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J Immunol 1998; 160:5098-5104; ; http://www.jimmunol.org/content/160/10/5098

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Regulation of TNF-α Production in Activated Mouse Macrophages by Progesterone

Lance Miller* and Joan S. Hunt2*†

The purpose of this study was to investigate the relationships between macrophage production of TNF-α and female hormones. Northern blot hybridization experiments showed that the female sex steroid hormone, progesterone, decreases steady state levels of TNF-α mRNA in LPS-activated mouse macrophages (RAW 264.7 and ANA-1 cells) in vitro. The production of intracellular and secreted TNF-α protein, as determined by ELISA, was decreased in both progesterone- and dexamethasone-treated, LPS-stimulated macrophages. Estrogen had no effect on expression of the TNF-α gene in mouse macrophages and did not alter progesterone-mediated suppression. Additional experiments conducted to investigate the mechanism of action of progesterone showed that this hormone, like dexamethasone, elevates steady state mRNA levels of IκBα and increases the levels of IκBα protein that are translocated from the cytoplasm to the nucleus. Thus, progesterone is a potent inhibitor of steady state levels TNF-α mRNA and TNF-α protein production in activated macrophages and may achieve this result through effects on an inhibitor of NF-κB.


Tumors of the breast and reproductive system rank second after tumors of the lung as the most common forms of cancer in women in the United States (1–3). Worldwide, cervical carcinoma is the leading cause of death by cancer among pregnant women (4). Macrophages are present in large numbers within the microenvironments of reproductive tissues and, when activated, could play an important role in host defense against tumors. The complex process of macrophage activation involves a series of function-enhancing steps induced by environmental signals that include various inflammatory cytokines and bacterial endotoxins such as LPS (5). Following activation, macrophages are capable of infiltrating tumors and releasing potent effector molecules such as TNF-α, a powerful cytokine that can initiate both local and systemic antitumor activities (6). TNF-α also has the ability to regulate tumor cell proliferation; in vitro studies have demonstrated both stimulation and inhibition of the growth of breast and ovarian cancer cells (7–9). Yet there is increasing evidence for the reciprocal pathway; hormones produced in and targeted to the female reproductive tract are major modulators of TNF-α production in LPS-activated macrophages (10).

The transcription factor NF-κB is a critical component of several signal transduction pathways, including those leading to TNF-α synthesis (11). Activation of NF-κB involves dissociation from inhibitory proteins, one of which is IκBα. Once dissociated, free NF-κB is transported into the nucleus, where it can activate transcription of certain genes, particularly those involved in immune and inflammatory responses (12). Anti-inflammatory agents such as corticosteroids induce IκBα gene and protein synthesis (13), whereas LPS, PMA, IL-1, and TNF-α induce IκBα to dissociate from NF-κB (14–16). The newly synthesized IκBα protein can either associate with free NF-κB in the cytoplasm and inhibit its translocation to the nucleus or translocate to the nucleus and sequester free NF-κB and promote the dissociation of DNA-bound NF-κB, resulting in a decrease in cytokine production (11, 17).

The purpose of this study was to investigate the relationships between macrophage production of TNF-α and female hormones so as to better understand immunity in women of child-bearing age. Our experiments show that the female sex steroid hormone, progesterone (P4), down-regulates the production of TNF-α mRNA and intracellular as well as released TNF-α protein. Like the effects of glucocorticoids, P4 increased levels of IκBα mRNA and increased the level of IκBα protein translocated from the cytoplasm to the nucleus in LPS-activated mouse macrophages, suggesting that the two steroid hormones may implement the same pathway leading to inhibition of TNF-α synthesis.

Materials and Methods

Cells and culture conditions

The mouse macrophage-like cell line, RAW 264.7, which was purchased from American Type Culture Collection (Rockville, MD) and was used in most experiments, and the mouse macrophage cell line, ANA-1, a gift from Dr. E. J. Kovacs (Loyola University, Chicago, IL), were cultured at 37°C in 5% CO2 in growth medium composed of phenol red-free MEM (Sigma, St. Louis, MO) containing 10% (v/v) FCS (HyClone, Logan, UT), 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma). For hormone treatment experiments, the FCS content was reduced to 1%.

TNF-α protein quantitation by ELISA

RAW 264.7 cells grown in 100-mm tissue culture dishes were used for dose-response and time-course experiments, in which TNF-α in the supernatant culture medium was measured using the Factor-Test-X mouse TNF-α ELISA kit (Genzyme, Cambridge, MA) according to the manufacturer’s procedure. Recombinant mouse TNF-α (Genzyme) was used to establish a standard curve. The cells (1 × 107 cells/dish) were activated with 500 ng/ml of LPS (the lipid A-rich fraction II of LPS phenol extracted from Escherichia coli 0111:B4, a gift from David Morrison, Department of Microbiology, Immunology and Molecular Genetics) as described previously (18). This concentration of LPS was used throughout the study. In two types of experiments, RAW 264.7 cell cultures were incubated either...
with a single bolus of hormone(s) (Sigma) or with a hormone-containing time-release pellet(s) (Innovative Research of America, Sarasota, FL). In the bolus dose-response experiments, RAW 264.7 cells were exposed to 0, 0.003, 0.03, 0.3, or 3 μg/ml of 17β-estradiol (E2), 1, P4, dexamethasone (DEX), E2 plus P4, E2 plus DEX, or P4 plus DEX with or without LPS for 40 min. In the bolus time-course experiments, RAW 264.7 cells were exposed to 0 or 3 μg/ml of E2, P4, DEX, E2 plus P4, E2 plus DEX, or P4 plus DEX for 0–6 h with or without LPS. Control cultures consisted of cells cultured in medium alone or in medium with LPS containing a matching concentration (0.01%) of the vehicle, DMSO, that was used to solubilize the hormones. In the continuous release pellet experiments, dose responses were determined by incubating the RAW 264.7 cells with time-release pellets containing 30, 45, and 60 μg of hormone for 2 h, 6 h, and 24 h, respectively. The membranes were incubated with 32P-labeled cDNA probes according to the manufacturer's instructions (Micron Separations, Westboro, MA). The murine TNF-α cDNA probe was a gift from Dr. C. Martens, Department of Internal Medicine, University of Alabama at Birmingham (Birmingham, AL). The TNF-α cDNA probe was a gift from Dr. I. M. Verma, The Salk Institute (San Diego, CA), and was a gift from Dr. C. Martens, Department of Internal Medicine, University of Alabama at Birmingham (Birmingham, AL). The TNF-α cDNA probe was a gift from Dr. I. M. Verma, The Salk Institute (San Diego, CA), and was a gift from Dr. C. Martens, Department of Internal Medicine, University of Alabama at Birmingham (Birmingham, AL).

Cell lysates and culture medium from experiments were collected and stored in polyethoxyl ethanol (Nonidet P-40) as described previously (19). Cell ly- lizin buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, and cells were harvested in Tris-buffered saline (TBS), pelleted, and lysed collected after 30, 45, and 60 min and 6 h. Culture medium was collected, step, the membranes were incubated with32 P-labeled cDNA probes speci- cifying according to the manufacturer’s instructions (Micron Separations, Westbor-ough, MA). The murine TNF-α was a gift from Dr. I. M. Verma, The Salk Institute (San Diego, CA), and was a gift from Dr. C. Martens, Department of Internal Medicine, University of Alabama at Birmingham (Birmingham, AL). The TNF-α cDNA probe was a gift from Dr. I. M. Verma, The Salk Institute (San Diego, CA), and was a gift from Dr. C. Martens, Department of Internal Medicine, University of Alabama at Birmingham (Birmingham, AL). The TNF-α cDNA probe was a gift from Dr. I. M. Verma, The Salk Institute (San Diego, CA), and was a gift from Dr. C. Martens, Department of Internal Medicine, University of Alabama at Birmingham (Birmingham, AL).

Hormone quantitation by RIA assay

Isolation of cytoplasmic and nuclear extracts and Western blotting

Total RNA was isolated from RAW 264.7 and ANA-1 cells exposed to the treatments described above using TRIZol reagent according to the manu- facturer’s instructions (Life Technologies, 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 ac- cording to the manufacturer’s instructions (Micron Separations, Westbor-ough, MA). Hybridization, autoradiography, and posthybridization proce- dures were performed using Quik-Hyb hybridization solution as described by the manufacturer (Stratagene, La Jolla, CA). During the hybridization step, the membranes were incubated with14C-labeled cDNA probes spe- cific for murine TNF-α, IxBo, or glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA, each labeled by the random primer DNA labeling method (Stratagene) using (α-32P)dCTP (ICN Biomedicals, Costa Mesa, CA). The murine TNF-α cDNA probe was a gift from Dr. C. Martens, Affymark Research Institute (Palo Alto, CA), the murine IxBo cDNA probe was a gift from Dr. J. M. Verma, The Salk Institute (San Diego, CA), and the murine G3PDH cDNA probe was a gift from Dr. R. W. Allen, American Red Cross Blood Services (St. Louis, MO) (20). The membranes probed with the TNF-α cDNA were autoradiographed on Kodak XAR-5 film (Eastman Kodak, Rochester, NY) at −70°C with intensifying screens and later washed and reprobed, first with the IxBo and then with the G3PDH cDNA control probe. The autoradiographic TNF-α and IxBo mRNA signals were quantitated by scanning densitometry and corrected relative to the G3PDH mRNA signal levels.

Isolation of cytoplasmic and nuclear extracts and Western blotting

Cytoplasmic and nuclear extracts were isolated according to methods de- scribed previously (19). Briefly, RAW 264.7 cells (1 x 107 cells/dish) were exposed to 3 μg/ml of P4 or DEX with or without LPS (500 ng/ml) for 5 or 15 min. Cells were harvested in TBS, pelleted, and lysed in a buffer contain- ing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, and 0.6% Nonidet P-40. Cells were centrifuged, and the nuclear pellet was isolated from the cytoplasmic extract supernatant and resuspended in buffer containing 20 mM HEPES (pH 7.9), 0.01 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF. The nuclear pellet mixture was incubated at 4°C for 15 min, and the nuclear extract was collected from the supernatant following a 5-min cen- trifugation. Cytoplasmic and nuclear extracts (25 μg/lane) were electro- phoresed on 4 to 15% gradient SDS-PAGE gels and transferred to nitro- cellulose (Schleicher and Schuell, Keene, NH). After electrophoretic protein transfer, nonspecific Ab binding sites on the nitrocellulose were blocked by incubation with 3% nonfat milk in Tris-buffered saline and 0.5% Tween-20 blocking buffer. The membranes were incubated with IxBo or TNF-α serum diluted in blocking buffer (1:2000) and then with anti-NF-κB (1/3000), and extracts were detected by chemiluminescence according to the manufacturer’s procedure (Pierce, Rockford, IL). The IxBo and NF-κB antisera were gifts from N. Rice, Frederick Cancer Research and Develop- ment Center (Frederick, MD). The m.w. were determined with prestained standards (Bio-Rad, Richmond, CA).

Hormone quantitation by RIA assay

The concentrations of E2 and P4 present in the culture medium were mon- itored using Coat-A-Count RIA kits (Diagnostic Products, Los Angeles, CA) according to the manufacturer’s procedure.

Isolation of RNA and Northern blotting

Results

Effects of continuous exposure to E2 and P4 on TNF-α mRNA levels in LPS-stimulated macrophages

The ability of female steroid hormones to modulate synthesis of TNF-α was first tested by determining the effects of E2 and/or P4 on the expression of TNF-α mRNA in LPS-activated macrophages. RAW 264.7 cells were pretreated with E2 and/or P4 using 100 μg of E2 and/or 100 μg of P4 time-release pellet(s) for 48 h followed by a 6- or 24-h exposure to LPS in the continued pres- ence of the pellets. As shown in Figure 1, A and B, steady state levels of TNF-α mRNA in LPS-activated RAW 264.7 cells exposed to P4 or to E2 plus P4 were consistently lower than levels in LPS-activated RAW 264.7 cells that were cultured in medium alone. This decrease was observed after 6 h and after 24 h. Shorter exposures for 1 or 3 h also caused a decrease in the steady state levels of TNF-α mRNA in LPS-activated RAW 264.7 cells exposed to P4 or to E2 plus P4 (data not shown). E2 did not affect TNF-α mRNA steady state levels (see Fig. 1, A and B) or relieve the suppression induced by P4. To be certain that this phenomenon was not unique to the RAW 264.7 cells, we tested the ANA-1 cells using identical techniques, and the results were essentially the same as those obtained using the RAW 264.7 cells (data not shown).

Table I shows the concentrations of E2 and P4 present in the culture supernatants of RAW 264.7 cells exposed to pellets containing 100 μg of E2 or 100 μg of P4. After 48 h, the levels of E2 or P4 present in the culture supernatants averaged 2.6 μg/ml for E2 and 3.3 μg/ml for P4. The concentrations of E2 or P4 present in the culture supernatants after an additional 6 h averaged 2.9 μg/ml for E2 and 3.7 μg/ml for P4. Activation by LPS had no effect on the levels of hormones in the supernatant culture medium. The viability of treated and untreated macrophage cultures was investigated in all experiments in this study, and no changes were observed (data not shown).
Effects of P4 on TNF-α production by LPS-stimulated macrophages

Having learned that P4, but not estrogen, decreases steady state levels of TNF-α mRNA, experiments were performed to determine whether E2 and/or P4 modulated TNF-α protein production or release by LPS-activated macrophages. Cell culture media were assayed for TNF-α protein following exposure to activators and various hormones using a commercial ELISA. As shown in Figure 2, RAW 264.7 macrophages displayed a dose-dependent decrease in TNF-α production following a 48-h pre-exposure to pellets containing various concentrations of P4. After 6 h of subsequent exposure to LPS, the concentration of TNF-α in the culture media of activated RAW 246.7 macrophages was decreased by 68 and 81% in cells exposed to P4 from time-release pellets containing 100 and 1000 µg of P4, respectively, compared with that in macrophages exposed to LPS alone. By contrast, E2 from pellets containing up to 1000 µg of the hormone did not affect RAW 264.7 cell production of TNF-α compared with that by the cell cultures stimulated with LPS alone (Fig. 2).

To determine the duration of effects of E2 and/or P4 on TNF-α production by activated macrophages, RAW 264.7 cells were pre-treated for 48 h with 100-µg E2 and/or 100-µg P4 pellet(s) and were stimulated for 6, 12, or 24 h with LPS. Culture media were assayed for the presence of TNF-α protein by ELISA. The results of this time-course study (Fig. 3) showed that E2 plus P4 or P4 alone reduced TNF-α production by 73% after 6 h and by 67% after 24 h of exposure.

Regulation of IkBa mRNA expression in unstimulated macrophages by P4

Gluocorticoids are known to inhibit TNF-α synthesis by stimulating the production of IkBa (13). Therefore, additional experiments were performed to learn whether P4 might act in a similar manner. To determine the effects of E2, P4, and the glucocorticoid, DEX, on steady state levels of IkBa mRNA in unstimulated macrophages, RAW 264.7 cells were exposed for 25, 40, or 55 min to a single bolus of 3 µg/ml of E2, P4, DEX, or combinations of the steroid hormones. Figure 4 and Table II show that unstimulated RAW 264.7 cells exposed to P4 and/or DEX displayed higher steady state levels of IkBa mRNA and that the increase over constitutive expression was maximal at 40 min. The presence of E2 did not affect IkBa mRNA expression and did not change the ability of P4 and DEX to increase steady state levels (data not shown).

Regulation of IkBa mRNA expression in LPS-stimulated macrophages by P4

RAW 264.7 cells were exposed to various concentrations of P4 and/or DEX (bolus form) in the presence of LPS for 40 min. As shown in Figure 5A, the expression of IkBa mRNA in LPS-activated RAW 264.7 cells given 3 µg/ml of P4 and/or DEX displayed

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Table I. Estrogen and progesterone concentrations in medium collected over time from RAW 264.7 macrophages exposed to either 100 µg estrogen or 100 µg progesterone time-release pellets with or without LPS

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Estrogen (ng/ml)</th>
<th>Progesterone (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- LPS + LPS</td>
<td>- LPS + LPS</td>
</tr>
<tr>
<td>1</td>
<td>55 ± 31</td>
<td>62 ± 26</td>
</tr>
<tr>
<td>3</td>
<td>172 ± 50</td>
<td>163 ± 48</td>
</tr>
<tr>
<td>6</td>
<td>315 ± 72</td>
<td>323 ± 79</td>
</tr>
<tr>
<td>12</td>
<td>679 ± 118</td>
<td>671 ± 150</td>
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<tr>
<td>24</td>
<td>1398 ± 184</td>
<td>1409 ± 175</td>
</tr>
<tr>
<td>48</td>
<td>2602 ± 301</td>
<td>2637 ± 368</td>
</tr>
<tr>
<td>54</td>
<td>2973 ± 416</td>
<td>3031 ± 462</td>
</tr>
</tbody>
</table>

* The concentrations of E2 or P4 present in the culture medium collected after 1, 3, 6, 12, 24, 48, and 54 h from RAW 264.7 macrophage cell cultures exposed to either 100 µg E2 or 100 µg P4 time-release pellets with or without LPS. Culture media were assayed for E2 or P4 using a RIA assay. The results shown (mean ± SE of triplicates) represent one of two separate experiments that had essentially the same outcome.
FIGURE 2. Dose-response study of the effects of E2 and/or P4 on TNF-α production by LPS-activated macrophages. RAW 264.7 cells were cultured in medium alone or in medium containing 0.001, 100, or 1000 µg of E2; 0.001, 100, or 1000 µg of P4; or a combination of 0.001, 100, or 1000 µg of E2 plus 0.001, 100, or 1000 µg of P4 time-released pellet(s) for 48 h followed by 6-h exposure to LPS or no treatment (control). Culture supernatants were assayed for TNF-α protein by ELISA. The results shown (mean ± SE of triplicate determinations) represent one of two separate experiments that had essentially the same results. * indicates p < 0.01 compared with LPS alone control.

FIGURE 3. Time-course study of the effects of E2 and/or P4 on TNF-α production by activated macrophages. RAW 264.7 cells were cultured in medium alone or in medium containing 100 µg of E2, 100 µg of P4, or 100 µg of E2 plus 100 µg of P4 time-release pellet(s) for 48 h followed by exposure for 6, 12, or 24 h to LPS or no treatment (control). Culture supernatants were assayed for TNF-α protein by ELISA. The results shown (mean ± SE of triplicate determinations) represent one of two separate experiments that had essentially the same results. * indicates p < 0.01 compared with LPS alone control.

Discussion

The female sex steroid hormones, estrogens and P4, exert both immunosuppressive and immunostimulatory effects on the complex process of macrophage activation, resulting in dramatic alterations in cell-mediated and cytokine effector functions (21). In this study we determined that P4 decreases steady state levels of TNF-α mRNA and inhibits TNF-α protein production while increasing the steady state levels of IκBα mRNA and IκBα protein translocation from the cytoplasm to the nucleus in LPS-stimulated macrophages. By contrast, E2 did not alter the steady state levels of TNF-α or IκBα mRNA or the production of TNF-α protein.
Analysis by Northern blot hybridization was used to investigate the effect of hormones on TNF-\(\alpha\) mRNA expression in LPS-activated macrophages. The dramatic reduction in steady state levels of TNF-\(\alpha\) mRNA achieved by P4 in these experiments, which included the testing of two different macrophage cell lines, supports the postulate that P4 inhibits TNF-\(\alpha\) gene transcription. Also, the P4-mediated suppression of cellular and released TNF-\(\alpha\) protein production observed in ELISA assays indicates that P4 is a potent modulator of post-transcriptional events, not just transcription.

Identifying the effects of P4 on macrophage TNF-\(\alpha\) mRNA required long term exposure to the hormone, which was accomplished using time-release pellets, while TNF-\(\alpha\) protein levels were decreased early after activation. Thus, mouse macrophages may not be sensitive to transient alterations in steroid concentrations but instead, be most profoundly influenced in situations such as pregnancy where P4 levels are continuously high. This contrasts sharply with our observation that short term exposure to pharmacologic levels of P4 inhibits iNOS gene activity and nitric oxide production by IFN-\(\gamma\) plus LPS-activated mouse macrophages (22). However, it is known that different activation pathways lead to the production of TNF-\(\alpha\) and nitric oxide by macrophages (23, 24).

We observed no effects of estrogen on macrophage production of TNF-\(\alpha\). This result differs from those of other experiments showing that E2 can either increase or decrease TNF-\(\alpha\) gene expression in a dose-dependent manner in human monocytes or rat peritoneal macrophages in vitro (25, 26). The results shown here indicate that high doses of E2 do not alter TNF-\(\alpha\) gene activity nor does E2 affect the P4-mediated inhibition of TNF-\(\alpha\) mRNA steady state levels. Differences in our results compared with those of other reports could be attributed to differences in species, culture techniques, or experimental design.

In general, P4 appears to have anti-inflammatory properties. Our observations of its effects on TNF-\(\alpha\) are consistent with reports from other investigators who have shown that pharmacologic

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**Table II. Northern blot hybridization study of the effects of P4 and/or DEX on I\(\kappa\)B\(\alpha\) mRNA expression by unstimulated RAW 264.7 macrophages exposed for 40 min to either medium alone, medium containing DMSO (vehicle), or medium containing 3 \(\mu\)g/ml of P4 and/or DEX**

<table>
<thead>
<tr>
<th>Expt</th>
<th>Medium</th>
<th>Vehicle</th>
<th>P4</th>
<th>DEX</th>
<th>P4/DEX</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.529</td>
<td>0.645</td>
<td>1.377</td>
<td>2.139</td>
<td>2.256</td>
</tr>
<tr>
<td>2</td>
<td>0.255</td>
<td>0.251</td>
<td>0.982</td>
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<td>1.103</td>
</tr>
<tr>
<td>3</td>
<td>0.266</td>
<td>0.211</td>
<td>0.550</td>
<td>0.649</td>
<td>0.702</td>
</tr>
<tr>
<td>Mean + SE</td>
<td>0.350 ± 0.09</td>
<td>0.369 ± 0.138</td>
<td>0.970 ± 0.239</td>
<td>1.287 ± 0.443</td>
<td>1.354 ± 0.466</td>
</tr>
</tbody>
</table>

*Total RNA was isolated from RAW 264.7 macrophages exposed for 40 min to either medium alone, medium containing DMSO (vehicle), or medium containing 3 \(\mu\)g/ml of P4 and/or DEX. Equal amounts (10 \(\mu\)g) of isolated total RNA were electrophoretically separated, transferred to nylon membranes, and hybridized with a \(^{32}\)P-labeled mouse I\(\kappa\)B\(\alpha\) probe. After autoradiography and densitometric analysis the nylon membranes were washed and reprobed with a \(^{32}\)P-G3PDH cDNA. After densitometric analysis the autoradiographic I\(\kappa\)B\(\alpha\) mRNA signals were corrected relative to the G3PDH mRNA signal levels (see Materials and Methods).
FIGURE 5. Northern blot hybridization study of the effects of P4 and/or DEX on IκBα mRNA expression (A) and TNF-α expression (B) by unstimulated and LPS-stimulated macrophages exposed to P4 and/or DEX. Total RNA was isolated from RAW 264.7 macrophages cultured for 40 min in medium alone or in medium containing DMSO (vehicle) as well as 3 µg/ml of P4 and/or DEX. Equal amounts (10 µg) of isolated total RNA were electrophoretically separated, transferred to nylon membranes, and hybridized with a 32P-labeled mouse IκBα probe. After autoradiography and densitometric analysis, the nylon membranes were washed, reprobed with 13P]G3PDH cDNA, and processed for Northern blot analysis. After densitometric analysis, the autoradiographic TNF-α mRNA signals were corrected relative to the G3PDH mRNA signal (see Materials and Methods). The results shown represent one of two separate experiments of each type that had essentially the same outcome.

Table III. TNF-α concentrations in cell lysate and culture medium collected over time from RAW 264.7 macrophages cultured in medium alone, medium containing DMSO (vehicle), or in medium containing 3 µg/ml of P4 or DEX with or without LPSa

<table>
<thead>
<tr>
<th>Time</th>
<th>Cell Lysate (TNF-α pg/ml)</th>
<th>Culture Medium (TNF-α pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Medium + LPS</td>
<td>P4 + LPS</td>
</tr>
<tr>
<td>30 min</td>
<td>38 ± 6</td>
<td>990 ± 12</td>
</tr>
<tr>
<td>45 min</td>
<td>41 ± 3</td>
<td>1447 ± 5</td>
</tr>
<tr>
<td>60 min</td>
<td>46 ± 8</td>
<td>2018 ± 9</td>
</tr>
<tr>
<td>1 h</td>
<td>79 ± 7</td>
<td>8026 ± 183</td>
</tr>
</tbody>
</table>

* The concentrations of TNF-α present in the cell lysates and culture medium collected after 30, 45, and 60 min and after 6 h from RAW 264.7 macrophage cell cultures exposed to either medium alone, medium containing DMSO (vehicle), or in medium containing 3 µg/ml of P4 or DEX with or without LPS. Cell lysates and culture medium were assayed for TNF-α using an ELISA assay. The results shown (mean ± SE of triplicates) represent one of three separate experiments which had essentially the same outcome.

$p < 0.05$, test compared with vehicle + LPS control.
In summary, the results of our study demonstrate that P4 is a potent inhibitor of macrophage TNF-α gene activity and TNF-α protein production. This P4-mediated suppressive effect on TNF-α production lends support to the concept that female sex steroid hormones such as P4 are capable of profoundly altering the outcome of an immune response by regulating macrophage activation and production of inflammatory cytokines in tissues proximal to their production of P4.

Acknowledgments

We thank Drs. Stephen W. Russell, William J. Murphy, Elizabeth J. Kovacs, Christine Martens, Tsuneo Suzuki, I. M. Verma, Nancy Rice, and Robert W. Allen for generously providing cell lines and reagents.

References

12. Chiao, P. J., S. Miyamoto, and I. M. Verma. 1994. Autoregulation of IκBα expression in the cytoplasm and nuclear extracts of unstimulated and LPS-stimulated macrophages exposed to P4 or DEX. Cytoplasmic or nuclear extracts (25 g/lane) were electrophoresed on 4 to 15% gradient SDS-polyacrylamide gels and transferred to nitrocellulose membranes. After electrophoretic protein transfer, nonspecific Ab binding sites on the polypeptide film were exposed to the nitrocellulose membranes, and protein extracts were detected by chemiluminescence. Nitrocellulose membranes were washed and incubated with secondary antibody to blocking buffer followed by a final wash. The x-ray film was exposed to the nitrocellulose membranes, and protein extracts were detected by chemiluminescence. Nitrocellulose membranes were washed and probed with NF-κB antiserum and processed for Western blot analysis. The m.w. were determined with prestained standards. The results shown represent one of two separate experiments of each type that had essentially the same outcome.

FIGURE 6. Western blot study of the effects of P4 or DEX on IκBa and NF-κB protein expression in the cytoplasm and nuclear extracts of unstimulated and LPS-stimulated macrophages exposed to P4 or DEX. Cytoplasmic or nuclear extracts (25 g/lane) were electrophoresed on 4 to 15% gradient SDS-polyacrylamide gels and transferred to nitrocellulose membranes. After electrophoretic protein transfer, nonspecific Ab binding sites on the polypeptide film were exposed to the nitrocellulose membranes, and protein extracts were detected by chemiluminescence. Nitrocellulose membranes were washed and incubated with secondary antibody to blocking buffer followed by a final wash. The x-ray film was exposed to the nitrocellulose membranes, and protein extracts were detected by chemiluminescence. Nitrocellulose membranes were washed and probed with NF-κB antiserum and processed for Western blot analysis. The m.w. were determined with prestained standards. The results shown represent one of two separate experiments of each type that had essentially the same outcome.