Kouji Matsuhashita, Kanazawa University, Kanazawa, Japan, see Ref. 29) and was ligated into the XhoI-HindIII site of the pGL3-basic plasmid upstream to the translation initiation site of the firefly luciferase gene. The resulting construct was verified by sequencing. Because deletion analysis has shown that the transcription of MCP-1 is manipulated by two 5′-flanking regions, the distal enhancer region (ENH) and the proximal promoter region (PRM) (30), we obtained the 167-bp human MCP-1 PRM fragment (from −107 to +60) by digesting with XhoI and HindIII of the pGL3-PRM (a gift from Professor Atsuhisa Ueda, Yokohama City University, Yokohama, Japan, see Ref. 31) and the 230-bp human MCP-1 ENH between −2742 and −2513 by digesting with KpnI and XhoI of the pGL3-ENH (also from Professor Atsuhisa Ueda, see Ref. 31). The juxtaposed MCP-1 ENH fragment (−2742/−2513) and PRM fragment (−107/+60) were then ligated into the KpnI-HindIII site of the pGL3-basic luciferase firefly reporter gene construct pGL3-MCP-1-ENH-PRM. The pmx vectors expressing wild-type PPARγ were the generous gift from Professor Mitchell Lazar (University of Pennsylvania) and were prepared previously (32).

Transient transfection was performed as described previously (16, 33) with minor modifications. Briefly, HASM cells in 24-well plates were grown to 60–70% confluence and then transfected with 0.4 μg/well of the reporter gene plasmids for 16 h. Transfected cells were pretreated...
with or without drugs for 0.5 h and then incubated with or without TNFα (10 ng/ml) for 6 h. To assess the roles of PPARγ agonists, cells were cotransfected with increasing concentrations of pCMX-PPARγ together with reporter gene plasmids for 16 h, and then treated with or without TNFα (10 ng/ml) for 6 h. Firefly luciferase activity was determined as described before (16, 33).

ChIP Assay—ChIP assay was performed as described previously (24, 33). Briefly, confluent and serum-deprived HASM cells in 90-mm dishes were pretreated with or without drugs for 0.5 h and then incubated with or without TNFα (10 ng/ml) for 2.5 h. The cells were then incubated with 1% formaldehyde to fix protein-DNA complexes. Chromatin pellets from these cells sheared by sonication were pre-cleaned with salmon sperm DNA-saturated protein A- and G-Sepharose. One portion of the soluble chromatin was used as DNA input control, and the remaining were sub- aliquoted and then precipitated using specific antibodies against acetylated histone H4 or transcription factors NF-κB p65, PPARγ, or GR (the most abundant GR isoform). The purified DNA from the immunoprecipitated complexes of antibody-protein-DNA was detected by semi-quantitative PCR (28 cycles) using the following specific primer pairs spanning promoter regions that contain major regulatory elements such as NF-κB: for eotaxin promoter, forward, 5′-CTCTACAGTTGGAGGCTGAAG-3′, and reverse, 5′-GAGTGTCGGAAGGTCTTCTGT-3′, and reserve, 5′-GAGGCCTGTGCTGCCTGCTG-3′.

Coimmunoprecipitation and Western Blotting—Confluent and serum-starved HASM cells were pretreated with or without the drugs for 0.5 h and then incubated with TNFα (10 ng/ml) for 1 h. Nuclear proteins were extracted from the cells, and protein concentrations were measured as described previously (16, 33). Immunoprecipitation (IP) was conducted as described previously (26, 34) with minor modifications. Briefly, the nuclear extracts were preclared with 20 μl of protein AG-agarose. After microcentrifugation, the supernatants were incubated with 5 μg/ml anti-PPARγ, anti-GR or rabbit polyclonal antibody, or normal rabbit IgG overnight at 4°C followed by incubation with protein AG-agarose beads for 3 h. The immune complexes were washed with PBS containing 0.02% Tween 20 and pelleted by gentle centrifugation, and then eluted in the resulting immunoprecipitates (IPs) was analyzed by Western blotting (19, 38).

Statistical Analysis—Data were expressed as means ± S.E. Statistical analysis was performed with Graphpad Prism 4. Analysis of variance (ANOVA) and unpaired two-tailed student t test were used to determine the significant differences between the means. p ≤ 0.05 was considered significant.

RESULTS

Effects of PPAR Agonists Flut and Salme on TNFα-induced Chemokine Production—Because only PPARα and PPARγ are expressed in HASM cells (16, 17), we first explored the effects of the synthetic PPARα agonist WY-14643, the synthetic PPARγ agonist TRO, and the natural PPARγ agonist 15d-PGJ2 on TNFα-induced chemokine production from HASM cells. As shown in Fig. 1, the production of eotaxin, IL-8, and MCP-1 was markedly increased by TNFα treatment (10 ng/ml, 8 h). Both 15d-PGJ2 and TRO markedly inhibited TNFα-induced eotaxin production (Fig. 1A) and partially but significantly inhibited MCP-1 production (Fig. 1E) in a concentration-dependent manner but had no effect on IL-8 production (Fig. 1C). In contrast, WY-14643 had a variable effect on the production of all three chemokines (Fig. 1A, C, and E). The glucocorticoid Flut concentration-dependently inhibited TNFα-induced eotaxin (Fig. 1B), IL-8 (Fig. 1D), and MCP-1 production (Fig. 2F), whereas the β2-agonist Salme partially inhibited eotaxin production (Fig. 1B) but had no effect on IL-8 (Fig. 1D) and MCP-1 production (Fig. 2F). These results suggest that TNFα-induced chemokine production in HASM cells is differentially regulated by PPARγ agonists as well as GCs and β2-agonists.

Effects of 15d-PGJ2 and Flut Combination on TNFα-induced Chemokine Production—As shown in Fig. 2A, 15d-PGJ2 alone markedly inhibited TNFα-induced eotaxin production in a concentration-dependent manner (p = 0.0001, ANOVA). Flut alone at 0.001 and 0.01 μM also inhibited the production. The concentration-dependency inhibition by 15d-PGJ2 (0.1–10 μM) was significantly enhanced by the addition of Flut at 0.001 and 0.01 μM. 15d-PGJ2 partially inhibited TNFα-induced MCP-1 production at high concentrations (5 and 10 μM, Fig. 2E, p = 0.0304 and p = 0.0029, respectively). Flut alone at 0.001 and 0.01 μM also reduced MCP-1 production. An additive inhibition was observed when Flut was used in combination with the highest concentration of 15d-PGJ2 tested (10 μM). 15d-PGJ2 had no effect on TNFα-induced IL-8 production (Fig. 2C). Although Flut (0.01 and 0.1 μM) significantly inhibited the production, the inhibition was not altered when it was used together with 15d-PGJ2 (0.1–10 μM). The results show that Flut augments the inhibitory effect of 15d-PGJ2 on eotaxin and MCP-1 production and suggest that that activated PPARγ and GR could interact with each other, resulting in additive inhibition on TNFα-induced chemokine production in HASM cells.

Effects of 15d-PGJ2 and Salme Combination on TNFα-induced Chemokine Production—To investigate if PPARγ agonists, like GCs, could interact with β2-agonists to regulate cytokine-induced chemokine production, we compared the effects of 15d-PGJ2 alone with the effects of 15d-PGJ2 + Salme on TNFα-induced chemokine production. As shown in Fig. 2B, 15d-PGJ2 alone inhibited TNFα-induced eotaxin production in a concentration-dependently manner (p = 0.0001, ANOVA). Salme at 0.01 μM, but not 0.001 μM, also significantly inhibited eotaxin production. The inhibition by 15d-PGJ2 was further enhanced with the addition of Salme at both 0.001 and 0.01 μM. TNFα-induced MCP-1 production was also concentration-dependently inhibited by 15d-PGJ2 (Fig. 2F); although Salme alone (0.001 and 0.01 μM) had no effect, it significantly enhanced the inhibition by 15d-PGJ2 when they were used in combination. 15d-PGJ2 and Salme, either alone or in combination, had no effect on TNFα-induced IL-8 production, and even the concentrations of Salme were 10 times higher than those used in eotaxin and MCP-1 production (Fig. 2D). The data strongly suggest that interactions between activated PPARγ and signal pathways activated by β2-agonists result in additive inhibition on TNFα-induced eotaxin production and synergistic inhibition on TNFα-induced MCP-1 production in HASM cells.

Effects of PPARγ Agonists, Flut and Salme, on TNFα-induced Chemokine mRNA Expression—To explore the molecular mechanisms by which PPARγ agonists GCs and β2-agonists differentially regulate TNFα-induced production of eotaxin, MCP-1, and IL-8, we then examined if the effects of these drugs on chemokine protein production were due to the alteration of the chemokine mRNA expression. As analyzed by RT-PCR, the level of eotaxin mRNA in control cells was low (Fig. 3A, lanes 1 and 12) and was markedly induced by TNFα treatment for 8 h (lanes 2 and 13). 15d-PGJ2 inhibited the mRNA expression in a concentration-dependent manner (Fig. 3A, lanes 5 and 6 and 14–16), and TRO also reduced the mRNA expression (lanes 7 and 8) as compared with TNFα alone (lanes 2 and 13). In contrast, WY-14643 had no effect (Fig. 3A, lane 9). Both Salme (Fig. 3A, lanes 3 and 4) and Flut (lanes 10 and 11) alone inhibited TNFα-induced eotaxin mRNA expression. An additive inhibition was observed when Salme and Flut were used in combination with increasing concentrations of 15d-PGJ2 (Fig. 3A, lanes 17–22 and 23–28, respectively) as compared with 15d-PGJ2 alone (lanes 14–16). The results were consistent with the effects of these drugs on eotaxin protein production (Figs. 1, A and B, and 2, A and B), strongly suggesting that PPARγ agonists, GCs, and β2-agonists when used together, additively and transcriptionally inhibit TNFα-induced eotaxin gene expression.

The IL-8 mRNA expression in the control cells was detectable (Fig. 3B, lane 1) and markedly induced by TNFα (lane 2).
FIG. 1. Effects of PPAR agonists Flut and Salme on TNFα-induced eotaxin, IL-8, and MCP-1 production. Confluent and serum-deprived HASM cells were pretreated with or without increasing concentrations of 15d-PGJ2, TRO, WY-14643, Flut, or Salme for 0.5 h and then incubated with or without TNFα for 8 h. Concentrations of eotaxin (A and B), IL-8 (C and D), and MCP-1 (E and F) in the media were measured by ELISA. Each point represents the mean ± S.E. from three or four independent experiments performed in duplicate or triplicate. *, p < 0.05; **, p < 0.01; ***, p < 0.001 compared with TNFα alone.
However, treatment with 15d-PGJ2 (Fig. 3B, lanes 3–5), TRO (lanes 6 and 7), WY-14643 (lanes 8 and 9), Salme (lanes 10 and 11), Flut (lanes 12 and 13), and the combinations of 15d-PGJ2 + Flut (lanes 16 and 17) had no effect on TNFα-induced IL-8 mRNA expression, which was consistent with the findings that 15d-PGJ2 and Salme had no effect on TNFα-induced IL-8 production and suggests that the inhibition by Flut occurs at post-transcriptional levels. In contrast to eotaxin and IL-8, the basal MCP-1 mRNA expression in control cells was relatively high (Fig. 3C, lane 1) and was further induced by TNFα (lane 2). However, the expression was not altered by the drugs tested, either alone or in combination (Fig. 3C, lanes 3–17), suggesting the effects of PPARγ agonists, GCs, and β2-agonists, either alone or in combination, on TNFα-induced MCP-1 production are due to post-transcriptional regulation.

Effects of 15d-PGJ2, Flut, and Salme on the Stability of TNFα-induced Chemokine mRNA—To clarify whether the effects of PPARγ agonists, GCs, and β2-agonists on TNFα-induced chemokine production resulted from accelerated mRNA degradation, mRNA stability assay was conducted with the semiquantitative RT-PCR. Fig. 4A showed that TNFα-induced eotaxin (lanes 1–5), IL-8 (lanes 6–10), and MCP-1 (lanes 11–15) mRNA expression at 4 and 8 h was strongly inhibited or abolished by the general transcription inhibitor Act D, indicating that Act D blocks the generation of new transcripts. The level of TNFα-induced eotaxin mRNA was constant from 2 to 20 h after the addition of Act D (Fig. 4B, lanes 3–6), and treatment with 15d-PGJ2 (lanes 7–10), Flut (lanes 11–14), and Salme (lanes 15–18) did not alter the mRNA level. In contrast, a time-dependent natural degradation of TNFα-induced IL-8 mRNA was observed after the addition of Act D (Fig. 4C, lanes...
2–6); 15d-PGJ2 (lanes 7–10) and Flut (lanes 11–14) did not alter the course, but Salme (lanes 15–18) inhibited the degradation. A time-dependent natural degradation of TNFα-induced MCP-1 mRNA was also observed after the addition of Act D (Fig. 4D, lanes 2–6), but treatment with the tested drugs did not affect the degradation (lanes 7–18). Collectively, results from Figs. 3 and 4 strongly suggest that the inhibition of TNFα-induced eotaxin production by PPARγ agonists, GCs, and β2-agonists is transcriptional and that the inhibition of TNFα-induced IL-8 production by GCs and the inhibition of TNFα-induced MCP-1 production by PPARγ agonists and GCs are via post-transcriptional regulations other than mRNA stability.

**Effects of PPARγ Agonists, Flut, and Salme on TNFα-induced Eotaxin Promoter Activity**—We then focused on the ef-
Effects of these drugs on the transcriptional regulation of the eotaxin gene, and we performed reporter gene assay to assess whether these drugs alter the eotaxin gene promoter activity. The full-length eotaxin promoter (-1363 bp) luciferase reporter plasmids were transiently transfected into HASM cells. TNFα markedly induced the eotaxin promoter activity by 6-fold, which was markedly inhibited by both 15d-PGJ2 and TRO, but not WY-14643, in a concentration-dependent manner (Fig. 5A). Flut and Salme alone also significantly inhibited TNFα-induced eotaxin promoter activity (Fig. 5B). The combined use of low concentration 15d-PGJ2 (1 μM) with Flut or Salme all further augmented the inhibition of the eotaxin promoter activity by the individual drugs alone (Fig. 5B), indicating an additive effect. These results suggest that these drugs suppress eotaxin gene transcription by inhibiting the eotaxin promoter activity and that interactions between these drugs occur upstream of the eotaxin promoter activation.

Because it has been reported that PPARγ agonists may exert...
their effects through PPARγ-independent mechanisms (35), to clarify the role of PPARγ in the inhibition of TNFα-induced eotaxin transcription by 15d-PGJ2 and TRO, we cotransfected HASM cells with the eotaxin promoter reporter plasmid and vectors that express human PPARγ. As shown in Fig. 5C, compared with control (0 μg/ml PPARγ), overexpression of PPARγ alone markedly and concentration-dependently inhibited TNFα-induced eotaxin promoter activity (p = 0.0001, ANOVA). The PPARγ agonist 15d-PGJ2 (1 μM) alone inhibited TNFα-induced eotaxin promoter activity and further enhanced the inhibition by PPARγ overexpression up to 0.025 μg/ml. In contrast, neither 15d-PGJ2, TRO, nor WY-14643 had any significant inhibition on TNFα-induced transcriptional activities of the IL-8 promoter (Fig. 6A) and the MCP-1 enhancer-promoter (Fig. 6B). Taken together, these data demonstrate that 15d-PGJ2 and TRO do not have any effect on TNFα-induced IL-8 and MCP-1 transcription, and their inhibition on TNFα-induced eotaxin expression is transcriptional and PPARγ-dependent.

Effects of 15d-PGJ2, Flut, and Salme on TNFα-induced Histone H4 Acetylation and p65 Binding with the Eotaxin Promoter—Chromatin remodeling following histone acetylation at specific gene promoter sites is a major transcriptional regulatory mechanism that allows transcription factors to bind to specific gene promoters, initiating gene transcription. As studies have shown that GCs inhibit histone H4 acetylation (24, 26) and we have demonstrated that TNFα-induced eotaxin transcription is NF-κB-dependent in HASM cells, we then applied ChIP assay to assess directly whether PPARγ agonists, GCs, and β2-agonists affect TNFα-induced histone H4 acetylation at

Fig. 5. Effects of PPAR agonists Flut and Salme on TNFα-induced eotaxin promoter activity. 60–70% confluent HASM cells were transfected with the eotaxin promoter reporter gene plasmid alone (A and B) or together with increasing concentrations of PPARγ expression vector (C) for 16 h. The transfected cells were pretreated with or without the drugs, either alone or in combination, for 30 min and then incubated with or without TNFα for 6 h. The firefly luciferase (Luci) activity was assayed and used to represent the transcriptional activities of the promoter. Each point represents the mean ± S.E. of two or three independent experiments performed in triplicate. *, p < 0.05; **, p < 0.01; ***, p < 0.001 compared with TNFα alone (A and B) or with corresponding PPARγ alone (C).
The firefly luciferase (Luci) activity was assayed and was used to represent the transcriptional activities of the promoters. Each point represents the mean ± S.E. of two or three independent experiments performed in triplicate.  

![Graph](https://example.com/graph.png)

**Fig. 6. Effects of PPAR agonists on TNFα-induced IL-8 and MCP-1 promoter activities.** 60–70% confluent HASM cells were transfected with the IL-8 promoter (A) or MCP-1-ENH-PRM (B) reporter gene plasmids for 16 h. The transfected cells were pretreated with the drugs for 30 min and then incubated with or without TNFα for 6 h. The firefly luciferase (Luci) activity was assayed and used to represent the transcriptional activities of the promoters. Each point represents the mean ± S.E. of two or three independent experiments performed in triplicate.

The eotaxin promoter and NF-κB p65 in vivo binding with the eotaxin promoter in a chromatin context. As shown in Fig. 7A, after TNFα treatment, IPs with antibody against acetylated histone H4 revealed a marked enrichment of the eotaxin promoter DNA (197 bp containing STAT6 and NF-κB-binding sites, lane 2) compared with the control (lane 1), indicating that histone H4 was acetylated specifically at the eotaxin promoter site. Pretreatment of the cells with 15d-PGJ2 (Fig. 7A, lanes 3 and 4), Flut (lanes 5–7), and Salme (lanes 8–10) markedly inhibited histone H4 acetylation in a concentration-dependent manner. Similarly, p65 IPs also showed a marked enrichment of the eotaxin promoter DNA after TNFα treatment (Fig. 7A, lane 2) compared with the control (lane 1), indicating p65 binding to the eotaxin promoter. The binding was also inhibited by 15d-PGJ2 (Fig. 7A, lanes 3 and 4), Flut (lanes 5–7), and Salme (lanes 8–10), suggesting that the changes in histone H4 acetylation are correlated with those of p65 binding to the eotaxin promoter.

In contrast, parallel experiments using primers spanning the IL-8 promoter regulatory region (289 bp containing NF-κB-, AP-1-, and PEA3-binding sites) showed that although TNFα induced a marked enrichment of IL-8 promoter DNA with acetylated histone H4 and p65 IPs (Fig. 7B, lane 2) compared with the control (lane 1), the effect was not affected by pretreatment with 15d-PGJ2, Flut, or Salme (lanes 3–10).

Taken together, these results are consistent with the findings on protein production, mRNA expression, and promoter activity of eotaxin and IL-8 and directly demonstrate that PPARγ agonists and β2-agonists, like GCs, suppress TNFα-induced eotaxin gene transcription in a chromatin-dependent manner through inhibition of histone H4 acetylation and in vivo NF-κB p65 binding to its promoter. The results also suggest that these drugs have no effect on TNFα-induced IL-8 gene transcription.

**Effects of 15d-PGJ2, Flut, and Salme on the Physical Interaction between PPARγ and GR and Its Association with the Eotaxin Promoter**—We then conducted coimmunoprecipitation (IP) and ChIP assays to explore whether activated PPARγ can directly interact with GR and whether PPARγ and GR are associated with the eotaxin promoter. As shown in Fig. 8, in control cell (treated with TNFα only) nuclear samples, GRα was detected only with GRα IPs (Fig. 8, lane 1) and not with PPARγ (lane 2) or normal rabbit IgG (lane 3) IPs. In cells treated with 15d-PGJ2 (5 μM) in addition to TNFα, GRα was detected not only with GRα IPs (Fig. 8, lane 4) but also with PPARγ IPs (lane 5), compared with normal rabbit IgG IPs (lane 6). In cells treated with both 15d-PGJ2 and Flut (0.01 μM), more GRα in GRα IPs (Fig. 8, lane 7) and PPARγ IPs (lane 8) were detected compared with control cells (lanes 1 and 2) and cells treated with 15d-PGJ2 only (lane 4 and 5). Treatment with 15d-PGJ2 and Salme (0.01 μM, Fig. 8, lanes 10 and 11) produced similar results as treatment with 15d-PGJ2 and Flut (lanes 7 and 8). These data provide direct evidence that PPARγ activation with 15d-PGJ2 results in physical interaction between PPARγ and GRα and that GR activation with Flut and signaling pathways activated by β2-agonists enhance the interaction. It is also of note that β2-agonists, together with PPARγ agonists, stimulate GRα nuclear translocation (activation).

Furthermore, by ChIP assay, PPARγ IPs revealed a marked enrichment of the eotaxin promoter DNA in cells treated with 15d-PGJ2 (Fig. 9, lanes 2 and 3), Flut (lanes 4 and 5), and Salme (lanes 6 and 7) compared with cells treated with TNFα alone (lane 1). GRα IPs showed a similar enrichment of the eotaxin promoter DNA in cells treated with these drugs (Fig. 9, lanes 2–7) compared with cells treated with TNFα alone (lane 1). These results suggest that the protein-protein interaction between PPARγ and GRα induced by PPARγ agonists alone or in combination with GCs and β2-agonists is associated with the eotaxin promoter, which may lead to the inhibition of histone H4 acetylation and p65 binding to the eotaxin promoter, resulting in the suppression of eotaxin gene transcription.
DISCUSSION

This study is the first to demonstrate that TNFα-induced chemokine production in HASM cells is differentially regulated by PPARγ agonists and that GCs and β2-agonists interact with PPARγ agonists in this process. PPARγ agonists inhibit TNFα-induced eotaxin production transcriptionally, which is additively enhanced by GCs and β2-agonists; PPARγ agonists inhibit TNFα-induced MCP-1 production post-transcriptionally, which is additively enhanced by GCs but synergistically enhanced by β2-agonists; PPARγ agonists have no effect on IL-8 production. The novel finding is that activated PPARγ physically interacts with GR and that GR activation and the signaling pathways activated by β2-agonists potentiate the interaction, which may provide an explanation for the observed additive and synergistic inhibition on chemokine production by these drugs. The interaction between PPARγ and GRα may be associated with the eotaxin promoter, resulting in the inhibition of histone H4 acetylation and p65 binding to the eotaxin promoter and the suppression of eotaxin gene transcription. These results are consistent with the view that PPARγ can act...
in combination with GR to inhibit inflammatory responses (36).

PPARs have complex regulatory effects on inflammatory responses. Studies so far have shown cell- and stimulus-specific effects of PPARγ agonists on MCP-1 and IL-8 production (14, 15), but their effects on eotaxin expression have not been explored. We have demonstrated previously that PPARγ is expressed in HASM cells, and PPARγ agonists up-regulate cyclooxygenase-2 expression (16). The present study is the first to demonstrate that PPARγ agonists differentially regulate TNFα-induced chemokine production in HASM cells, providing further evidence for the complex regulatory effects of PPARγ on chemokine production. Although PPARα is also expressed in HASM cells (16), no effect of the PPARγ agonist WY-14643 on TNFα-induced chemokine production in these cells has been detected in the present study, which is consistent with previous findings that WY-14643 has no effect on cyclooxygenase-2 expression in these cells (16) and on IL-1β-induced IL-8 and MCP-1 expression in colon epithelial cells (14).

More recent evidence has shown that PPARγ agonists also promote their biological effects through PPARγ-independent mechanisms. For example, 15d-PGJ2 induces IL-8 mRNA and protein through a mitogen-activated protein kinase and NF-κB pathway rather than PPARγ activation in human T-cells as synthetic PPARγ agonists do not mimic the effect of 15d-PGJ2 (37). Imazumil et al. (38) demonstrated that 15d-PGJ2 inhibits lipopolysaccharide-induced GM-CSF expression through a mechanism unrelated to PPARγ. In this study, however, the effects of the synthetic PPARγ agonist TRO are consistent with those of the natural PPARγ agonist 15d-PGJ2, and overexpression of human PPARγ mimics the effect of 15d-PGJ2 (39). Our results therefore indicate that the inhibitory effects of 15d-PGJ2 and TRO on TNFα-induced eotaxin expression are PPARγ-dependent.

PPARγ and GR are both nuclear receptors that share highly conserved ligand-binding domain activation function-2, essential for transcriptional regulatory effects (19). Activated nuclear receptors induce transcriptional activity by recruiting a variety of coactivators. It has been shown that PPARγ and GR may interact with each other by sharing the same coactivators such as the steroid receptor coactivator-1 (SRC-1) (40, 41) and cAMP-response element-binding protein-binding protein (42). It has been observed that estrogen, progesterone, and GCs could interfere with the functions of each other (20) and that PPARγ activation enhances GC-mediated transcription and GCs in turn modulate PPARγ-mediated gene expression in osteoblasts, suggesting complex interactions between PPARγ and GR signaling pathways (42). Our current study is the first to provide concrete evidence that PPARγ and GR physically interact with each other to regulate chemokine expression in any cell system.

β2-Agonists are mainly used as bronchodilators in asthma therapy. However, we have demonstrated recently that they synergistically and additively enhance the inhibition by GCs on TNFα-induced IL-8 (8) and eotaxin production (7), respectively, in HASM cells in a cAMP-dependent manner. Increasing evidence has shown that GCs can interact with β2-agonists through the cross-talk between GR and the cAMP signaling pathways activated by β2-agonists. For instance, GR can up-regulate β2-receptor expression (44), whereas β2-agonists can cause GR nuclear translocation (activation) even in the absence of GCs in vascular smooth muscle cells (45) and HASM cells (46). The finding in the present study that Salme enhances the effect of 15d-PGJ2 on increasing GR nuclear presence is consistent with the previous finding that β2-agonists can cause GR activation in HASM cells (46). Because of the structural and functional similarities between PPARγ and GR, we proposed that β2-agonists could also interact with PPARγ agonists to regulate chemokine production. Indeed, we have found in the present study that the β2-agonist Salme additionally enhances the inhibition of TNFα-induced eotaxin production by 15d-PGJ2, in a similar way as it enhances the inhibition of eotaxin production by GCs (8), and that Salme enhances 15d-PGJ2-induced physical interaction between PPARγ and GR. We have also found that Salme synergistically enhances the inhibition of TNFα-induced MCP-1 production by 15d-PGJ2 when it has no effect on its own. We have shown in the present study that PPARγ activation induces physical interaction between PPARγ and GR even in the absence of GCs, and that this interaction is further enhanced by GR activation with GCs as well as the signaling pathways activated by β2-agonists. This may have important clinical implications as airway inflammation is a main feature of asthma.

Formation of a permissive chromatin environment by hyperacetylation of histone is a prerequisite for gene trans-activation, whereas hypoacetylation is correlated with reduced transcription or gene silencing (21, 24, 25, 47). Targeted acetylation of histone H4 plays an important role in allowing regulatory proteins to access DNA and is likely to be a major factor in the regulation of gene transcription (24, 25). It has been demonstrated that activated transcription factors such as NF-κB p65 form complexes with cAMP-response element-binding protein-binding protein, which has intrinsic histone acetyltransferase (HAT) activity (26, 48, 49), and induce histone acetylation of relevant lysine residues, resulting in local unwinding of DNA, increased transcription factor binding to the promoter, and gene transcription (24, 26). GCs have been shown to repress p65-activated HAT activity and consequently inflammatory gene expression (26), but whether PPARγ agonists have similar effects has not been known. NF-κB is a major transcription factor involved in the regulation of many genes and is implicated in the pathogenesis of a large number of diseases, particularly inflammatory diseases such as asthma and arthritis (50). We have recently identified that TNFα-induced eotaxin expression in HASM cells is NF-κB-dependent. p65 is also involved in TNFα-induced IL-8 expression (51). In this study, we have demonstrated that TNFα induces histone H4 acetylation and p65 binding to the eotaxin and IL-8 promoters and that the effects on eotaxin, but not those on IL-8, are suppressed by 15d-PGJ2, Flut, and Salme. We have also found that these drugs stimulated the association of both PPARγ and GR with the eotaxin promoter, which may be explained by the physical interactions between PPARγ and GRs induced by these drugs. To our best knowledge, this is the first demonstration that shows PPARγ and GR are associated with the eotaxin promoter even though there is no peroxisome proliferator-response element and glucocorticoid-response element within the region of the eotaxin promoter we detected in the study. This association could result in the inhibition of TNFα-induced histone H4 acetylation and p65 binding to the eotaxin promoter. As NF-κB p65 is likely to be responsible for histone H4 acetylation and the transcription of the eotaxin gene, it is reasonable to speculate that PPARγ and GR may directly affect p65 transactivation as there is evidence that the PPARγ agonist ciglitazone increases the physical interaction of PPARγ with p65, leading to the inhibition of NF-κB in colon cancer cells (52). However, we have not found any physical interaction of PPARγ and GR with p65 in our current study (data not...
shown). It is likely that PPARγ, like GR (26), suppresses eotaxin gene expression by direct inhibition of p65-HAT activity and/or by recruiting histone deacetylase to the p65-HAT complex. But why PPARγ agonists and GCs have no effects on TNFα-induced HAT activity in the IL-8 promoter site is unknown, and the detailed mechanisms, particularly how PPAR γ and GRs physically interact with each other and associate with the eotaxin promoter, remain to be elucidated.

A recent study (53) has demonstrated that PPARγ activation reduces antigen-induced lung inflammation, eosinophilia (53). Because airway inflammation, particularly in asthma, may offer novel opportunities for new therapies in asthma and other inflammatory diseases.

Acknowledgment—We thank Colin Clelland for providing us with specimens of human trachea.

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