Progesterone inhibits inducible nitric oxide synthase gene expression and nitric oxide production in murine macrophages

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Abstract: The purpose of this study was to determine whether the female hormones estradiol-17β (E2) and progesterone (P4) influence inducible nitric oxide synthase (iNOS) and the production of nitric oxide (NO) by interferon-γ (IFN-γ)- and lipopolysaccharide (LPS)-activated mouse macrophages. Treatment with P4 alone caused a time- and dose-dependent inhibition of NO production by macrophage cell lines (RAW 264.7, J774) and mouse bone marrow culture-derived macrophages as assessed by nitrite accumulation. RAW 264.7 cells transiently transfected with an iNOS gene promoter/luciferase reporter-gene construct that were stimulated with IFN-γ/LPS in the presence of P4 displayed reduced luciferase activity and NO production. Analysis of RAW 264.7 cells by Northern blot hybridization revealed concurrent P4-mediated reduction in iNOS mRNA. These observations suggest that P4-mediated inhibition of NO may be an important gender-based difference within females and males that relates to macrophage-mediated host defense. *J. Leukoc. Biol.* 59: 442–450; 1996.

Key Words: mouse · female sex steroids · effector molecules · host defense

INTRODUCTION

Activated macrophages form a first line of defense in host resistance to disease. Macrophage activation is a complex process that occurs in multiple sequential stages and results in an enhanced potential to complete a wide array of functions such as chemotaxis, phagocytosis, processing and presentation of antigens, regulation of hematopoiesis, tissue repair, and the production of effector molecules [1]. Nitric oxide (NO) is one of the effector molecules produced at high levels by activated mouse macrophages and is believed also to be an important product of human macrophages [2].

NO is an inorganic free radical gas synthesized in mammalian tissues from the guanido nitrogen group of L-arginine and molecular O2 by various isoforms of NO synthase (NOS) [3]. NO has many biological activities. It is a second messenger for neuronal activity, participates in the regulation of blood pressure and in the inhibition of blood clotting by preventing platelet aggregation, and is essential for the killing of microbes and lysis of tumor cells [4]. The neuronal or brain NOS and endothelial cell isoforms are constitutively expressed and synthesize NO in response to external stimuli. Synthesis of the major isoform found in macrophages, neutrophils, and hepatocytes, inducible nitric oxide synthase (iNOS), is stimulated by activators such as interferon-γ (IFN-γ) and bacterial lipopolysaccharide (LPS) [5].

Evidence has accumulated to support the concept that female sex steroid hormones have a regulatory role in macrophage gene expression and effector functions. In vivo, regulation may be either indirect or direct. For example, macrophages migrate into the estrogen-stimulated mouse uterus [6], possibly as an indirect response to chemoattractive cytokines such as colony-stimulating factor-1 and granulocyte-macrophage colony-stimulating factor produced by estrogen-targeted uterine cells [7]. However, in vitro studies show that estrogen and progesterone have direct effects as well. The hormones may inhibit or stimulate, in a dose-dependent manner, the Fc-mediated clearance of antibody-coated erythrocytes by guinea pig splenic macrophages [8, 9] and may regulate expression of MHC class II (Ia) antigen and interleukin-1β (IL-1β) protein synthesis by mammalian monocytes, macrophages, and macrophage cell lines [10–13].

The purpose of this study was to determine whether or not the female reproductive hormones estradiol-17β (E2) and/or progesterone (P4) have a regulatory role in murine macrophage activation for the production of NO. First, we determined that P4 but not E2 affected NO production.

Abbreviations: iNOS, inducible nitric oxide synthase; NO, nitric oxide; IFN-γ, interferon-γ; LPS, lipopolysaccharide; IL-1β, interleukin-1β; HEPES *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MEM, minimum essential medium; FCS, fetal calf serum; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcribe; PCR, polymerase chain reaction; ER, estrogen receptor; PR, progesterone receptor; AE, analogue of estrogen; PGE_2, prostaglandin E_2.

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Subsequent experiments indicated that modulation of NO production is achieved by direct inhibition of iNOS gene transcription.

MATERIALS AND METHODS

Cell culture

The mouse macrophage-like cell lines RAW 264.7 and J774 were purchased from the American Type Culture Collection (Rockville, MD). Bone marrow culture-derived macrophages were prepared from 6-week-old male C3H/HeN mice. The procedures for harvesting and culturing the mouse bone marrow cells have been described previously [14]. Macrophage cell lines and bone marrow cultured-derived macrophages used for this study were cultured in growth medium composed of 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered phenol red-free minimum essential medium (MEM; Sigma, St. Louis, MO) containing 10% (vol/vol) fetal calf serum (FCS; Hyclone Laboratories Inc., Logan, UT), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml of streptomycin (Sigma). For hormone modulation experiments the FCS content was reduced to 2%. The electroporation medium was composed of 25 mM HEPES-buffered RPMI 1640 medium (Sigma) containing 10% (vol/vol) FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Each type of experiment was conducted at least three times with three replicates for each group.

Nitrite assay

RAW 264.7 and J774 cells and bone marrow culture-derived macrophages (7 × 104 cells/treatment incubated in 96-well plates) were used for dose-response and time course experiments. Macrophages were activated with 10 U/ml IFN-γ (Schering-Plough through the American Cancer Society, Atlanta, GA) and 100 ng/ml LPS (the lipid A-rich fraction II of LPS phenol extracted from *Escherichia coli* 0111:B4, a gift from David Morrison, Dept. of Microbiology, Immunology and Molecular Genetics) as described earlier [15]. In dose-response experiments, RAW 264.7 and J774 cells were incubated for 24 h with IFN-γ/LPS and 0, 0.01, 0.1, 1.0, 2.0, 3.0, or 6 μg/ml of E2 and/or P4 in phenol red-free MEM supplemented with 2% FCS. RAW 264.7 and J774 cells and bone marrow culture-derived macrophages, used in time course experiments, were incubated as above for 24, 36, and 48 h with IFN-γ/LPS with or without E2 (3.0 μg/ml) and/or P4 (3.0 μg/ml) in phenol red-free MEM supplemented with 2% FCS. Controls of each cell type were cultured in medium alone or in medium containing appropriate concentrations of the dimethyl sulfoxide (DMSO) vehicle and IFN-γ/LPS. The concentration of 3.0 (μg/ml E2 and/or P4 was used in all subsequent experiments. Additionally, each cell type was cultured for 24 h with IFN-γ/LPS with or without E2 and/or P4 in medium containing the steroid antagonist RU486 (0.75 μg/ml), dexamethasone (0.3 μg/ml), 25-hydroxycholesterol (3.0 μg/ml), the E2 analogue estradiol-17α (3.0 μg/ml), the P4 analogue 4-pregnen-20α-ol-3-one (3.0 μg/ml), the E2 steroid antagonist tamoxifen (3.0 μg/ml), or the prostaglandin inhibitor indomethacin (3.0 μg/ml) (all from Sigma). After treatments, supernatants were collected and analyzed for the production of NO using the Griess reaction assay, which measures the concentration of nitrite, a stable product of the reaction of NO with O2 [16]. Supernatants (100 μl) were incubated with equal volumes of the Griess reagent [1% sulfanilamide, 2.5% H2PO4, 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride] for 10 min at room temperature. The absorbance at 570 nm was determined by using a Dynatech MR5000 microplate reader (Dynatech Laboratories Inc., Chantilly, VA). Sodium nitrite dilutions were used as standards.

Cell viability assay

The viability of macrophages exposed to the various treatments was determined in all experiments by the MTT colorimetric assay [17], which measures the mitochondrial enzymatic cleavage of the tetrazolium ring of MTT (3-[4,5,-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) into the blue-colored product, formazan. The absorbance of formazan product is measured on a microplate reader at 570 nm. A comparison of the absorbance values from each treated group to untreated and vehicle controls was used to determine cell viability.

Transfection procedures

RAW 264.7 cells were used to investigate iNOS gene promoter activity. Electroporetic transfection of RAW 264.7 cells with DNA constructs was conducted using previously developed methods [18, 19]. Logarithmically growing RAW 264.7 cells were harvested, washed, and resuspended at 2 × 107 cells/ml in electroporation medium. Aliquots of 250 μl of cells were electroporated with 2.7 pmol of purified plasmid DNA in a 0.4-cm electroporation cuvette at 300 V, 960 μF using a Bio-Rad Gene Pulser (Bio-Rad, Hercules, CA) with a capacitance extender. The cells were cultured in growth medium for 72 h before the exposure to activators and hormones. RAW 264.7 cells were transfected with an iNOS regulatory region-reporter construct containing both the region I (LPS response region) and region II (IFN and LPS response region) response elements cloned into a pGL2-Basic GeneLight luciferase vector (Promega, Madison, WI, construct H/H in ref. 19). In addition to the above transfections, RAW 264.7 cells were also transfected with the pGL2-Control construct (Promega, Madison, WI), which contains the luciferase gene placed under the control of the SV40 promoter/enhancer. Transfected RAW 264.7 cells were stimulated for 8 h with IFN-γ/LPS with or without E2 and/or P4. Additionally, control cells were cultured in medium alone or in medium containing the DMSO vehicle and IFN-γ/LPS. After transfected macrophages were exposed to their respective treatments, cell monolayers were washed with sterile phosphate-buffered saline (PBS), lysed, and assayed for luciferase activity in a luminometer according to the manufacturer's instructions (Enhanced Luciferase Assay, Monolight 2010 luminometer, Analytical Luminescence Laboratory, San Diego, CA). In parallel studies, transfected RAW 264.7 cells were incubated in 96-well plates for 24 h and culture supernatants were spectrophotometrically analyzed for NO production using the Griess assay and sodium nitrite as standards. Additionally, the viability of treated, transfected RAW 264.7 cells was determined by using the MTT assay.

Northern blot analysis of iNOS mRNA

Total RNA was isolated from RAW 264.7 cells (1 × 107 cells/treatment) incubated from 0 to 24 h with IFN-γ/LPS with E2 and/or P4 or vehicle

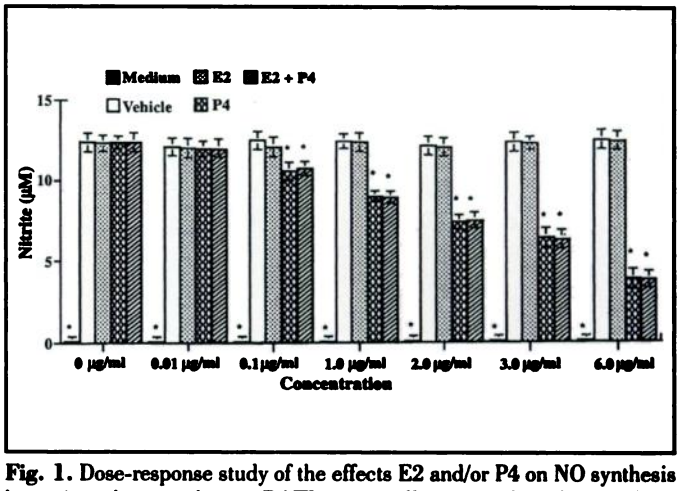

Fig. 1. Dose-response study of the effects E2 and/or P4 on NO synthesis by activated macrophages. RAW 264.7 cells were cultured in medium alone or in medium containing IFN-γ (10 U/ml) and LPS (100 ng/ml) as well as DMSO (vehicle) or 0, 0.01, 0.1, 2.0, 3.0, or 6.0 μg/ml of E2 and/or P4 for 24 h. Culture supernatants were assayed for accumulated nitrite concentration using the Griess reaction. The results shown (mean ± SE of triplicates) are representative of one of three separate experiments. ($*P <$ 0.01, test compared with vehicle control).

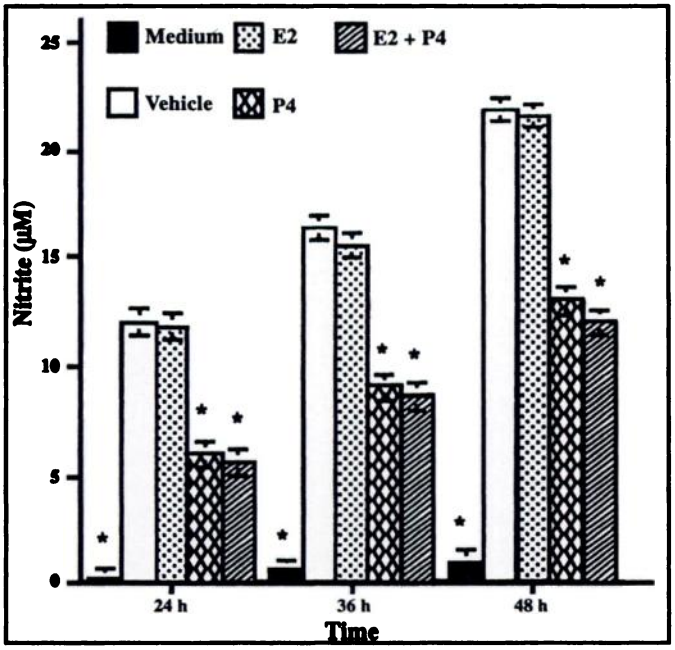

Fig. 2. Time course study of the effects of E2 and/or P4 on NO synthesis by activated macrophages. RAW 264.7 cells were cultured in medium alone or in medium containing IFN-γ (10 U/ml) and LPS (100 ng/ml) as well as DMSO (vehicle) or 3.0 μg/ml E2 and/or P4 for 24, 36, and 48 h. Culture supernatants were assayed for accumulated nitrite concentration using the Griess reaction. The results shown (mean ± SE of triplicates) are representative of one of three separate experiments. ($*P < 0.01$, test compared with vehicle control).

controls using the TRIzol reagent according to the manufacturer's instructions (GIBCO-BRL, Grand Island, NY). Isolated RNA (10 μg/lane) was separated by electrophoresis on 1% agarose gels containing 2.2 M formaldehyde and transferred to nylon membranes according to the manufacturer's instructions (Schleicher and Schuell, Kenne, NH). Prehybridization, hybridization, and posthybridization procedures were performed using Quik-Hyb hybridization solution as described by the manufacturer (Stratagene, La Jolla, CA). During the hybridization step the membranes were incubated with 32P-labeled cDNA probes specific for iNOS or control glyceraldehyde-3-phosphate dehydrogenase (G3PDH) [20] each labeled by the random primer DNA labeling method (Stratagene) with the use of [α-32P]dCTP (ICN Biomedicals Inc., Costa Mesa, CA). The membranes probed with the iNOS cDNA were autoradiographed on Kodak XAR-5 film at -80°C with intensifying screens and later washed and reprobed with G3PDH cDNA. The autoradiographic iNOS mRNA signals were quantitated by scanning densitometry and corrected relative to the G3PDH mRNA levels.

Identification of estrogen and progesterone receptor mRNA by reverse-transcribed polymerase chain reaction

Total RNA was extracted as described above from macrophage monolayers (1 × 107 cells) cultured in medium alone or in medium containing E2 and/or P4 for 0–24 h and the RNA (1μg) was reverse transcribed (RT) into first-strand cDNA in a 20-μl RT mixture containing 50 mM KCl, 10 mM tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 8.3 at 25°C), 5 mM MgCl2, 0.1% Triton X-100, 40 units of RNase inhibitor (Perkin-Elmer, Norwalk, CT), 1 mM each of dATP, dCTP, dGTP, and dTTP, 12.5 μM random hexamers, and 200 units of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). The RT mixture was incubated at 42°C for 1 h, heated to 99°C for 5 min to inactivate the reverse-transcriptase activity and chilled to 4°C for 5 min. A portion of the RT product (5 μl) was then subjected to polymerase chain reaction (PCR) in a DNA thermal cycler (model 9600, Perkin-Elmer). A 50-μl PCR reaction mixture included 50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25°C), 4 mM MgCl2, 0.1% Triton X-100, 1 mM of each dNTP, 2.5 units of *Taq* DNA polymerase (Promega) and 15 μM of each primer. The cycle profile was 94°C, 1 min; 62°C, 1 min; 72°C, 1 min for 50 cycles followed by 1 cycle of 72°C for 7 min. The PCR products (15 μl) were fractionated in a 2% agarose gel and visualized by ethidium bromide staining. The two sets of primers specific for the steroid-binding domain of the estrogen receptor (ER) and progesterone receptor (PR) were synthesized (Oligos Etc., Wilsonville, OR) from published sequences [21]. The presence of a 478-bp band identified ER mRNA and a 327-bp band identified PR mRNA.

Statistical analysis

All methods that yielded numerical values were subjected to one-way analysis of variance and differences between individual means were determined using Duncan's multiple range test. The data presented in each figure of the following studies is one of three representative experiments performed separately at different times. Each experiment was conducted with three replicates for each treated or untreated macrophage culture. Results were analyzed using the Super ANOVA Statistics Package (Abacus Concepts, Berkeley, CA).

RESULTS

P4 inhibits NO production by maçrophages stimulated with IFN-γ/LPS

To determine whether or not E2 and/or P4 altered NO production by IFN-γ/LPS-activated macrophages, supernatants from macrophage cultures were assayed for their concentrations of accumulated nitrite following exposure to activators and various levels of E2 and/or P4.

As shown in **Figure 1**, RAW 264.7 macrophages displayed a dose-dependent reduction in nitrite accumulation

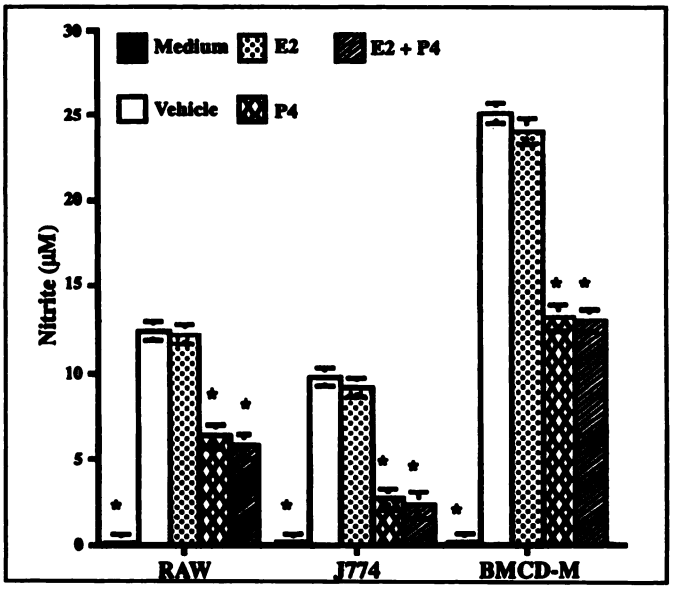

Fig. 3. Comparative study of the effects of E2 and/or P4 on NO synthesis by different activated macrophages cell types. RAW 264.7, J774 and bone marrow culture-derived (BMCD-M) macrophages were cultured in medium alone or in medium containing IFN-γ (10 U/ml) and LPS (100 ng/ml) as well as DMSO (vehicle) or 3.0 μg/ml E2 and/or P4 for 24 h. Culture supernatants were assayed for accumulated nitrite concentration using the Griess reaction. The results shown (mean ± SE of triplicates) are representative of one of three separate experiments. ($*P < 0.01$, test compared with vehicle control).

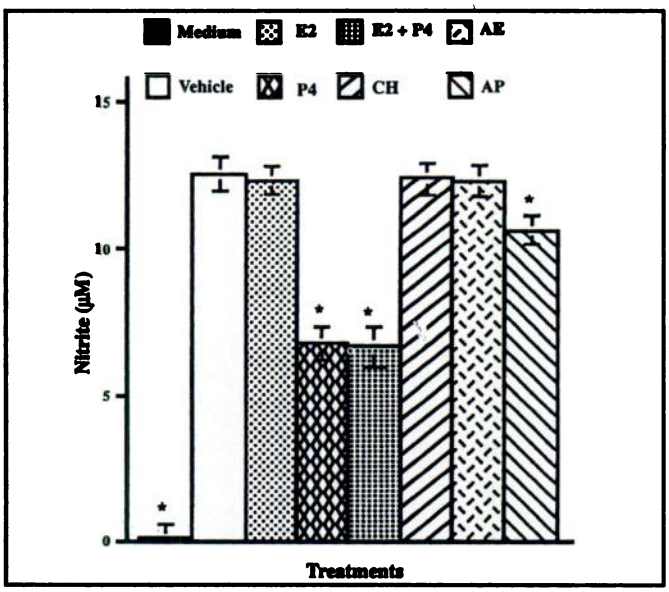

Fig. 4. Comparative study of hormone analogues and other compounds on NO synthesis by activated macrophages. RAW 264.7 cells were cultured in medium alone or in medium containing IFN-γ (10 U/ml) and LPS (100 ng/ml) as well as DMSO (vehicle) or 3.0 μg/ml E2 and/or P4, the progesterone analogue 4-pregnen-20α-ol-3-one (AP), the E2 analogue 17α-estradiol (AE), or 25-hydroxycholesterol (CH) for 24 h. Culture supernatants were assayed for accumulated nitrite concentration using the Griess reaction. The results shown (mean ± SE of triplicates) are representative of one of three separate experiments. (*$P < 0.01$, test compared with vehicle control).

following simultaneous incubation with IFN-γ/LPS and various concentrations of E2 and/or P4. After 24 h, the level of nitrite accumulated in culture supernatants of IFN-γ/LPS-activated RAW 264.7 macrophages was significantly reduced 11% compared with vehicle-treated controls when culture medium included 0.1 μg/ml and by 64% in cultures containing 6.0 μg/ml of P4. Doses of E2 of up to 6.0 μg/ml did not significantly reduce nitrite accumulation when compared with vehicle-treated RAW 264.7 macrophages. Pretreatment of macrophages with hormones for up to 24 h had no additional effect (data not shown). As a result of these experiments the concentration of 3.0 μg/ml of E2 and P4 was used in all subsequent experiments.

To determine the duration of effects of E2 and/or P4 on NO production in activated macrophages, RAW 264.7 cells were stimulated with IFN-γ/LPS and exposed to E2 and/or P4 for 24, 36, and 49 h. Culture supernatants were then assayed for the accumulation of nitrite. The results of this time course study (**Fig. 2**) indicated that maximum inhibition by P4 alone or in combination with E2 was achieved by 24 h. P4 reduced nitrite accumulation by 45% after 24 h and by 38% after 48 h of exposure.

A study comparing the effects of E2 and/or P4 exposure on the production of NO by various macrophage cell types was conducted using RAW 246.7 and J774 macrophage cell lines and bone marrow culture-derived macrophages. The results (**Fig. 3**) show that, following a 24-h exposure to IFN-γ/LPS with or without E2 and/or P4, all three sources of macrophages exhibited reduced production of nitrite in response to P4 or E2 plus P4, but not with E2 alone. However, the degree to which nitrite accumulation was suppressed due to P4 co-treatment varied somewhat, with accumulation ratios of the vehicle-treated-to-P4-treated macrophages being 43% in RAW 264.7 cells, 66% in J774 cells, and 44% in bone marrow culture-derived macrophages.

Effects of hormone analogues and other compounds on NO production by macrophages stimulated with IFN-γ/LPS

To evaluate the specificity of effects of female hormones on NO production by activated macrophages, RAW 264.7 cells were stimulated for 24 h with IFN-γ/LPS in the presence of an analogue of estrogen (AE), estradiol-17α, or an analogue of progesterone (AP), 4-pregnen-20α-ol-3-one. The results of this study (**Fig. 4**) showed no significant reduction of nitrite concentrations when macrophages were treated with AE. AP caused a modest but significant reduction in nitrite accumulation (11%) when compared with the nitrite concentrations produced by vehicle-treated cells.

Additionally, 25-hydroxycholesterol was used as a control to investigate the effects of a biologically non-active steroid on NO production by activated macrophages. IFN-γ/LPS-activated RAW 264.7 cells concurrently exposed to 25-hydroxycholesterol for 24 h (Fig. 4) did not show any

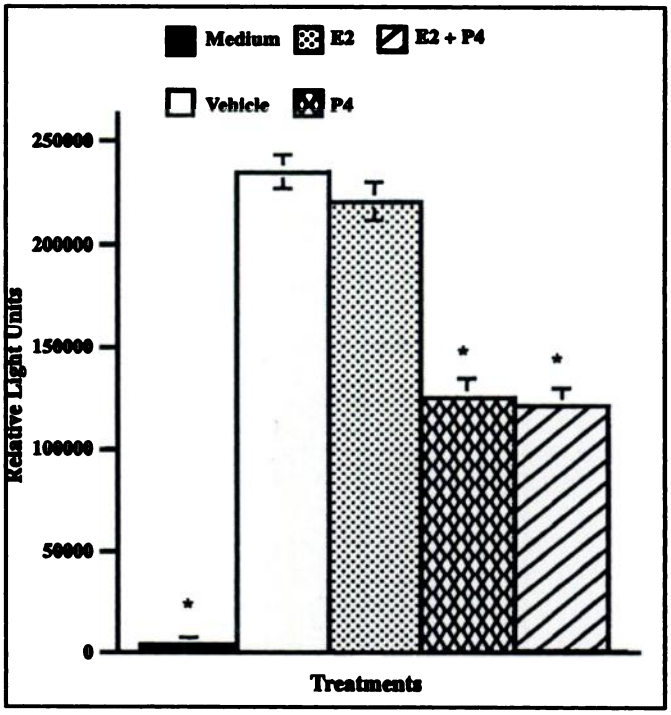

Fig. 5. iNOS promoter activity study on the effects of E2 and/or P4 on activated macrophages transiently transfected with an iNOS promoter-luciferase reporter construct. RAW 264.7 macrophages transfected with an iNOS regulatory region-luciferase construct were cultured in medium alone or in medium containing IFN-γ (10 U/ml) and LPS (100 ng/ml) as well as DMSO (vehicle) or 3.0 μg/ml E2 and/or P4. After 8 h, iNOS promoter activity was determined by assay of lysates for luciferase activity. Results shown (mean ± SE of triplicates) are representative of one of three separate experiments. (*$P < 0.01$, test compared with vehicle control).

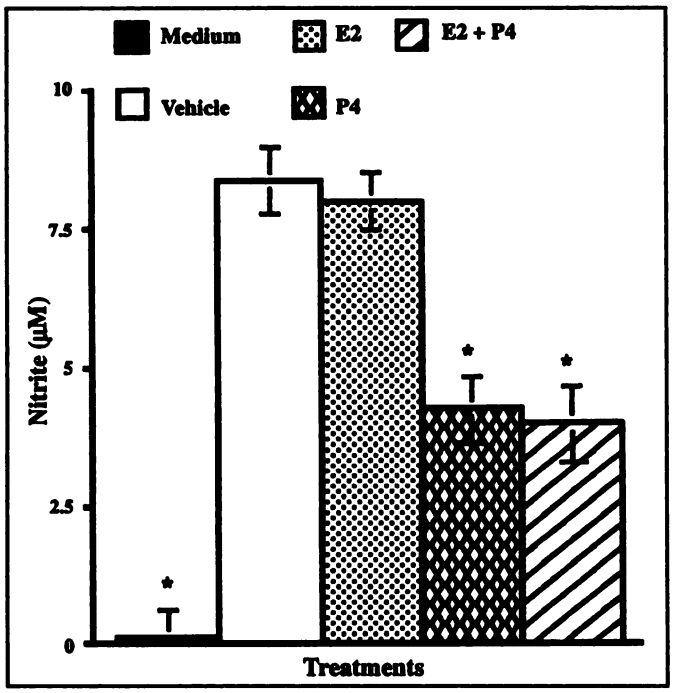

Fig. 6. NO production by activated, hormone-treated macrophages transiently transfected with an iNOS promoter-luciferase reporter construct. RAW 264.7 macrophages transfected with an iNOS regulatory region-luciferase construct were cultured in medium alone or in medium containing IFN-γ (10 U/ml) and LPS (100 ng/ml) as well as DMSO (vehicle) or 3.0 μg/ml E2 and/or P4 for 24 h. Culture supernatants were assayed for accumulated nitrite concentration using the Griess reaction. The results shown (mean ± SE of triplicates) are representative of one of three separate experiments. (*$P < 0.01$, test compared with vehicle control).

alteration in their level of nitrite accumulation when compared with vehicle control values, indicating that steroid toxicity did not contribute to the P4-mediated reduction of nitrite accumulation.

IFN-γ/LPS-stimulated RAW 264.7 cells were also exposed to the E2 antagonist tamoxifen, with or without E2 and/or P4, to examine the effect of blocking E2 receptor binding on P4-mediated inhibition of nitrite accumulation. P4 enhances prostaglandin E2 (PGE2) production by macrophages [22] and PGE2 is a known inhibitor of macrophage NO synthesis [23]. The prostaglandin inhibitor indomethacin was also investigated in culture treatments similar to those described above to determine if the inhibition of nitrite accumulation was due to a P4-mediated enhancement of PGE2 production. The results of these studies indicated that tamoxifen and indomethacin do not affect the P4-mediated inhibition of nitrite accumulation (data not shown).

P4 decreases iNOS gene promoter activity in IFNγ/LPS-stimulated macrophages

An examination of the effects of E2 and/or P4 on iNOS gene promoter activity in activated macrophages was conducted using RAW 264.7 cells transiently transfected with an iNOS gene promoter/luciferase reporter-gene construct. Transfected RAW 264.7 cells were stimulated with IFN-γ/LPS in the presence of E2 and/or P4 for 8 h. Luciferase activity was assayed as an indication of iNOS promoter activity.

The results of this study (**Fig. 5**) show that IFN-γ/LPS-stimulated RAW 264.7 cells exposed to P4 displayed a significant decrease (44% reduction) in the level of luciferase activity when compared with activated vehicle controls. The results obtained when cells were treated with E2 plus P4 were the same. However, the luciferase activity of the IFN-γ/LPS-stimulated RAW 264.7 cells was not modulated by E2 alone.

In addition, parallel cultures of transfected RAW 264.7 were stimulated with IFN-γ/LPS with E2 and/or P4 as described above for 24 h and culture supernatants were assayed for nitrite accumulation. As shown in **Figure 6**, P4 reduced the accumulation of nitrite by 42% in parallel with the reduction of iNOS promoter activity.

To investigate the potential toxic effects of P4 on activated macrophages, cell viability was determined in parallel cultures of each of the various treatments described above, using the MTT colorimetric assay. The results in **Figure 7** show that the viability of transfected RAW 264.7 cells was not affected by exposure to hormones. The viability of treated and untreated macrophage cultures was investigated in all experiments throughout the present study with results similar to those described above (data not shown).

In addition to the above transfections we also transfected RAW 264.7 cells with the pGL2-control construct, which

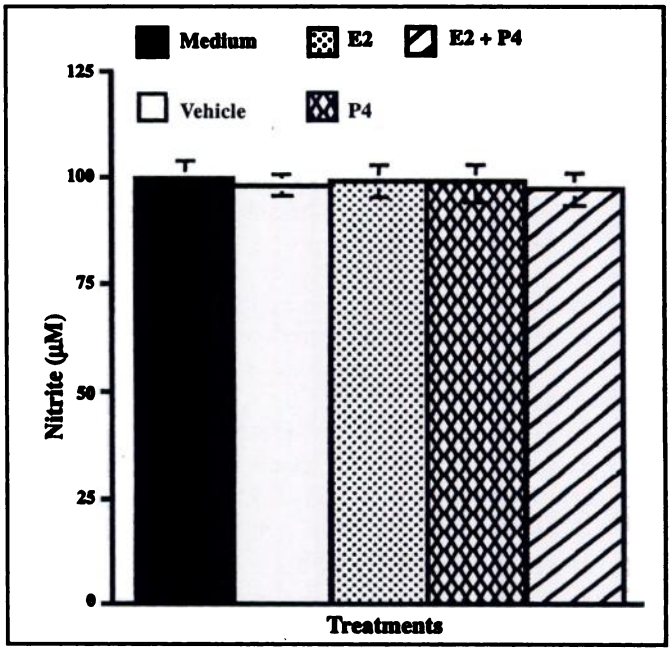

Fig. 7. Viability of activated, hormone-treated macrophages transiently transfected with an iNOS promoter-luciferase reporter construct. RAW 264.7 macrophages transfected with an iNOS regulatory region-luciferase construct were cultured in medium alone or in medium containing IFN-γ (10 U/ml) and LPS (100 ng/ml) as well as DMSO (vehicle) or 3.0 μg/ml E2 and/or P4. After 24 h, the viability of medium-, vehicle-, and hormone-treated macrophages was determined using a mitochondrial enzyme (MTT) assay. The results shown (mean ± SE of triplicates) are representative of one of three separate experiments. (*$P < 0.01$, test compared with vehicle control).

has the luciferase gene placed under control of the SV40 promoter/enhancer. When these transfected cultures were treated with IFNγ/LPS in the presence or absence of E2 and/or P4, no difference in luciferase activity was detected between cultures treated with hormones compared with those that were not (data not shown). This result supports our hypothesis that P4 has a specific effect on the iNOS promoter at the level of transcription initiation rather than a nonspecific effect on luciferase-mRNA stability or translational activity.

P4 decreases iNOS mRNA expression in IFNγ/LPS-stimulated macrophages

To determine the effects of P4 on the expression of iNOS mRNA in IFN-γ/LPS-activated macrophages, RAW 264.7 cells were stimulated with IFN-γ/LPS with or without exposure to P4 for 0 to 24 h and total cellular RNA was isolated for Northern blot analysis. As shown in **Figure 8**, iNOS mRNA was detectable 3 h after IFN-γ/LPS stimulation and peaked at about 6 h with a lower but continued expression thereafter. Steady-state levels of iNOS mRNA in P4-treated RAW 264.7 cells were consistently lower than those in the untreated cells. The presence of E2 in cultures of IFN-γ/LPS-stimulated RAW 264.7 cells did not affect iNOS mRNA expression (data not shown).

Murine macrophages contain estrogen receptor but not progesterone receptor mRNA

In an investigation of the potential response of mouse macrophages to E2 and P4, RT-PCR was used to identify the expression of ER and PR mRNA by these cells. Electrophoresis of the PCR products on agarose gels (**Fig. 9**) showed that RAW 264.7, J774 and bone marrow culture-derived macrophages contained the expected 478-bp ER product. However, the 327-bp PR product was not observed in any of the macrophages. The 478-bp ER product and 327-bp PR product were both obtained when mRNA isolated from murine ovarian tissue, used as a positive control, was amplified. Because expression of the ER gene is believed to be required for the expression of the PR

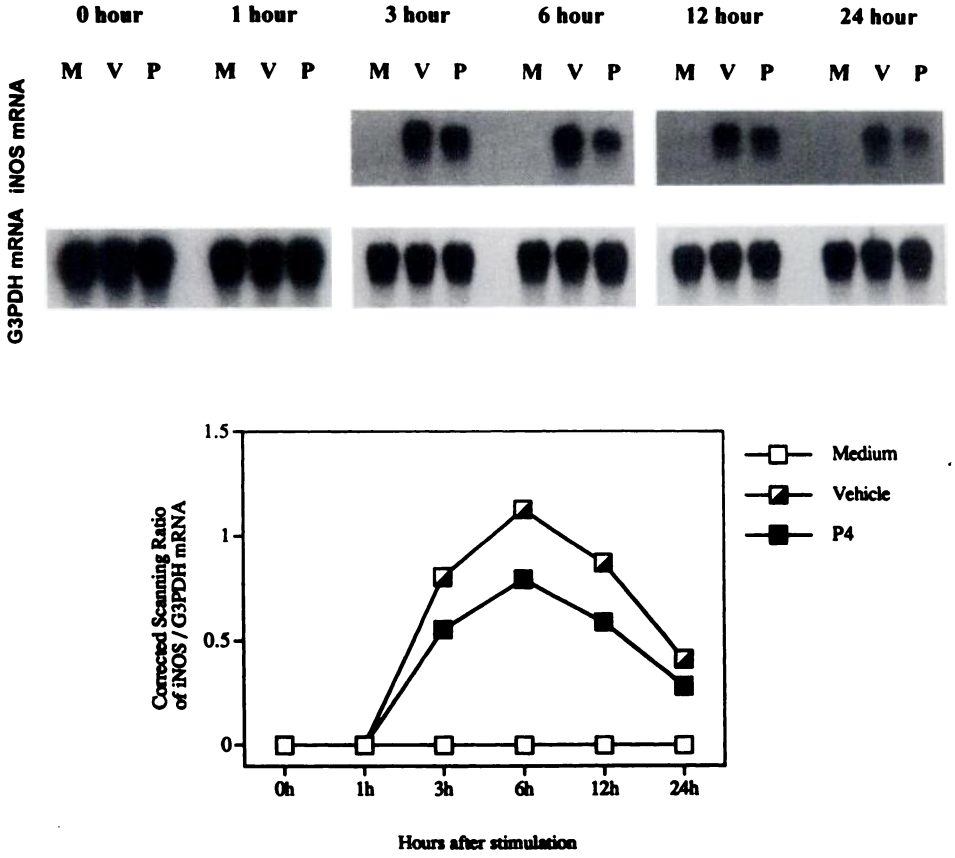

Fig. 8. Time course study of the effects of progesterone on iNOS mRNA expression by activated macrophages by use of Northern blot analysis. Total RNA was isolated from RAW 264.7 macrophages cultured from 0 to 24 hours in medium alone or in medium containing IFN-γ (10 U/ml) and LPS (100 ng/ml) as well as DMSO (vehicle) or 3.0 μg/ml P4. Equal amounts (10 μg) of isolated RNA were electrophoretically separated, transferred to nylon membranes, and hybridized with a ^{32}P-labeled mouse iNOS probe. After autoradiography and densitometric analysis the nylon membranes were washed and reprobed with a [^{32}P]G3PDH cDNA and processed for Northern blot analysis. After densitometric analysis the autoradiographic iNOS mRNA signals were corrected relative to the G3PDH mRNA levels (see MATERIALS AND METHODS). The results shown are representative of one of three separate experiments all of which had essentially the same outcome.

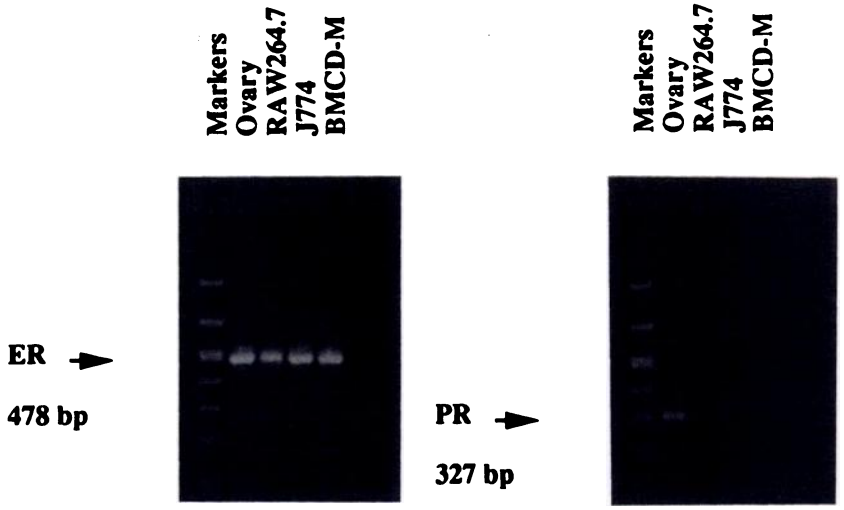

Fig. 9. Identification of ER and PR mRNA in RAW 264.7, J774 and bone marrow culture-derived (BMCD-M) macrophages by RT-PCR. Agarose gels showing the PCR products amplified with ER and PR primers. One microgram total cellular RNA was included in the RT-PCR reaction for each sample. Ovary RNA was used as positive control. The presence of a 478-bp band indicates the presence of ER mRNA and the PR mRNA is indicated by a 327-bp band. The results shown are representative of one of three separate experiments, all of which had essentially the same outcome.

gene, macrophages were preincubated with E2 or P4 for 0–24 h and total RNA was analyzed for the presence of ER or PR receptor. Again, the ER product but not PR product was observed in the RAW 264.7, J774, and bone marrow-culture-derived macrophages (data not shown).

Dexamethasone and P4 independently inhibit NO production by macrophages stimulated with IFN-γ/LPS

To determine whether the mechanism by which P4 inhibits NO production by activated macrophages might involve binding of P4 to glucocorticoid receptors, the steroid antagonists RU486 and the glucocorticoid dexamethasone were tested. RAW 264.7 cells were pretreated with RU486 for 2 h. Afterwards, the RAW 264.7 cells were stimulated with IFN-γ/LPS in the presence of E2 and/or P4 with or without RU486 and/or dexamethasone. **Figure 10** shows that the addition of dexamethasone to P4 and E2 plus P4-treated cells had no further inhibitory effect. RU486 (0.75 μg/ml) completely reversed the 25% decrease in nitrite levels mediated by 0.30 μg/ml dexamethasone but did not alter reductions by P4 (3.0 mg/ml) or E2 (3.0 mg/ml) plus P4 (3.0 mg/ml) of 44%.

DISCUSSION

Regulation of the immune system by sex hormones as well as the effects of gender on humoral and cell-mediated immune responses have been studied in detail and reviewed (refs. 24 and 25). Female sex hormones, estrogen and progesterone, modulate a variety of effector functions in macrophages [10–13]. However, a definitive investigation of the effects of sex hormones on the cellular and molecular mechanisms involved in the induction of iNOS and NO production in activated macrophages has yet to be done. Here, we show clearly that P4 strongly inhibits iNOS gene activity and NO synthesis in IFN-γ/LPS-stimulated mouse

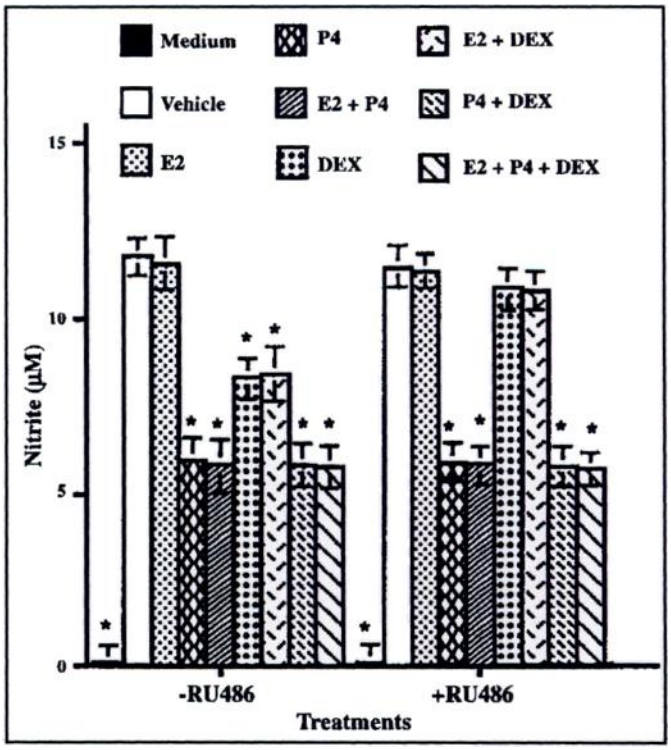

Fig. 10. Comparative study on the effects of dexamethasone and the steroid antagonist RU486 on activated macrophages exposed to E2 and/or P4. RAW 264.7 cells were pretreated for 2 h with or without 0.75 μg/ml of RU486 then cultured in medium alone or in medium containing IFN-γ (10 U/ml) and LPS (100 ng/ml) as well as DMSO (vehicle) or 3.0 μg/ml E2 and/or P4 with or without 0.3 μg/ml dexamethasone (DEX) with or without 0.75 μg/ml of RU486 for 24 h. Culture supernatants were assayed for accumulated nitrite concentration using the Griess reaction. The results shown (mean ± SE of triplicates) are representative of one of three separate experiments. (*$P < 0.01$, test compared with vehicle control).

macrophages, whereas E2 does not modulate NO production or have any effect on P4-mediated inhibition.

Levels of circulating E2 and P4 in female mice during the estrus cycle and pregnancy have been reported to reach 60.0 pg/ml and 39.0 ng/ml, respectively [26]. In human placentas P4 levels can vary from 0.5 μg/ml to 5.1 μg/ml [27, 28]. In the present study concentrations greater than 0.1 μg/ml of P4 inhibited iNOS gene activity and NO production in activated mouse macrophages. Reduced production of NO was not due to a toxic effect of added steroid; macrophage viability was unaffected.

Production of effector molecules by activated macrophages can be either inhibited or stimulated by E2 and P4. Synthesis of mRNA for human monocyte chemoattractant protein-1 and its mouse homologue, JE, by LPS-stimulated macrophages is not affected by P4 but is greatly reduced by E2. This effect is reversed by the E2 antagonist tamoxifen [29]. Our results show that E2, the E2 analogue estradiol-17α, and the E2 antagonist tamoxifen, do not alter iNOS activity or NO production in IFN-γ/LPS-stimulated macrophages. A limited study published earlier failed to identify any P4 effects [30]; however, we observed a P4-mediated time- and dose-dependent suppression of NO production. Differences between the results of the previous study and ours could be due to differences in the source or the stage of differentiation of the macrophages, to culture or stimulation conditions, or to steroid concentrations. The idea that macrophage production of effector molecules is modulated by P4 is supported by the results of other experiments in which macrophage production of IL-1β and tumor necrosis factor-α could be either increased or decreased in a dose-dependent manner by P4 [10, 11, 31].

Northern blot analysis and transient transfection using an iNOS gene promoter/luciferase reporter-gene construct were both used to investigate the mechanistic basis of P4-mediated inhibition. The profound reduction in iNOS promoter activity of transfected RAW 264.7 cells and decrease in the steady-state levels of iNOS mRNA strongly support the postulate that P4 inhibits iNOS gene transcription. Differences in the decreased levels of iNOS mRNA and promoter activity might be attributable to long-term changes in expression of mRNA in contrast to the immediate change in promoter activity. Other substances inhibit the induction of iNOS and NO production by activated macrophages, including transforming growth factor-β (TGF-β1, -2, -3) and interleukins IL-4, -8, and -10 [32–35]. TGF-β destabilizes and decreases translation of iNOS mRNA and increases degradation of iNOS protein [36], whereas IL-4 appears to down-regulate iNOS enzymatic activity and decrease iNOS transcription without affecting iNOS mRNA stability [37].

PGE2 is one of many biological factors produced by activated macrophages and is one of several inhibitors of NO production [23]. P4 enhances macrophage PGE2 production [22]. We investigated the possible effects of PGE2 on P4-mediated inhibition of NO production using the prostaglandin inhibitor indomethacin. Our data suggest that P4 does not inhibit NO production via prostaglandin-related pathways.

The classic steroid receptor-mediated modulation of immune cells by sex hormones implies the presence of specific sex hormone receptors. Consistent with previous studies [38, 39], we here show that the macrophage cell lines, RAW 264.7 and J774, and bone marrow culture-derived macrophages express mRNA for the ER but we were unable to identify PR. Although a computer search (Silicon Graphics IRIS-4D transcription factor database, Madison, WI) indicated that the promoter region of iNOS gene does not contain the classical progesterone and estrogen response elements used as steroid hormone receptor binding sequences, the absence of classical hormone binding sites does not preclude the possibility of hormonal modulation of cytokine genes [40]. P4 modulation of macrophage iNOS effector functions might be due to a nonspecific membrane-mediated mechanism as suggested in studies on human lymphocytes [41]. Alternatively, the methods used in this study may not have been sufficiently sensitive or extensive to detect low or transient P4 receptor expression.

Another possibility is that P4 inhibited iNOS through binding to the glucocorticoid response element. Glucocorticoids, dexamethasone, hydrocortisone, and cortisol act through this element to inhibit the induction of iNOS and decrease NO production in activated macrophages [30]. Under some conditions, P4 can bind to these same receptors [42, 43]. To test this possibility, we performed experiments using dexamethasone as well as the steroid analogue RU486, which has a high affinity for P4 and glucocorticoid receptors and has been reported to reverse the immunosuppressive effects of glucocorticoids [44]. The results indicate that P4 inhibition of NO production is likely to have been mediated through binding to the glucocorticoid receptor. If separate pathways existed for P4 and glucocorticoids, exposure of activated macrophages to a combination of P4 and dexamethasone would result in an additive inhibitory effect but we did not observe this. Our ability to inhibit the dexamethasone but not the P4 effect with RU486 could have been due to the use of higher concentrations of P4 (3.0 μg/ml) than of dexamethasone (0.3 μg/ml). An increase in the concentration of RU486 above 0.75 μg/ml decreased cell viability (data not shown). Alternatively, there may be subtle differences in the binding of P4 and dexamethasone to the glucocorticoid receptors.

In summary, this work shows that P4 inhibits a major effector system in activated macrophages, reducing induction of the iNOS promoter gene, iNOS mRNA expression, and synthesis of NO. Tumors of the female reproductive system are the most common forms of cancer in women and sex hormones have been implicated in sexual dimorphic characteristics of certain autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis, which occur predominantly in females [45–47]. Because iNOS is a potent cytotoxic effector molecule against tumors and infections, our results have important implications regarding gender-based differences within females and males related to macrophage-mediated host defense.

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