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Cytokine and nitric oxide release by J774A.1 macrophages is not regulated by estradiol

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Abstract

Estradiol is able to regulate the release of inflammatory mediators by macrophages; however, the presence, extent, and direction of this modulation varies with species, tissue of origin, and cell culture conditions. This study examines the effects of 17-β-estradiol (E_2) on the release of inflammatory mediators by the J774A.1 mouse macrophage cell line. For experiments, cells were plated in phenol red-free DMEM containing 5% charcoal-dextran stripped calf serum. Western analysis showed that J774A.1 cells contain the estrogen receptor α (ERα) protein. We found that physiological and pharmacological levels of E_2 (10^{-12} M – 10^{-6} M) have no effect on the release of nitric oxide (NO), tumor necrosis factor–α (TNF–α), interleukin-6 (IL-6), or monocyte chemoattractant protein-1 (MCP-1). This suggests that J774A.1 cells grown under these culture conditions would be useful for the investigation of non-estrogen-dependent mechanisms by which certain endocrine disruptors may affect their targets in macrophages.

Keywords: Macrophage; Estrogen; Nitric oxide; Tumor necrosis factor-a; Interleukin-6; Monocyte chemoattractant protein-1; Endocrine disruptors

Introduction

The existence of sexual dimorphism with respect to immune function is well established. Females tend to have a higher immune reactivity and a greater incidence of autoimmune diseases than do males [1]. Such sexually dimorphic responses appear to be, in part, a consequence of the presence or absence of reproductive hormones such as estrogens. Evidence supporting this derives from the observation that estrogens regulate the functioning of lym-

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phoid and myeloid cells both *in vivo* and *in vitro* [2, 3]. In addition, estrogen receptors have been found in B lymphocytes, T lymphocytes, and macrophages of male and female animals [4].

A number of studies have shown that macrophage function can be modulated by E_2. The presence, extent, and direction of this effect varies with species, tissue of origin, and culture conditions [5–8]. For example, physiological concentrations of E_2 inhibit the release of nitric oxide by activated rat peritoneal macrophages [9] but have no effect on NO release or inducible nitric oxide synthase (iNOS) promotor activity in the mouse macrophage cell line RAW 264.7 [10]. E_2 enhances TNF–α release by rat peritoneal macrophages [11] but inhibits TNF–α production by activated mouse splenic macrophages, RAW 264.7 cells, and ANA–1 cells [12, 13].

The J774 mouse cell lines, derived from a female BALB/c mouse [14], have been used extensively as models of macrophage function. E_2 has been shown to regulate some processes of these cells, including cholesterol hydrolysis [15, 16], cholesterol oxidation [17], and the reversal of multidrug resistance [18]. However, knowledge of the effects of E_2 on the release of cytokines and other inflammatory mediators by J774 lineage cells is not complete. This study was undertaken to more fully characterize the effects of physiological and pharmacological levels of E_2 on the release of NO, TNF–α, IL-6, and MCP-1 by J774A.1 cells.

Methods

Chemicals

E_2 was obtained from Steraloids (Newport, RI), dexamethasone (Dex) was obtained from Sigma (St Louis, MO), and bacterial lipopolysaccharide from *E. coli* B5:055 (LPS) was obtained from Difco (Detroit, MI). Stock solutions of steroids were made in 200 proof ethanol (EtOH).

Cells

J774A.1 cells (ATCC, Manassas, VA) were maintained in a humidified incubator at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 4 mM L-glutamine (10% FBS). Steroid-depleted experimental medium was phenol red-free DMEM containing 5% sulfatase-treated, charcoal dextran-stripped calf serum [19], 4 mM glutamine, 100 U/ml penicillin and 100 ug/ml streptomycin. For experiments, cells were plated in 5% stripped calf serum at a density of 3.33×10^5 cells/ml (1.52×10^5 cells/cm^2) in 24-well plates. After 24 hours, the medium was changed to 5% stripped calf serum containing either EtOH vehicle, E_2, or Dex such that the final EtOH concentration was always 0.01%. This EtOH concentration was chosen because we found that it had no effect on LPS-stimulated cytokine or NO release and did not mask the effect of Dex on these parameters in J774A.1 cells. LPS was added 24 hours later. After 22 hours of LPS exposure, culture plates were centrifuged at $1200\times g$ for 10 minutes and supernatants were collected and stored at −20°C (for NO analysis) or −80°C (for cytokine analysis).

Western blot analysis

J774A.1 cells were plated in 10% FBS or 5% stripped calf serum as described above. Whole cell lysates were made by washing cells twice with ice-cold Hank's Balanced Salt Solution (HBSS) then lysing in a buffer containing 5 mM Tris-HCl pH 7.5, 3 mM EDTA, 2 mM DTT,

0.02 mM leupeptin, and 0.1 mM PMSF. Protein in lysates was measured using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Whole cell lysates were kept frozen at −80°C. 50 μg of whole cell lysate protein or 100 ng recombinant human ERα (Pan Vera, Madison, WI) per lane were subjected to SDS-PAGE and transferred to Hybond ECL nitrocellulose membrane (Amersham-Pharmacia, Piscataway, NJ). Blots were incubated with 0.4 μg/ml anti-ERα sc-542 (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 hours at room temperature followed by secondary antibody and detection by ECL enhanced chemiluminescence (Amersham-Pharmacia).

Analysis of NO production

The amount of NO produced was indexed by measuring nitrate and nitrite levels in the supernatants. Nitrate was first reduced to nitrite according to the method of Grisham et al. [20] with the following modifications. Nitrate reductase (Roche Molecular Biochemicals, Indianapolis, IN) was used at a final concentration of 0.1 U/ml. Samples were incubated for 30 minutes at 37°C then were diluted 1:20 with deionized water. Nitrite was measured by the method of Greiss [21, 22] modified for use in a QuickChem Flow Injection Analyzer using Nitrate/Nitrite Method #60-107-04-1 (Lachat, Milwaukee, WI).

Cytokine analyses

TNF-α, IL-6, and MCP-1 in supernatants were measured using ELISA kits (BioSource International, Camarillo, CA).

Statistical analyses

A Kruskal-Wallis one way ANOVA on ranks (SigmaStat, SPSS Inc., Chicago, IL) was used for data analysis.

Results

Whole cell lysates of J774A.1 cells contain detectable ERα (Fig. 1). This receptor is present when cells are cultured in either 10% FBS or 5% stripped calf serum.

E_2 has no effect on the LPS-stimulated release of NO by J774A.1 cells (Fig. 2). Dexamethasone (10^{-6} M) decreases NO release when J774A.1 cells are stimulated with 10 μg/ml, 0.1 μg/ml, or 0.01 μg/ml LPS. E_2 alone (10^{-12} M – 10^{-8} M) has no effect on unstimulated NO release (data not shown).

E_2 has no effect on the LPS-stimulated release of IL-6, MCP-1, or TNF-α by J774A.1 cells (Fig. 3). Dexamethasone (10^{-6} M) decreases the release of all three cytokines. E_2 alone (10^{-6} M) has no effect on unstimulated release of IL-6, MCP-1, or TNF-α (data not shown).

Discussion

Our study indicates that E_2 does not regulate the release of NO, IL- 6, MCP-1, or TNF-α by J774A.1 cells. However, the release of these inflammatory mediators is clearly under some steroidal control, as dexamethasone significantly decreases the production of each substance.

The lack of effect of E_2 on NO release by J774A.1 cells agrees with the results of Miller et al.

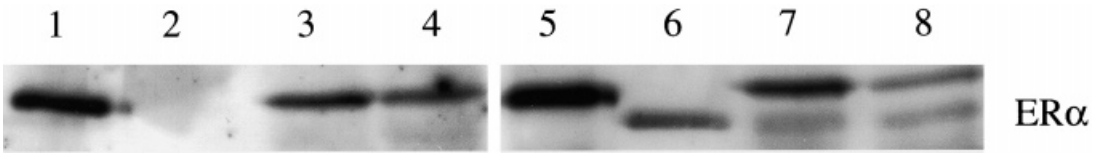

Fig. 1. Detection of ERα protein in J774A.1 cells. Whole cell lysates from J774A.1 cells were subjected to SDS-PAGE. Western blot analysis was performed using specific antibodies for ERα (lanes 5 – 8) or pre-immune rabbit serum as a control for nonspecific secondary antibody binding (lanes 1 – 4). Lane 1: 66.2 kDa protein standard; lane 2: human recombinant ERα; lane 3: J774A.1 cells cultured for 24 hours in 10% FBS; lane 4: J774A.1 cells cultured for 24 hours in 5% stripped calf serum; lane 5: 66.2 kDa protein standard; lane 6: human recombinant ERα; lane 7: J774A.1 cells cultured for 24 hours in 10% FBS; lane 8: J774A.1 cells cultured for 24 hours in 5% stripped calf serum. The upper band in lanes 3, 4, 7, and 8 represents nonspecific binding of the secondary antibody. The lower band in lanes 7 and 8 is ERα.

[10] in J774 cells. In contrast, Hayashi et al. [23, 24] found that E_2 (10^{-10} M – 10^{-6} M) decreased NO release by J774.2 cells. Similar to our findings, Frazier-Jessen and Kovacs [25] also found no effect of E_2 (10^{-11} M – 10^{-9} M) on TNF-α release by J774A.1 cells. In contrast, they found that E_2 exposure decreased MCP-1 mRNA levels.

Several explanations exist for the discrepancies between our results and some of the other reports in the literature. The most obvious of these is that different cell lines were used. Although J774, J774A.1, and J774.2 are all of the same lineage, it is possible that significant differences in signaling pathways may exist between these cell lines. Such differences have been observed by several laboratories with various cell lines [26, 27, 28]. A second potential explanation for these conflicting results is differences between the cell culture conditions that were used. Previously published experiments were performed in the presence of 10% calf serum [23, 24] or 2% FBS [25, 10]. Both of these conditions do not provide steroid-depleted environments, thus the actual concentration of E_2 which was present is unknown. A third explanation is that sulfatase-treated, charcoal-dextran-stripped calf serum lacks some serum component that is required for E_2 modulation of NO or cytokine release. For instance, certain hormones such as testosterone, insulin, T3, T4, and PTH, in addition to E_2, are dramatically decreased by the charcoal-dextran stripping process [29].

The existence of an estrogen-nonresponsive macrophage system is significant in that it may be used to expand our understanding of the mechanism of action of certain endocrine disruptors on the immune system. Recent reports suggest that like E_2, the estrogenic detergent breakdown product 4-octylphenol can inhibit IL-1β expression in a macrophage cell line transfected with genes for the estrogen receptor and IL-1β [30, 31]. However, it is also possible that the classical estrogenic endocrine disruptors may affect their targets through non-estrogen-dependent pathways. For example, in rat Leydig cells, 4-tert-octylphenol decreases human chorionic gonadotropin-stimulated testosterone synthesis whereas E_2 itself has no effect. Furthermore, addition of the pure antiestrogen ICI 182,780 does not reverse the effect of octylphenol [32]. Such results suggest that it would be valuable to examine the immune effects of estrogenic endocrine disruptors in an estrogen-nonresponsive system such as J774A.1 cells grown under steroid-depleted conditions.

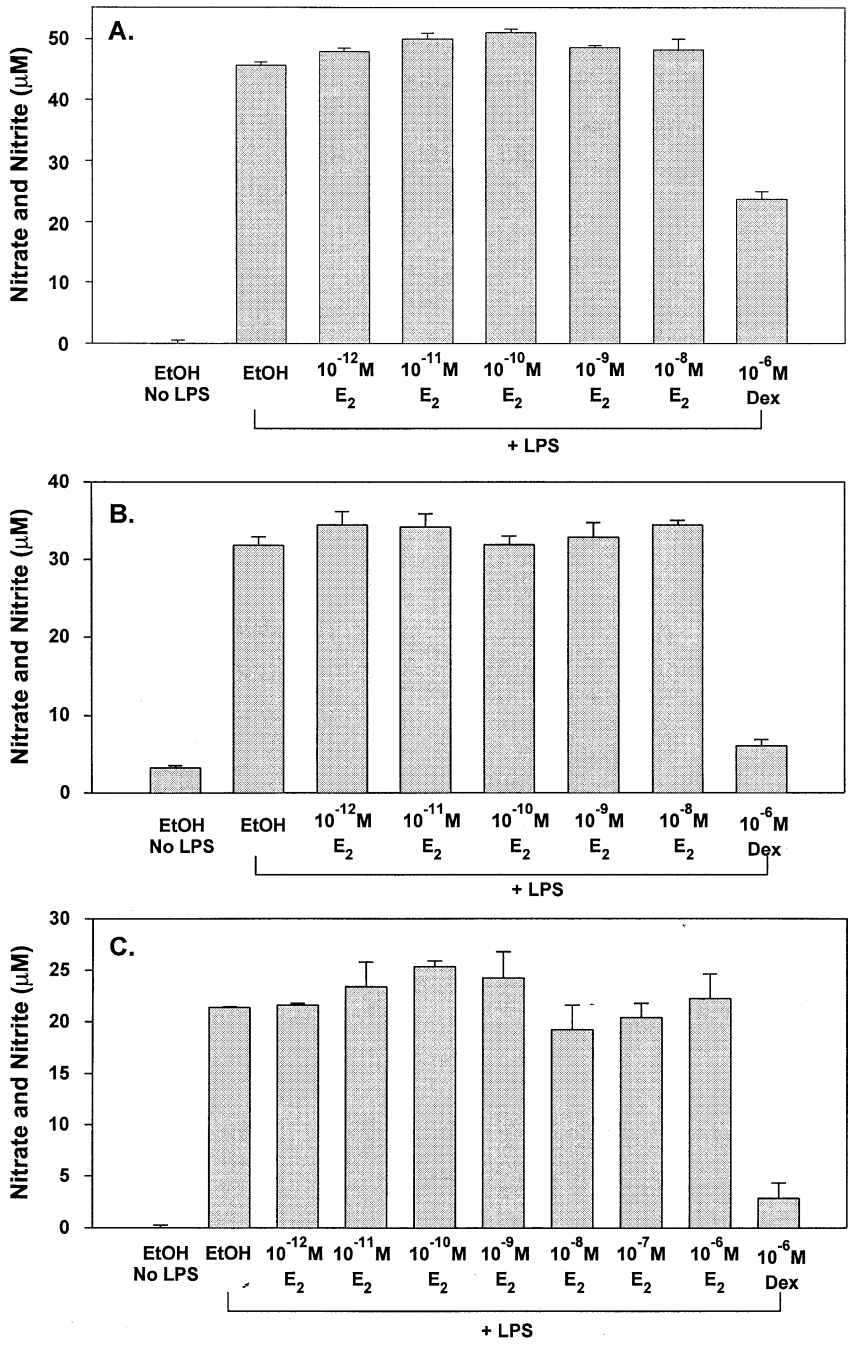

Fig. 2. Effect of E_2 on LPS-stimulated NO production by J774A.1 cells. Cells were plated in 5% stripped calf serum then changed to 5% stripped calf serum containing 0.01% EtOH, E_2, or Dex as described in Materials and Methods. After 24 hours, LPS was added at 10 μg/ml (A), 0.1 μg/ml (B), or 0.01 μg/ml (C), then supernatants were collected 22 hours later and nitrate and nitrite levels in the supernatants were measured. Data shown are the mean +/− SE of three samples and are representative of three or more separate experiments.

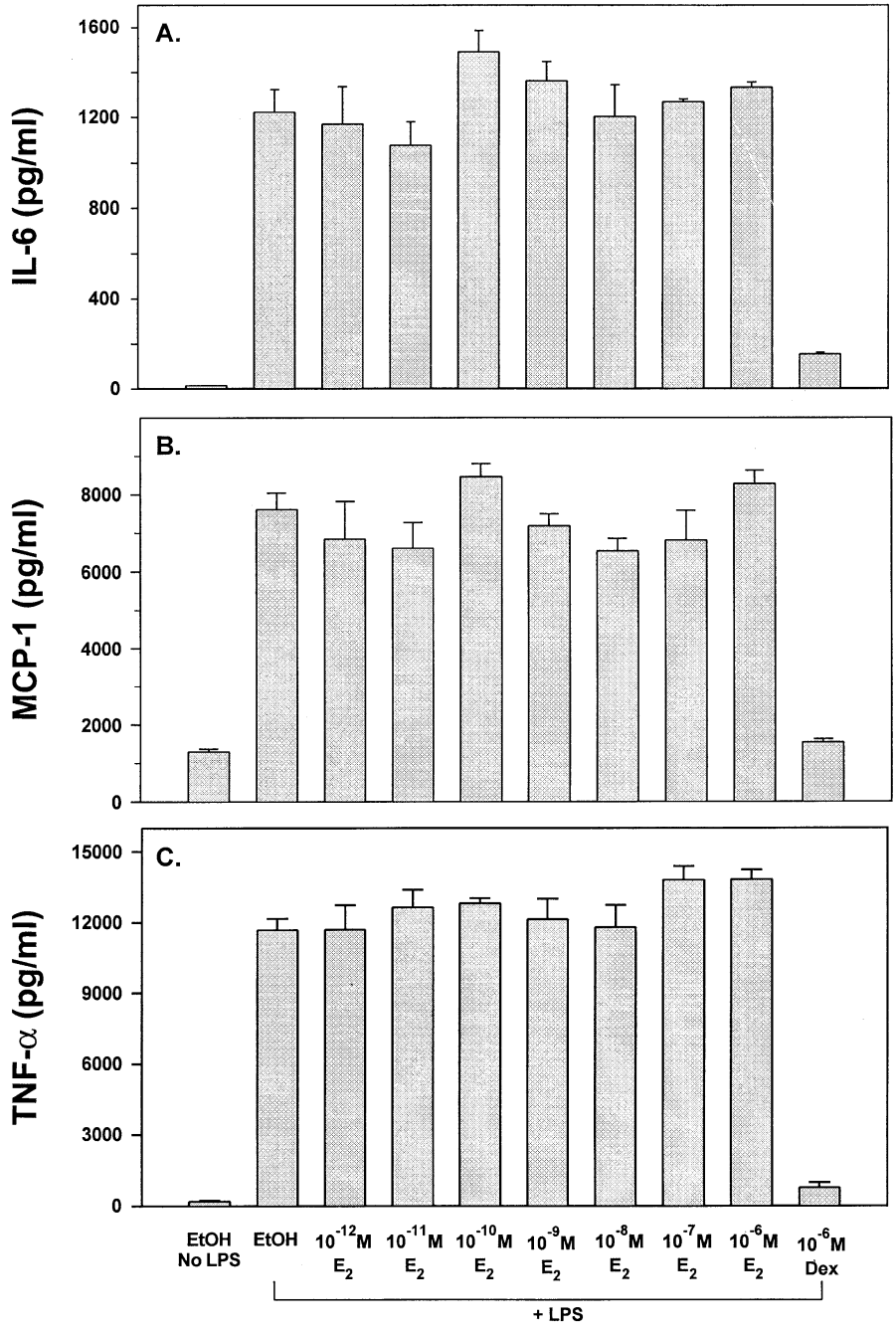

Figure 3. Effect of E_2 on LPS-stimulated cytokine production by J774A.1 cells. Cells were plated in 5% stripped calf serum then changed to 5% stripped calf serum +0.01% EtOH, E_2, or Dex as described in Materials and Methods. After 24 hours, 0.01 μg/ml LPS was added, then supernatants were collected 22 hours later. IL-6 (A), MCP-1 (B), and TNF-α (C) were measured by ELISA. Data shown are the mean +/− SE of three samples and are representative of three or more separate experiments.

Conclusion

Although J774A.1 cells contain the ERα protein, the LPS-stimulated release of NO, TNF-α, IL-6, and MCP-1 by these cells is not regulated by E_2 under the conditions described above. The cell culture system of J774A.1 cells grown in 5% stripped calf serum may be used as a model to expand the knowledge of possible non-estrogen-dependent mechanisms of action of certain endocrine disruptors on the immune system.

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