Medroxyprogesterone Acetate Differentially Regulates Interleukin (IL)-12 and IL-10 in a Human Ectocervical Epithelial Cell Line in a Glucocorticoid Receptor (GR)-dependent Manner*

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Background: Little is known about the mechanism of action of MPA in the female genital tract.

Results: GR mediates MPA-induced up-regulation of IL-12 and down-regulation of IL-10 mRNA and protein levels.

Conclusion: MPA favors a pro-inflammatory milieu in ectocervical epithelial cells.

Significance: MPA used in hormonal therapy may modulate inflammation in the ectocervical environment via this genomic mechanism.

Medroxyprogesterone acetate (MPA), designed to mimic the actions of the endogenous hormone progesterone (P₄), is extensively used by women as a contraceptive and in hormone replacement therapy. However, little is known about the steroid receptor-mediated molecular mechanisms of action of MPA in the female genital tract. In this study, we investigated the regulation of the pro-inflammatory cytokine, interleukin (IL)-12, and the anti-inflammatory cytokine IL-10, by MPA versus P₄, in an in vitro cell culture model of the female ectocervical environment. This study shows that P₄ and MPA significantly increase the expression of the IL-12p40 and IL-12p35 genes, whereas IL-10 gene expression is suppressed in a dose-dependent manner. Moreover, these effects were abrogated when reducing the glucocorticoid receptor (GR) levels with siRNA. Using a combination of chromatin immunoprecipitation (ChIP), siRNA, and re-ChIP assays, we show that recruitment of the P₄- and MPA-bound GR to the IL-12p40 promoter requires CCAAT enhancer-binding protein (C/EBP)-β and nuclear factor κB (NFκB), although recruitment to the IL-10 promoter requires signal transducer and activator of transcription (STAT)-3. These results suggest that both P₄ and MPA may modulate inflammation in the ectocervix via this genomic mechanism.

Medroxyprogesterone acetate (MPA) has previously been shown to regulate cytokine/chemokine gene expression in epithelial cell lines of the female genital tract in a ligand-, promoter-, and cell-specific manner (39). The possibility thus exists that MPA may disrupt normal immune responses in the female genital tract, thereby influencing inflammation at this site. This is consistent with some latter mimic the progestogenic activity of P₄ and have been used in a number of therapeutic applications, such as contraception, hormone replacement therapy, and treatment of some gynecological disorders (1–3). Medroxyprogesterone acetate (MPA or Depo-Provera®) is an example of a synthetic progestin extensively used as a progestin-only injectable contraceptive in South Africa (4–7). At the molecular level, MPA elicits its biological effects by binding not only to the progesterone receptor (1, 8) but also to other members of the steroid receptor family such as the glucocorticoid receptor (GR), androgen receptor, and mineralocorticoid receptor (9–13). The subsequent off-target biological effects via these receptors may contribute to the undesirable side effects observed with its clinical use. For example, androgen receptor-mediated effects of MPA have been associated with an increased risk of breast cancer (14), although its activity via the GR has been linked to immunosuppression (1, 2, 15, and apoptosis (16).

Clinical and epidemiological evidence suggests that the use of MPA as a contraceptive may increase the risk of acquiring genital tract infections such as herpes simplex virus type (HSV)-2 (17), Chlamydia (18), gonorrhea (19), and HIV-1 (6, 20–22). The lower female genital tract is the primary site of exposure to the majority of these sexually transmitted pathogens (23–26). Epithelial cells lining the female genital tract play a protective role when this site is exposed to pathogens (27–29). In addition to providing a physical barrier against sexually transmitted pathogens, these epithelial cells are also capable of producing a wide variety of cytokines and chemokines that regulate both innate and acquired local immune responses (27–31). This cytokine milieu in the female genital tract is a vital determinant of inflammation (30–32) and most likely susceptibility to infections (32–38).

MPA has previously been shown to regulate cytokine/chemokine gene expression in epithelial cell lines of the female genital tract in a ligand-, promoter-, and cell-specific manner (39). The possibility thus exists that MPA may disrupt normal immune responses in the female genital tract, thereby influencing inflammation at this site. This is consistent with some
reports suggesting that hormonal contraception, such as MPA, is associated with an increase in inflammation at this site (18, 40, 41). Modulation of inflammation by MPA in the female genital tract is likely to affect susceptibility to sexually transmitted infections by altered recruitment of inflammatory cells (42). In the light of the above, this study investigated the effects of MPA, relative to P₄, on cytokine gene expression in a human ectocervical epithelial cell line. Specifically, we used gene-specific mRNA analysis, siRNA, and chromatin immunoprecipitation (ChIP) assays to explore the gene regulation of the pro-inflammatory cytokine, interleukin (IL)-12p40, and anti-inflammatory cytokine, IL-10, in response to P₄ and MPA, and ELISA to determine IL-12 and IL-10 protein levels. IL-12, a 70-kDa heterodimeric protein composed of two disulfide-linked subunits, p40 and p35, is a key cytokine that promotes cellular immunity and the subsequent production of other pro-inflammatory cytokines (43, 44). However, IL-10 inhibits cellular immunity by suppressing the production of pro-inflammatory cytokines such as IL-12 and IL-8 (45, 46). Our results indicate that both P₄ and MPA increase the expression of the IL-12p40 and IL-12p35 genes, although the IL-10 gene expression is decreased. A detailed investigation into the molecular mechanism, using a combination of chromatin immunoprecipitation (ChIP), siRNA, and re-ChIP assays, show that the GR is needed for the regulation of these cytokine genes and that recruitment of the P₄ and MPA-bound GR to the IL-12p40 promoter requires CCAAT enhancer-binding protein (C/EBP)-β and nuclear factor κB (NFκB), whereas recruitment to the IL-10 promoter requires signal transducer and activator transcription (STAT)-3.

EXPERIMENTAL PROCEDURES

Cell Culture—The human Ect1/E6E7 ectocervical epithelial cell line was purchased from the ATCC and cultured and prepared as described previously (39, 47). The MDA-MB-231 human breast cancer cell line was a generous gift from Prof. Guy Haegemann (University of Gent, Belgium) and was cultured as described previously (48). Only mycoplasma-negative cells were used in experiments.

Materials—P₄, MPA, cortisol, and tumor necrosis factor (TNF) were purchased from Sigma. [³H]Dexamethasone (specific activity of 82.8 Ci/mmol) was from AEC-Amersham Biosciences.

Immunoblotting—Ect1/E6E7 and MDA-MB-231 cells were seeded in 12-well plates at a density of 1 × 10⁵ cells per well. The cells were washed with ice-cold 1× PBS before lysis with sample buffer (100 mM Tris-HCl, pH 6.8, 20% glycerol, 5% SDS, 0.1% bromphenol blue, and 2% β-mercaptoethanol) (49). Protein samples were resolved by 10% SDS-PAGE, transferred to PVDF membranes (Millipore), and then blocked in 10% fat-free milk powder. The membranes were first probed with the primary antibodies, followed by HRP-conjugated secondary antibodies (goat anti-rabbit or anti-mouse) (Santa Cruz Biotechnology). Proteins were visualized using enhanced chemiluminescence (Pierce Thermo Scientific Inc.) and x-ray film (Africa X-Ray Industrial and Medical). The following primary antibodies all from Santa Cruz Biotechnology were used: anti-GR (H-300), anti-C/EBPβ (C-19), anti-NFκB p65 (C20), anti-STAT-3 (C-20), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (0411), and anti-heat shock protein (Hsp)-90α/β (H-114).

Whole Cell Binding Assay—Competitive whole cell binding assays were performed as described previously (11), with a few modifications. Briefly, Ect1/E6E7 cells were seeded in 24-well plates at a density of 1 × 10⁵ cells per well. After 48 h, the cells were washed three times with PBS and incubated for 6 h at 37 °C with 10 nM [³H]dexamethasone, in the absence (total binding) and presence of 1 μM unlabeled P₄, MPA, or cortisol (nonspecific binding). Cells were washed three times with ice-cold PBS containing 0.2% bovine serum albumin (BSA), before lysis with reporter lysis buffer (Promega). Total binding was measured as counts/min (cpm), whereas the specific binding was determined by subtracting nonspecific binding from total binding. Specific binding was normalized to the protein concentration, determined using the Bradford protein assay method (50).

Quantitative Real Time PCR (qPCR)—Ect1/E6E7 and MDA-MB-231 cells were seeded in 12-well plates at a density of 1 × 10⁵ cells per well and were incubated with test compounds for 6 h, before total RNA was isolated using Tri-Reagent (Sigma) according to the manufacturer’s instructions. Duration of hormone treatment was chosen based on time course studies in Ect1/E6E7 cells showing maximum TNF-induced mRNA expression of these genes at this time (data not shown). Total RNA was reverse-transcribed using the Roche Applied Science transcriptor first strand cDNA synthesis kit. Real time qPCR was performed by using the LightCycler-FastStart DNA Master™ plus SYBR Green I system (Roche Applied Science) according to the manufacturer’s instructions. The mRNA expression of IL-12p40, IL-12p35, IL-10, and GAPDH (used as an internal standard) was measured using the primer sets as indicated in Table 1.

siRNA Transfections—Ect1/E6E7 and MDA-MB-231 cells were seeded in 12-well plates at a density of 1 × 10⁵ cells per well. Cells were transfected with 10 nm siRNA using HiPerfect transfection reagent (Qiagen), according to the manufacturer’s

### Table 1

| Target gene / Clonedown | Primer sequence (5′-3′) | Strand |
|-------------------------|-------------------------|--------|
| IL-12p40                | CACATGCACTTACTCTTCCC   | Forward |
|                         | CTGTCGCTGGAGACCTCAT    | Reverse |
| IL-12p35                | TGGAGGCTGAGTCAAGGCGC   | Forward |
|                         | ATCCGCTTCTACAAGGGAG    | Reverse |
| IL-10                   | AAGGCGATCTACAAAGGCA    | Forward |
|                         | TTGTCTAGTATGGCTCTATGAGT| Reverse |
| GAPDH                   | TGAGGGAGAACTGTCCTG     | Forward |
|                         | TCCACACCGTCTGGTCCTA    | Reverse |
| IL-12p40 promoter       | NFeB/C-EBPβ            | Forward |
|                         | GTCTATGCTCCCCCTCCCTTT | Reverse |
|                         | GTCTTCTCTCGCTCTGTTGA  | Reverse |
| Sp1                     | TGGCCCTCGGAGGTTACCTTT | Forward |
|                         | ATCCTGCTATAGGTTACTGCTT| Reverse |
| AP-1                    | GCATCCTCCATCTTCTCTATT | Forward |
|                         | CGAGGGAGAACATGACATC    | Reverse |
| IL-10 promoter          | Sp1/STAT-3             | Forward |
|                         | TACAGAGAGAGGCGCTCTTAAGG| Reverse |
|                         | AGGAGGAGCCTTCCTTACATT | Reverse |
| AP-1                    | GTTGCTGAGATGCTGGTGACATG| Forward |
|                         | AGTAGTTGACGCTTGCACTGCTG| Reverse |
| GRE / Sp1               | GGAGACGCTGAAAGGGTGGA   | Forward |
|                         | CCTCAAGITCACCAGAGCAGC  | Reverse |
instructions, and incubated for 24 h (GR, C/EBPβ, and STAT-3) or 48 h (NFκB). Cells were subsequently treated for 6 h (qPCR) or 24 h (ELISA) with 0.02 μg/ml TNF in the absence and presence of 0.1% ethanol (control) or 1 μM test compound. For the quantification of mRNA expression by qPCR, RNA was harvested, and cDNA was synthesized. For the quantification of protein levels by ELISA, cell culture supernatants were collected and analyzed as described above. The following siRNAs were used: nonsilencing scrambled sequence control (NSC) or GR HS_NR3C1_6 or GR_HS_NR3C1_5 (all from Qiagen), or C/EBPβ, STAT-3, or NFκB p65 (all from Santa Cruz Biotechnology). Reduction in the protein levels was confirmed by Western blot analysis.

Chromatin Immunoprecipitation (ChiP) and Re-ChiP Assays—ChiP assays were performed as described earlier with minor modifications (51, 52). Briefly, Ect1/E6E7 cells were seeded in 14-cm² dishes at a density of 1 × 10⁷ cells. After 72 h, the supplemented KSFM was replaced with unsupplemented KSFM. Twenty four hours later, the cells were treated with 0.02 μg/ml TNF in the absence and presence of 0.1% ethanol (control) or 1 μM P₄, MPA, or cortisol for 2 h, and then the proteins and the chromatin were cross-linked using 1% formaldehyde.

Cells were washed twice with ice-cold PBS, harvested in PBS containing protease inhibitors (1 × Complete Mini Protease Inhibitor Mixture tablet; Roche Applied Science), lysed, and sonicated. The sonicated chromatin was centrifuged at 15,000 × g for 10 min at 4 °C to pellet the cell debris. An aliquot of the lysate (30 μg) was removed and used as input, and 100 μg of the chromatin was immunoprecipitated with antibodies against GR (H-300) or anti-IgG (Santa Cruz Biotechnology). The immunoprecipitated chromatin was collected on protein A/G-agarose beads preblocked with salmon sperm DNA, extensively washed, and eluted with elution buffer (1% SDS and 100 mM NaHCO₃). The cross-linking was reversed by adding NaCl (final concentration 300 mM) and incubating the samples overnight at 65 °C. Thereafter, the proteins were digested by treating the samples with proteinase K (Roche Applied Science, South Africa). Both immunoprecipitated and input DNA were purified using the NucleoSpin® Extract II kit (Thermo Scientific), and the purified immunoprecipitated DNA was quantified by qPCR, normalizing against input chromatin. Locations of primers used to amplify the DNA are shown in Figs. 4A and 5A, and the primer sequences are shown in Table 1.

For the chromatin reimmunoprecipitation (re-ChiP) assay, the immunoprecipitated DNA-protein complexes were eluted at 37 °C in elution buffer (1% SDS and 10 mM DTT). An aliquot of the supernatant was removed and used as a control for the first immunoprecipitation. The remaining sonicated chromatin was reimmunoprecipitated with antibodies specific for C/EBPβ, NFκB, or STAT-3 and analyzed as above.

ELISA—Ect1/E6E7 cells were seeded in 12-well plates at a density of 1 × 10⁵ cells per well and incubated with test compounds for 24 h. Culture supernatants were collected and assayed for IL-12p70 and IL-10 by high sensitivity kits purchased from eBioscience according to the manufacturer’s instructions. The optical density readings and standard concentrations were plotted, and the optical densities were converted to picograms/ml using linear regression analysis. The linearity range of the specific immunoassay kits used in this study were 0.16 to 10 pg/ml for IL-12p70 and 0.39 to 25 pg/ml for IL-10.

Data Manipulation and Statistical Analysis—GraphPad Prism® version 5 (GraphPad Software) was used for data manipulation, graphical presentations, and statistical analysis. One-way ANOVA, with Dunnett’s (compares all columns versus control column) or Bonferroni’s (compares all pairs of columns) post-tests, were used. Statistically significant differences are indicated by either *, **, and *** or #, ##, and ###, to indicate p < 0.05, p < 0.01, or p < 0.001, respectively, whereas p > 0.05 indicates no statistical significance (ns). The error bars represent the mean ± S.E. of at least three independent experiments.

RESULTS

Progestogen-activated GR Regulates mRNA Expression of IL-12p40, IL-12p35, and IL-10 Genes—To assess the effects of P₄ and MPA on inflammation in the female genital tract, the mRNA expression levels of endogenous IL-12p40, IL-12p35, and IL-10 were measured in the Ect1/E6E7 cells. These cells were used as an in vitro cell culture model for mucosal immunity in the female ectocervical environment, as they closely resemble the characteristics of their tissue of origin and primary cells (39, 47). An increase in pro-inflammatory cytokines such as IL-12 is critical for the progression of inflammation, although anti-inflammatory cytokines such as IL-10 control the course of the inflammatory process (45, 53–56). The cells were treated with increasing concentrations of P₄ or MPA, and gene expression was measured using qPCR. P₄ and MPA increased the gene expression of both IL-12p40 and IL-12p35 in a dose-dependent manner (Fig. 1, A and B), although these ligands dose-dependently decreased the expression of IL-10 (Fig. 1C). The potencies (EC₅₀ values) of P₄ and MPA were in the nanomolar range and are similar on all three genes (Fig. 1D).

To gain insight into the mechanism whereby the progestogens regulate the mRNA expression of IL-12p40, IL-12p35, and IL-10, we investigated the involvement of the GR, because it is known that both P₄ and MPA can bind to the GR (11). Competitive whole cell binding assays in this cell line confirmed that P₄ and MPA bind to the native GR (Fig. 2A). To establish the involvement of the GR, cells were transfected with two different GR-specific siRNAs, or an NSC siRNA, prior to treatment with 0.02 μg/ml TNF in the absence and presence of 1 μM test compound for 24 h. Western blot analysis confirmed that both GR6 and GR5 siRNA reduced GR protein levels to a similar extent (p > 0.05) (Fig. 2B). Gene expression analysis by qPCR showed that the P₄- and MPA-induced effects on IL-12p40, IL-12p35, and IL-10 were significantly reversed when the GR levels were decreased (Fig. 2, C–E). As expected, the effects of the natural glucocorticoid, cortisol, on IL-12p40, IL-12p35, and IL-10 gene expression were also significantly reduced by the decrease in GR protein levels (Fig. 2, C–E). Although similar GR-dependent effects were observed for P₄ and MPA on IL-12p40 and IL-12p35 gene expression (Fig. 2, C and D), cortisol displayed differential effects on the expression of these genes, suggesting different mechanisms of regulation.

To determine whether the effects of the progestogens on IL-12 and IL-10 gene expression are specific to the Ect1/E6E7...
FIGURE 1. Effect of P₄ and MPA on the TNF induced expression of IL-12p40, IL-12p35, and IL-10 in the human ectocervical cell line. The human Ect1/E6E7 cell line was incubated for 6 h with 0.02 μg/ml TNF in the absence or presence of 0.1% EtOH (vehicle control) or increasing concentrations of P₄ or MPA. Total RNA was isolated and reverse-transcribed to cDNA. Real time qPCR was performed to determine the mRNA expression levels of IL-12p40 (A), IL-12p35 (B), and IL-10 (C), using GAPDH as the internal standard. Results shown are the average of at least four independent experiments (±S.E.). Relative IL-12p40, IL-12p35, and IL-10 mRNA expression of treated samples was calculated relative to the vehicle control (EtOH), which was set as 1.

D, relative potency (EC₅₀ ± S.E.) for each ligand for activation of the IL-12p40 and IL-12p35 genes and repression of the IL-10 gene was obtained from the data shown in A–C. Statistical analysis of the EC₅₀ values for all genes indicated P₄ versus MPA (p < 0.05).

FIGURE 2. Decreasing GR protein levels by siRNA indicates a role for the GR in mediating the effects of P₄ and MPA on IL-12p40, IL-12p35, and IL-10 mRNA expression in the Ect1/E6E7 cell line. A, human Ect1/E6E7 cell line was incubated with 10 nM [3H]dexamethasone in the absence (total binding) and presence of 0.02 μg/ml TFN in the absence or treated with 0.02 μg/ml TNF in the absence or presence of 0.1% EtOH (vehicle control) or 1 μM P₄, MPA, or cortisol for 6 h. The percentage of specific binding (total binding minus nonspecific binding) is plotted. Binding of the test compounds to the GR is shown relative to binding of cortisol set as 100%. One-way ANOVA analysis of variance and Dunnett’s test (compare all columns versus control (cortisol) column) were performed as post-test. B–E, untransfected human Ect1/E6E7 cells, as well as cells transfected with 10 nM NSC or two GR siRNA oligonucleotides, were either left untreated or treated with 0.02 μg/ml TNF in the absence or presence of 0.02 μg/ml TNF in the absence or presence of 0.1% EtOH (control) or 1 μM P₄, MPA, or cortisol for 6 h. B, for verification of GR knockdown, total protein from the untreated cells was harvested to perform Western blotting, using antibodies specific for the GR and GAPDH. The latter was used as a loading control. A representative blot is shown. GR expression levels relative to GAPDH were quantified using UN-SCAN-IT. Western blots of three independent experiments were quantified to determine the percentage GR protein knockdown. C–E, total RNA was isolated and reverse-transcribed to cDNA. Thereafter, real time qPCR was performed to determine the mRNA expression levels of IL-12p40 (C), IL-12p35 (D), and IL-10 (E), using GAPDH as the internal standard. Relative IL-12p40, IL-12p35, and IL-10 gene expression of treated samples was calculated relative to vehicle control (EtOH) of the NSC siRNA, which was set as 1. Statistically significant differences are indicated by *, **, or *** p < 0.05, p < 0.01, or p < 0.001, respectively, for GR6; #, p < 0.05; ##, p < 0.01; ###, p < 0.001, respectively, for GR5; ns, no statistical significance; UT, untransfected.
GR-mediated Regulation of Cytokine Genes by MPA

A

FIGURE 3. MPA regulates IL-12p40 and IL-10, but not IL-12p35, mRNA levels in the MDA-MB-231 cell line in a GR-dependent manner. Human MDA-MB-231 cells transfected with 10 nM NSC or GR6 siRNA oligonucleotides were either left untreated or treated with 0.02 μg/ml TNF in the absence or presence of 0.1% EtOH (control) or 1 μg/ml P₄, MPA, or cortisol for 6 h. A, for verification of GR knockdown, total protein from the untreated cells was harvested to perform Western blotting, using antibodies specific for the GR and GAPDH. The latter was used as a loading control. A representative blot is shown. GR expression levels in both cell lines, in a GR-dependent manner (Figs. 2 and 3) are quantified using UN-SCAN-IT. Western blots of at least two independent experiments were quantified to determine the percentage of GR protein knockdown. B-D, total RNA was isolated and reverse-transcribed to cDNA. Thereafter, real time qPCR was performed to determine the mRNA expression levels of IL-12p40 (B), IL-12p35 (C), and IL-10 (D) using GAPDH as the internal standard. Relative IL-12p40, IL-12p35, and IL-10 gene expression of treated samples was calculated relative to vehicle control (EtOH) of the NSC siRNA, which was set as 1. Results shown are the average (± S.E.) of at least two independent experiments. One-way ANOVA and Dunnett’s (compares all pairs of columns versus control column) post-tests were used for statistical analysis. ns, no statistical significance; *, **, and ***, p < 0.05, p < 0.01, or p < 0.001, respectively.

cell line, the experiments were repeated in the MDA-MB-231 breast cancer cell line (Fig. 3). We used this cell line as it has previously been reported that the MDA-MB-231 cell line expresses IL-12p40, IL-12p35, and IL-10 mRNA (57). The results show that the effects of the progestogens on IL-12 gene expression are cell-specific in that P₄ has no effect on IL-12p40 (Fig. 3B) and IL-12p35 (Fig. 3C) gene expression, although MPA decreases the mRNA expression of IL-12p40, but not IL-12p35, via a GR-mediated mechanism. A recent study by Hapgood and co-workers (58) observed similar GR-mediated anti-inflammatory effects of MPA in a human endocervical cell line. Interestingly, all the progestogens repressed IL-10 mRNA levels in both cell lines, in a GR-dependent manner (Figs. 2E and 3D), suggesting that the regulatory mechanisms for IL-10 are not cell-specific, unlike those for IL-12.

P₄ and MPA Promote the Recruitment of the GR to the Endogenous IL-12p40 and IL-10 Gene Promoters—Next, we wanted to elucidate the mechanism involved in GR-mediated regulation of IL-12 and IL-10 in response to P₄ and MPA. Because IL12p35 is expressed in most cell types, unlike IL-12p40 (59, 60), we focused our attention on investigating promoter occupancy on IL-12p40, with a view to understanding the cell-specific mechanism of IL12 gene regulation. Thus, to investigate whether the GR is recruited to the endogenous IL-12p40 and IL-10 promoters, the Ect1/E6E7 cells were incubated with 0.02 μg/ml TNF in the absence or presence of 1 μM test compound for 2 h. The cell lysates were immunoprecipitated with a GR-specific antibody or anti-IgG (negative control), followed by qPCR analysis. Although it is generally accepted that the ligand-bound GR activates transcription of target genes by binding to glucocorticoid-response elements (GREs) (61) in the promoter region of these genes, no consensus GRE sequences are present within the proximal promoter region (−880 bp relative to the transcription start site) of the IL-12p40 gene (62, 63). Alternative cis-elements such as C/EBPβ and specific protein 1 (Sp1) were thus investigated, as previous studies have indicated that tethering of the GR to C/EBPβ or Sp1 transcription factors can activate transcription of genes containing C/EBPβ- (64, 65) or Sp1 (66, 67)-binding sites, respectively. For IL-10, we investigated the binding sites such as STAT-3 and activator protein (AP)-1, as tethering of the GR to STAT-3 and AP-1 has previously been associated with suppression of some genes (68, 69). A schematic diagram of all the cis-elements investigated in this study and the position of the primers are presented in Figs. 4A and 5A. Of note, some elements are located in close proximity to each other, and thus some primers span more than one cis-element.

Results showed that the GR occupies the NFκB/C/EBPβ region (Fig. 4B), but not the Sp1- or AP-1-binding sites (Fig. 4, C and D), of the IL-12p40 promoter when cells were treated with P₄ and MPA. Interestingly, cortisol treatment resulted in GR recruitment to the NFκB/C/EBPβ- and Sp1-binding sites (Fig. 4, B and C). For IL-10, results show that the GR occupies the Sp1/STAT-3 region of the promoter (Fig. 5B) but not the AP-1 (Fig. 5C) or GRE/Sp1 (Fig. 5D)-binding sites. Collectively, the
results suggest that in the Ect1/E6E7 cell line, P₄, MPA, and cortisol-bound GR interact with the NFκB/C/EBPβ region of the IL-12p40 promoter to activate transcription of this gene, whereas recruitment of the P₄, MPA, and cortisol-bound GR to the Sp1/STAT-3 region of the IL-10 promoter causes suppression of gene transcription.
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**A** GR-C/EBPβ  
**B** GR-NFκB

**C** C/EBPβ  
**D** NFκB

**FIGURE 6.** Recruitment of the progestogen-bound GR to the IL-12p40 promoter is dependent on both the transcription factors C/EBPβ and NFκB. A and B, human Ect1/E6E7 cells were incubated with 0.02 μg/ml TNF in the absence or presence of 0.1% EtOH or 1 μM P₄, MPA, or cortisol for 2 h, followed by the re-ChIP assay. Cell lysates were subjected to immunoprecipitation with the GR-specific antibody and then with the C/EBPβ (A) or NFκB (B) antibody or anti-IgG (negative control). The immunoprecipitated DNA fragments and input DNA were analyzed by real time qPCR. Data shown are normalized to input and expressed as the fold-response relative to EtOH (IgG control), which was set as 1. Results shown are the average (± S.E.) of at least three independent experiments. One-way ANOVA analysis of variance and Dunnett’s (compares all columns versus control) post-tests were used for statistical analysis. *p < 0.05; **p < 0.01; ***p < 0.001.

**GR Recruitment to the IL-12p40 Promoter in Response to P₄ and MPA Is Dependent on Both the C/EBPβ and NFκB Transcription Factors**—As we showed that the GR interacts with the NFκB/C/EBPβ region of the IL-12p40 promoter, we next performed re-ChIP assays to determine whether the GR forms a complex with the C/EBPβ and/or NFκB on the IL-12p40 promoter. Intact Ect1/E6E7 cells were treated with 0.02 μg/ml TNF in the absence or presence of 1 μM P₄, MPA, or cortisol for 2 h. Cell lysates were subjected to immunoprecipitation with a GR-specific antibody and then with either the C/EBPβ- or NFκB-specific antibodies. Immunoprecipitation with anti-IgG served as negative control. Results in Fig. 6, A and B, suggest that in response to P₄, MPA, and cortisol, the GR, C/EBPβ, and NFκB are co-localized on the endogenous IL-12p40 promoter.

Both C/EBPβ and NFκB have previously been shown to be critical in the transcriptional activation of the IL-12p40 gene (70, 71). As the re-ChIP results indicate that the liganded GR interacts with both C/EBPβ and NFκB, we investigated whether both transcription factors are involved in the progestogen-induced up-regulation of IL-12p40 gene expression. The Ect1/E6E7 cell line was transfected with 10 nM NSC or validated C/EBPβ- or NFκB-specific siRNA oligonucleotides, followed by treatment with 0.02 μg/ml TNF in the absence or presence of 1 μM P₄, MPA, or cortisol for 6 h. Western blot analysis showed ~54 and ~68% reduction in endogenous C/EBPβ (Fig. 6C) and NFκB (Fig. 6D) protein levels, respectively. Reducing C/EBPβ (Fig. 6C) and NFκB (Fig. 6D) protein levels significantly abolished the ligand-induced up-regulation of IL-12p40 gene expression, indicating that the progestogen-bound GR requires both transcription factors to activate transcription of the human IL-12p40 gene.

**STAT-3 Is Required for GR-mediated Suppression of IL-10 Gene Expression in Response to P₄ and MPA**—The results indicating that the GR is recruited to the Sp1/STAT-3 region of the IL-10 promoter in response to P₄ and MPA (Fig. 5B), but not the GRE/Sp1-binding sites (Fig. 5D), suggest that the STAT-3 and not the Sp1 element is important for the transcriptional suppression of IL-10 gene expression. Thus, re-ChIP assays were used to investigate whether the liganded GR forms a complex with STAT-3 on the IL-10 promoter. Intact Ect1/E6E7 cells
cell line was transfected with 10 nM NSC or validated STAT-3-induced suppression of specific siRNA oligonucleotides, followed by treatment with 0.02 μg/ml TNF in the absence or presence of 0.1% EtOH (control) or 1 μM P₄, MPA, or cortisol for 2 h, followed by the re-ChIP assay. Cell lysates were subjected to immunoprecipitation with the GR-specific antibody and then with the STAT-3-specific antibody or anti-IgG (negative control). The immunoprecipitated DNA fragments and input DNA were analyzed by real time qPCR. Data shown are normalized to input and expressed as the fold-change relative to vehicle control (EtOH) for IL-12p70 (~1.5 pg/ml) and IL-10 (~5 pg/ml) were set as 1, and the relative IL-12p70 and IL-10 protein levels of treated samples were calculated relative to this. Results shown are the average (± S.E.) of at least three independent experiments. Two-way ANOVA and Bonferroni (compares all pairs of columns) post-tests were used for statistical analysis. ns, no statistical significance; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

were treated with 0.02 μg/ml TNF in the absence or presence of 1 μM P₄, MPA, or cortisol for 2 h. Cell lysates were subjected to immunoprecipitation with a GR-specific antibody and subsequently a STAT-3-specific antibody. Immunoprecipitation with anti-IgG served as negative control. Results showed that in response to P₄, MPA, and cortisol, the GR and STAT-3 were co-recruited to the endogenous IL-10 promoter (Fig. 7A).

To further confirm a role for STAT-3 in the progestogen-induced suppression of IL-10 gene transcription, the Ect1/E6E7 cell line was transfected with 10 nM NSC or validated STAT-3-specific siRNA oligonucleotides, followed by treatment with 0.02 μg/ml TNF in the absence or presence of 1 μM P₄, MPA, or cortisol for 6 h. Western blot analysis showed 55% reduction in the endogenous STAT-3 protein levels (Fig. 7B). Reducing STAT-3 levels significantly attenuated the P₄, MPA, and cortisol-induced suppression of IL-10 gene expression. Interestingly, STAT-3 knockdown appears to lift this suppression. In summary, these results suggest that the progestogen-bound GR and STAT-3 bind as a complex to the human IL-10 promoter, thereby suppressing transcription of the IL-10 gene.

Progestogen-activated GR Also Regulates IL-12 and IL-10 Secreted Protein Levels—Finally, we evaluated the effects of the progestogens on the secreted protein levels of these cytokines in the human ectocervical cell line. The Ect1/E6E7 cells were transfected with a control or GR-specific siRNA and treated with 0.02 μg/ml TNF in the absence and presence of 1 μM test compound for 24 h. Western blot analysis confirmed efficient reduction of GR protein levels (Fig. 8, A and B). IL-12, measured as the p70 heterodimer, and IL-10 protein levels secreted in the supernatants of the Ect1/E6E7 cells were quantified using commercially available ELISA kits. Consistent with the mRNA results, we show that P₄ and MPA increases the protein levels of IL-12p70 (Fig. 8A), while decreasing IL-10 (Fig. 8B) protein levels. These responses were abrogated when the GR levels were reduced indicating that the GR dependence observed on the mRNA level is mimicked at the protein level.
GR-mediated Regulation of Cytokine Genes by MPA

**DISCUSSION**

In this study, we investigated the effects of the progestin-only injectable contraceptive MPA relative to natural P₄ on the transcriptional regulation of cytokine genes in a human ectocervical epithelial cell line treated with TNF to mimic infection. Our study is the first to show that P₄ and MPA increase the mRNA and secreted protein levels of the pro-inflammatory cytokine IL-12, while decreasing the expression of the anti-inflammatory IL-10 gene, in the ectocervical cell line in a dose-dependent manner (Fig. 1). These pro-inflammatory effects are in line with our previous results in the ectocervical epithelial cell line, showing that P₄ up-regulates the expression of the pro-inflammatory IL-6, IL-8, and RANTES (39) genes. However, in these cells MPA has either no effect (IL-6) or up-regulates (IL-8), or down-regulates (RANTES) gene expression. Furthermore, we show that the effects of the progestogens on IL-12 gene expression are cell-type-specific, as similar effects were not observed in the MDA-MB-231 breast cancer cell line. However, the effects of the progestogens on IL-10 gene expression do not appear to be cell-specific, as similar GR-mediated effects were shown for the Ect1/E6E7 and MDA-MB-231 cell lines.

To delineate the molecular mechanism underlying the differential regulation of IL-12p40 and IL-10 by P₄ and MPA, we investigated the role of the GR and its subsequent recruitment to these promoters. As the promoter of the IL-12p40 gene contains functional cis-acting sequences, such as response elements for NFkB, AP-1, Sp1, and C/EBP (62, 63, 70, 71), we used a combination of siRNA technology and ChIP assays to understand how P₄ and MPA modulate IL-12p40 at the transcriptional level. Similar experiments were performed for IL-10 as its promoter also contains numerous cis-elements for AP-1, GRE, Sp1, and STAT-3 (72–77). Our results show that the GR is recruited to both the IL-12p40 (Fig. 4B) and IL-10 (Fig. 5B) promoters in response to P₄ and MPA. Consistent with a role for the GR, we also demonstrate that the GR is recruited to these promoters in the presence of cortisol, the natural glucocorticoid (Figs. 4B and 5B), and that cortisol showed similar effects on the expression of these genes (Fig. 2, C and F). Moreover, we show for the first time that the liganded GR co-localizes with C/EBPβ and NFkB on the endogenous IL-12p40 promoter (Fig. 6). For IL-10, re-ChIP assays showed co-localization of the ligand-bound GR and STAT-3 on the endogenous IL-10 promoter (Fig. 7). Taken together, our investigations regarding the mechanism underlying the differential effects of P₄ and MPA on IL-12p40 and IL-10 gene expression have revealed the role of different transcription factors, in particular the GR, NFkB, and C/EBPβ, in up-regulating IL-12p40 expression, although the GR and STAT-3 play a role in down-regulating IL-10 mRNA expression.

GR agonists such as cortisol and partial GR agonists such as P₄ and MPA are usually reported to exert anti-inflammatory actions when acting via the GR by the classical mechanism of down-regulating pro-inflammatory genes and up-regulating anti-inflammatory genes (10–12, 69, 78). In agreement with this mechanism, some studies in human peripheral blood mononuclear cells show that glucocorticoids (62, 79, 80), P₄ (81), and MPA (82) decrease IL-12p40 protein levels. However, evidence for the effects of these ligands on IL-10 gene expression is contradictory. Some studies show no effect (79, 82, 83), whereas others are in agreement with the classical mechanism showing an increase in IL-10 mRNA and protein expression (67, 79, 84–86), and some deviate from the classical mechanism by showing a decrease in IL-10 mRNA and protein levels (79, 87–90). Clearly, our results in the ectocervical epithelial cell line showing pro-inflammatory GR-mediated effects by P₄, MPA, and cortisol via a unique tethering mechanism deviate from the classically accepted GR mechanism. Consistent with our results however, emerging evidence suggests that glucocorticoids can also elicit pro-inflammatory effects (91). Moreover, MPA has previously been shown to elicit pro-inflammatory effects in the cervix of mice by suppressing IL-10 mRNA and protein levels (90), although the mechanism and receptor mediating the response was not determined. Considering that P₄ and MPA have previously been shown to have different binding affinities and transcriptional activities via the GR (11, 92), it was surprising that these progestogens displayed similar GR-mediated effects in the Ect1/E6E7 cell line. However, the relative affinities of P₄ and MPA for the GR may be different in this cell line compared with other cell lines, as it has previously been shown that the concentration of GR determines the binding affinity of a ligand for the receptor (93). Moreover, as we have previously shown that P₄ and MPA differentially regulate cytokine gene expression in a cell- and promoter-specific manner (39), discrepancies between the results from this study and others using synthetic GRE-containing promoters in COS-1 cells, for example (11), may be due to either cell- or promoter-specific effects.

The precise signal transduction pathways leading to the activation of IL-12p40 gene transcription and the inhibition of IL-10 gene expression in the ectocervical cell line are not clear. However, some hypotheses can be formulated and are illustrated in Fig. 9. First, because TNF has previously been shown to activate and induce nuclear translocation of C/EBPβ and NFkB (94–96), as well as STAT-3 (97, 98), it is plausible that C/EBPβ and NFkB are recruited to the IL-12p40 promoter, and STAT-3 to the IL-10 promoter, upon TNF treatment. We further propose that the liganded GR interacts with the following: 1) NFkB, which tethers to C/EBPβ, the latter bound to its binding site in the IL-12p40 promoter, or 2) both NFkB and C/EBPβ, each bound to their respective binding sites. NFkB tethering to C/EBPβ bound to its binding site, and NFkB or C/EBPβ each bound to their respective sites are both mechanisms that have previously been proposed for the up-regulation of the pro-inflammatory IL-8 cytokine gene by TNF (99). Furthermore, at least two previous studies have shown that target gene expression is enhanced when glucocorticoid-bound GR tethers to DNA-bound C/EBPβ (64, 65). Further support of this mechanism is the fact that the IL-12p40 promoter does not appear to contain a functional GRE (62, 63) and that tethering of the GR to C/EBPβ bound to its binding site has previously been proposed as a mechanism for transactivation of glucocorticoid-responsive genes that lack functional GREs (100). In terms of IL-10 gene regulation, we propose that the liganded GR tethers to STAT-3 bound to its binding site within the IL-10 promoter. Tethering of the GR to DNA-bound STAT-3 has
previously been implicated in transcriptional repression of genes, although direct binding of the GR to a STAT-3-binding site is associated with transcriptional activation of genes (68).

Taken together, this biochemical study provides a novel mechanism whereby P₄ and MPA are likely to modulate local immune function in the female genital tract.

Trying to understand the physiological implications of these results is not a simple task. Both IL-12 and IL-10 are key cytokines that play major roles in regulating inflammatory responses (54, 101–103). The IL-12 p40/p35 heterodimer is essential for the initiation of an effective immune response, although IL-10 protects the host from excessive inflammation (54, 101). Support for the critical role of these cytokines in regulating inflammatory responses is gained from studies showing that IL-10-deficient mice display dysregulated inflammatory responses and develop chronic inflammatory disorders, possibly due to their inability to counteract IL-12-driven inflammation (104, 105). Interestingly, enhanced production of IL-12p40 has been shown to prevent chronic enterocolitis in the intestinal epithelium of IL-10-deficient mice (56), due to the formation of IL-12p40 homodimers, suggesting that the IL-12 heterodimer is critical for chronic inflammatory responses. At first glance, it thus appears that our data showing increased levels of IL-12 and decreased levels of IL-10 by P₄ and MPA suggest that these ligands would lead to increased inflammation in the ectocervical environment. However, it is important to remember that the defense function in the ectocervical environment is not only dependent on IL-12 and IL-10 but on a number of regulatory factors. Thus, the observed effects of P₄ and MPA on IL-12 and IL-10 expression should be considered in the light of the fact that there is a constant release of various pro- and anti-inflammatory mediators in the cervical environment.

Another crucial point to ponder is whether the dosage of MPA used in hormonal therapy will exert similar effects on local immune function in the ectocervical environment in vivo. Serum concentrations of MPA range between 4.5 and 65 nM a few days after administration of the intramuscular injection, followed by a gradual decrease to 2.6 nM for about 3 months (15). As our dose response analysis shows that the potency (EC₅₀ values) for MPA regulation of the IL-12p40, IL-12p35, and IL-10 genes in the ectocervical epithelial cells is in the nanomolar range (1.4, 7.47, and 3.25 nM, respectively), it is likely that the pro-inflammatory effects of MPA in these cells are relevant at serum doses of the injectable contraceptive Depo-Provera. Indeed, increased IL-12 protein levels have been reported in the vaginal lavage fluid of adolescent females using Depo-Provera as a contraceptive compared with non-users (106). In contrast to our in vitro data however, these authors reported increased levels of IL-10 for Depo-Provera users (106). The increased levels of both IL-12 and IL-10 may be due to increased concentrations of IL-12 stimulating the production of IL-10, a mechanism previously proposed for increasing levels of these cytokines in cervical specimens (107, 108). Serum concentrations of endogenous P₄ have been reported to be low during the follicular phase (~0.65 nM), increasing to ~80 nM during the luteal phase, and ~600 nM during pregnancy (1). As our dose response analysis shows that the potency for P₄ regulation of IL-12p40, IL-12p35, and IL-10 is 4.58, 0.31, and 5.67 nM, respectively, it is probable that P₄, like MPA, would modulate immune responses in the ectocervical environment in vivo.
Evidence in the literature suggests that high P₄ concentrations, such as those in the luteal phase and during pregnancy, are associated with increased HIV-1 shedding in cervical secretions (21, 109, 110) and increased susceptibility to HIV-1 infections (21, 111–113). Similarly, both human and animal studies suggest a link between high P₄ levels and increased risk of sexually transmitted diseases like HSV-2, Chlamydia, and Candidiasis (24, 114). Considering that the concentrations of P₄ fluctuate due to reproductive processes (27, 114, 115), whereas the serum levels of MPA used as contraceptive peak after injection (4.5–65 nm) but then stay constant (~2.6 nm) for approximately 3 months, P₄ may induce transient inflammation of the ectocervical environment during times of high P₄ concentrations, although MPA may cause a more sustained inflammation. Our results are consistent with a model whereby pregnant and pre-menopausal nonpregnant women in the luteal phase could be vulnerable to sexually transmitted diseases due to pro-inflammatory actions of P₄ via the GR, although women on depo-medroxyprogesterone acetate could be vulnerable at all times, but especially after injection, via the same mechanism.

Taken together, our study shows a GR-dependent mechanism for the differential regulation of IL-12 and IL-10 by both P₄ and MPA in the ectocervical epithelial cell line and reveals the role of different transcription factors, including NFkB and C/EBPβ to enhance transcription of the IL-12p40 gene and STAT-3 to suppress IL-10 gene transcription. Furthermore, we suggest that both P₄ and physiological doses of Depo-Provera may disrupt normal immune function in the ectocervix via this mechanism. Although we have not investigated how these progesterone-induced effects would impact on HIV infectivity, our results, taken together with evidence in the literature indicating that modulation of mucosal immunity in the female genital tract may increase susceptibility to HIV-1 (35, 38, 42), suggest that P₄ and MPA could increase susceptibility to genital tract infections. The clinical implications of these results may be significant and warrant further investigation.

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