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# Female sex steroids: effects upon microglial cell activation

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**Abstract**

Multiple sclerosis occurs more commonly in females than males. However, the mechanisms resulting in gender differences in multiple sclerosis are unknown. Activated microglia are believed to contribute to multiple sclerosis pathology, perhaps in part due to production of nitric oxide (NO) and TNF-α, molecules which can be toxic to cells including oligodendrocytes. The current study demonstrates that the female sex steroids estriol, β-estradiol and progesterone inhibit lipopolysaccharide (LPS) induction of nitric oxide (NO) production by primary rat microglia and by the mouse N9 microglial cell line. These hormones act by inhibiting the production of inducible nitric oxide synthase (iNOS) which catalyses the synthesis of NO. Estriol likely inhibits iNOS gene expression since the hormone blocks LPS induction of iNOS RNA levels. The pro-inflammatory cytokines IFN-γ and TNF-α are believed to be important modulators of multiple sclerosis. Here, we demonstrate that estrogens and progesterone also inhibit NO production by microglial cells activated in response to these cytokines. Activated microglia elicit TNF-α in addition to NO and we further demonstrate that estrogens and progesterone repress TNF-α production by these cells. Finally, estriol and progesterone, at concentrations consistent with late pregnancy, inhibit NO and TNF-α production by activated microglia, suggesting that hormone inhibition of microglial cell activation may contribute to the decreased severity of multiple sclerosis symptoms commonly associated with pregnancy. 

*Keywords:* Microglia; Nitric oxide; TNF-α; Estrogen; Progesterone

## 1. Introduction

Multiple sclerosis occurs more frequently in females than males (Duquette and Girard, 1993). This is consistent with many other autoimmune diseases including rheumatoid arthritis, systemic lupus erythematosus and Hashimoto's thyroiditis, in which women are also disproportionately affected (Beeson, 1994; Jacobson et al., 1997). Several studies have suggested that sex steroids influence the development and severity of multiple sclerosis. For example, pregnancy influences multiple sclerosis symptomology, with remission in the third trimester of gestation, followed by exacerbation in the postpartum period (Confavreux et al., 1998). In addition, oral contraceptives containing female sex steroids have been associated with a lower risk of developing multiple sclerosis and decreased disability (Villard-Macintosh and Vessey, 1993).

Experimental autoimmune encephalomyelitis (EAE) is an autoimmune disorder characterized by central nervous system (CNS) inflammation and demyelination and remittent paralysis; features consistent with multiple sclerosis (Martin et al., 1992). Recent studies have suggested that female sex steroids may modulate EAE, at least in part, through effects upon T-cells. For example, investigators have demonstrated that not only are female mice more susceptible than males to EAE, but also that T-cells from female mice produce more severe disease when adoptively transferred into recipients (Dalal et al., 1997; Ding et al., 1997). In addition, estrogens (Arnason and Richman, 1969; Jansson et al., 1994; Kim et al., 1999) and testosterone (Dalal et al., 1997) have been demonstrated to repress EAE. Sex steroids also shift T-cells toward a Th2 phenotype in vitro (Dalal et al., 1997; Gilmore et al., 1997; Correale et al., 1998) and cytokines produced by Th2 cells generally suppress EAE (Olsson, 1995). Collectively, these studies suggest that female sex steroids modulate EAE, at least in part, through effects upon T-cell phenotype.

In addition to autoreactive T-cells, activated microglia participate in pathology associated with multiple sclerosis (reviewed in Benveniste, 1997; Sriram and Rodriguez, 1997). However, the effect of sex steroids upon activation

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of microglia has not been previously investigated. Microglia are resident CNS cells that function in host defense. These cells may serve as antigen-presenting cells and can be phagocytic. Upon CNS injury or inflammation, microglia become activated, resulting in increased proliferation and altered morphology. Activated microglia also produce a variety of cytokines including TNF-α, as well as increased MHC class II and NO (Benveniste, 1997). NO is a gaseous molecule which performs a wide variety of cellular functions. This molecule is produced by a series of three enzymes termed nitric oxide synthases (NOS). Endothelial NOS (eNOS) and neuronal NOS (nNOS) occur constitutively, are $Ca^{2+}$-dependent and mediate vasodilation and neuro-signaling, respectively. Inducible NOS (iNOS) was first demonstrated in monocytes, but is now known to be expressed in a variety of cells including microglia. iNOS is a $Ca^{2+}$- and calmodulin-independent enzyme which is induced by a variety of inflammatory cytokines and bacterial products including lipopolysaccharide (LPS) (MacMiking et al., 1997). TNF-α is also produced by cells of monocyte origin including microglia, in response to inflammatory stimuli (Benveniste, 1997). Although molecules including NO and TNF-α are toxic to pathogens, these agents can also be toxic to CNS cells including myelin-producing oligodendrocytes (reviewed in Benveniste, 1997), which are compromised in the course of multiple sclerosis (reviewed in Raine, 1997). These molecules also may be toxic to neurons and thus, may contribute to axonal degeneration characteristic of multiple sclerosis (Trapp et al., 1998). It should be noted that NO and TNF-α have alternatively been reported to protect CNS cells (reviewed in Mattson et al., 1997; Munoz-Fernandez and Fresno, 1998). Inhibition of NO and TNF-α synthesis blocks development of EAE (Ruddle et al., 1990; Selmaj et al., 1991; Zhou et al., 1996; Ding et al., 1998). Thus, agents which inhibit NO and TNF-α synthesis may be effective in the treatment of multiple sclerosis.

The current study describes the novel observation that sex steroids including β-estradiol, estriol and progesterone inhibit production of NO and TNF-α by microglial cells. Thus, female sex steroids may decrease the development of EAE, at least in part, by repressing microglial cell activation. These studies support the concept that sex steroids may be therapeutic in the treatment of multiple sclerosis.

## 2. Materials and methods

### *2.1. Cell culture*

Pure rat microglia cultures were obtained through a modification of the McCarthy and deVellis protocol (1980). Briefly, cerebral cortices from 1 to 2-day-old rats were excised, meninges removed and cortices minced into small pieces. Cells were separated by trypsinization followed by trituration of cortical tissue. Cells were plated into tissue culture flasks and allowed to grow to confluence (~1 week) in DMEM media containing 10% FBS and 1.4 mM glutamine. Flasks were shaken overnight (200 rpm at 37°C) in a temperature controlled shaker to loosen microglia and oligodendrocytes from the more adherent astrocytes. These non-adherent cells were plated for 2 h and then lightly shaken to separate oligodendrocytes from the relatively more adherent microglia. These panning procedures were repeated, as necessary, to obtain primary microglia of greater than 95% purity as determined by immunohistochemistry with antibodies prepared against microglia specific proteins Mac-1 (Boehringer Mannheim Biochemicals) and OX-42 (Serotec) and by staining with the lectin, *Griffonia simplicifolia* (Sigma). The N9 cell line is derived from myc-immortalized mouse microglia (Corradin et al., 1993) and was graciously provided by P. Ricciardi-Castagnoli (U. Milan, Italy). Cells were cultured in MEM medium containing 10% FBS (Sigma, St. Louis, MO), 1.4 mM glutamine and 20 μM 2-mercaptoethanol (Sigma, St. Louis, MO). Where indicated, cells were treated with steroid hormones, lipopolysaccharide (LPS) (Sigma, St. Louis MO) or the cytokines IFN-γ or TNF-α (R&D Systems, Minneapolis, MN).

### *2.2. Nitric oxide production and cell viability assays*

Levels of the NO derivative nitrite were determined in the culture medium by Griess reaction as described previously (Barger and Harmon, 1997). Cells grown in 96-well plates were treated with steroid hormones as indicated. After 1 h cells were then treated as indicated with LPS or with the cytokines IFN-γ and TNF-α and nitrite levels determined following a 24-h incubation. A standard curve using $NaNO_2$ was generated for each experiment for quantitation. Cell viability was determined in the same cultures by MTT reduction assay as described previously (Chang et al., 1998).

### *2.3. Western blot analysis*

Cellular proteins (20 μg) were separated electrophoretically on 10% polyacrylamide gels containing the denaturant SDS. Proteins were transferred electrophoretically to nitrocellulose membranes (NitroBind, MSI, Westborough, MA) and then incubated with polyclonal rabbit anti-murine iNOS antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C at a 1:3000 dilution. Blots were then incubated with horseradish peroxidase conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) for 45 min at room temperature at a 1:2000 dilution. iNOS protein was detected by ECL Western Blotting Detection Reagents as described by the manufacturer (Amersham Life Sciences, Arlington Heights, IL), followed by autoradiography.

### 2.4. Northern blot analysis

RNA isolation and Northern blot analysis were performed as described previously (Drew et al., 1993). Briefly, total RNA was isolated from cells using RNAzol (Tel-Test Inc., Friendswood, TX) as described by the manufacturer. RNA (40 μg total) was separated on a 1.2% agarose gel containing 0.66 M formaldehyde in the presence of 1X MOPS buffer. RNA was transferred electrophoretically to nylon membranes (Micron Separations, Inc., Westboro, MA). cDNA probes were labeled with $^{32}P$ by random priming (Prime-It, Stratagene, La Jolla, CA) and used to hybridize Northern Blots at $10^6$ cpm/ml hybridization solution. Blots were hybridized with mouse macrophage iNOS cDNA (Alexis, San Diego, CA). Blots were also hybridized with human G3PDH cDNA (Clontech, Palo Alto, CA) to control the amount of RNA present in individual lanes. Following hybridization, blots were washed twice for 30 min in 2×SSC, 0.1% SDS at room temperature and then twice in 0.1×SSC, 0.1% SDS at 65°C and autoradiography was performed.

### 2.5. ELISA

TNF-α levels in tissue culture media were determined by ELISA as described by the manufacturer (mouse TNF-α OptEIA Set, Pharmingen, San Diego, CA). TNF-α concentrations in media were determined by spectrophotometer and calibrated from standards containing known concentrations of TNF-α.

### 2.6. Statistics

Data were analyzed by ANOVA followed by a Bonferroni test to determine the significance of difference.

## 3. Results

### 3.1. Effect of sex steroids upon NO production by microglial cells

Microglia are cells of monocyte origin which function as resident brain macrophages. These cells become activated in response to a variety of stimuli including the bacterial surface molecule LPS. Activated microglia produce NO which may be toxic to oligodendrocytes, which are compromised in multiple sclerosis. Female sex steroids were previously demonstrated to inhibit NO production by monocyte/macrophages (Miller et al., 1996; Hayashi et al., 1998). This suggested that these hormones may inhibit NO production by CNS microglial cells. In the present studies we demonstrated that LPS increased the production of nitrite in N9 microglial cells (Fig. 1). The female sex steroids estriol, β-estradiol and progesterone significantly repressed LPS-induced nitrite production in these cells, in a dose-dependent manner, at supraphysiologic concentrations (Fig. 1A, C and E). The sex steroids were not toxic to the cells at these concentrations as demonstrated by MTT reduction assays (Fig. 1B, D and F). Thus, female sex steroid inhibition of nitrite production was not due to effects on cell viability. As observed in N9 microglial cells, LPS induced nitrite production in primary microglia (Fig. 2), while the sex steroids estriol and progesterone alone had no effect upon constitutive nitrite production by these cells (data not shown). These female sex steroids did, however, markedly inhibit LPS-induced nitrite production in primary microglia (Fig. 2A). As seen with N9 microglial cells, these steroid hormones were not toxic to primary microglia (Fig. 2B). Serum levels of estrogens and progesterone rise markedly in late-pregnancy, which commonly correlates with remission of multiple sclerosis. Next, the effect of steroid hormones at concentrations present in the serum at late pregnancy (100 nM estriol+1 μM progesterone) (Wilson and Parsons, 1996) upon NO production by microglia was assessed. A combination of estriol and progesterone at concentrations common to late-pregnancy inhibited LPS induction of NO in N9 microglia cells (Fig. 3). These studies suggested that multiple sclerosis may remit in late pregnancy, at least in part, due to female sex steroid inhibition of microglial cell activation. Collectively, these studies indicated that female sex steroids significantly inhibited LPS-induced nitrite production in both primary microglia and the N9 microglial cell line. Subsequent analyses were performed with N9 cells, since many of these analyses require large cells numbers, which are not easily obtained with primary cultures of microglia.

### 3.2. Effect of female sex steroids upon iNOS protein in microglial cells

In activated monocytes, the enzyme iNOS is principally responsible for generation of NO. Western blot analyses were performed to determine the effect of female sex steroids upon iNOS protein levels in microglial cells. These studies demonstrated that LPS induced the production of the iNOS protein in N9 microglial cells and that estriol, estradiol and progesterone inhibited this induction. Thus, the female sex steroids inhibited nitrite production in these cells, at least in part, by inhibiting production of iNOS protein (Fig. 4).

### 3.3. Effect of female sex steroids upon iNOS RNA

The above studies demonstrated that female sex steroids inhibited iNOS protein production by N9 microglial cells. In order to begin to determine the molecular mechanisms resulting in this inhibition, we investigated the effect of estriol on iNOS RNA levels by Northern blot analyses. These studies indicated that these cells constitutively expressed little or no iNOS RNA, but that the level of the RNA was induced by LPS. In addition, estriol inhibited the

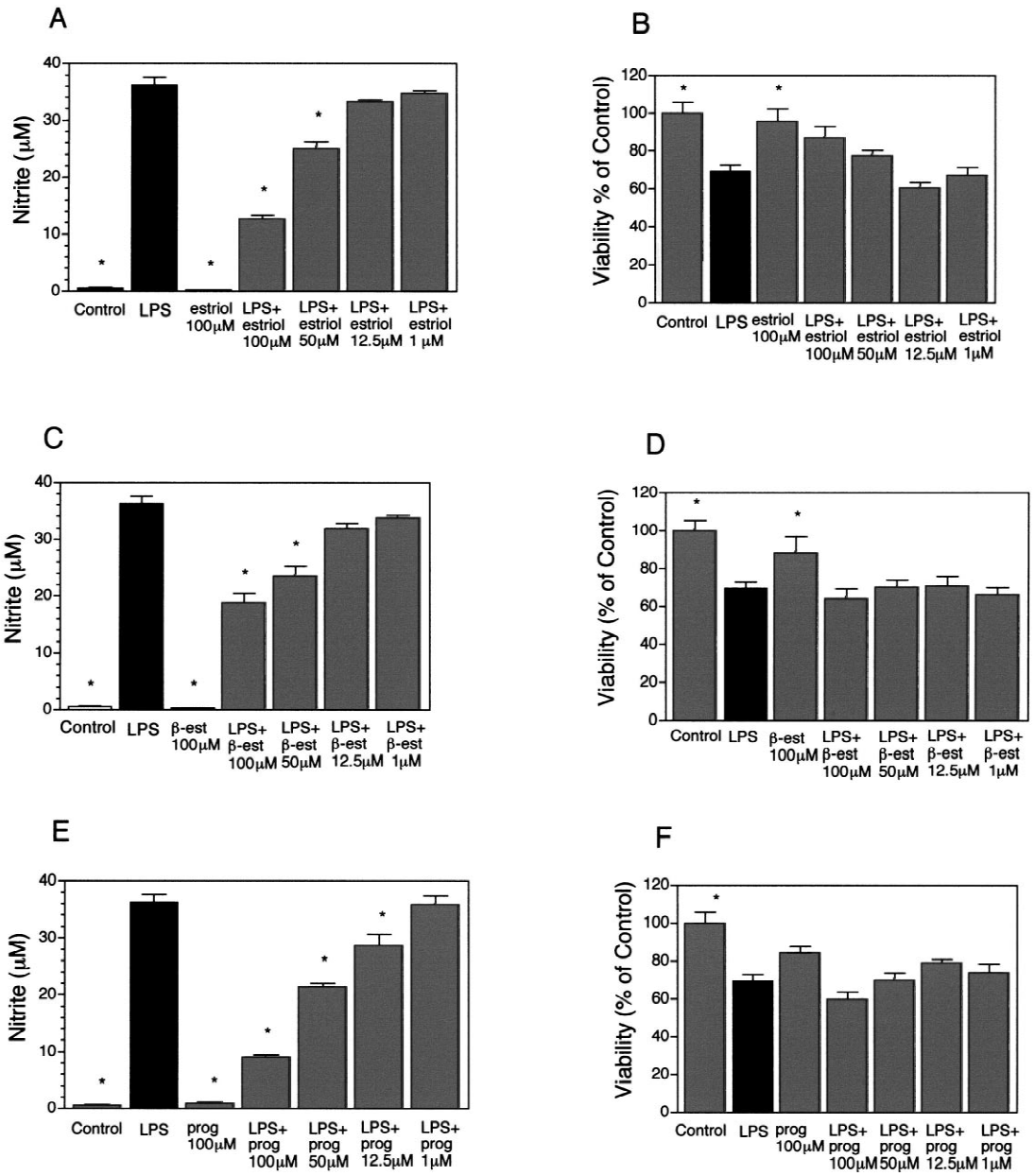


Fig. 1. Female sex steroids inhibit LPS induction of nitrite in N9 microglia cells. Cells were pre-treated for 1 h with the indicated concentrations of the steroid hormones estriol (A and B); β-estradiol (C and D); or progesterone (E and F). LPS (2 μg/ml) was added as indicated and 24 h later the concentration of nitrite in the culture medium was determined (A, C and E). Cell viability was determined by MTT assay (B, D and F). Values represent the mean±s.e.m. for quadruplicate cultures. *$P<0.001$ vs. LPS treated cultures.

production of iNOS RNA levels in the N9 microglial cells (Fig. 5). This suggests that estriol inhibited transcription of the iNOS gene which encodes this RNA. However, estriol effects on iNOS mRNA stability have not been investigated.

### *3.4. Effect of female sex steroids upon cytokine induction of NO in microglial cells*

The pro-inflammatory cytokines IFN-γ and TNF-α are important modulators of multiple sclerosis. These cytokines are also capable of activating microglial cells. Therefore, we wished to determine if female sex steroids effect microglial activation in response to these cytokines. The studies demonstrated that estriol and progesterone each inhibited NO production by IFN-γ and TNF-α treated microglial cells (Fig. 6A and C), without effecting cell viability (Fig. 6B and D). In addition, levels of estriol and progesterone present in the serum at late pregnancy also inhibited IFN-γ and TNF-α induction of NO production by microglial cells (Fig. 6E), without effecting cell viability (Fig. 6F). These studies support the hypothesis that female

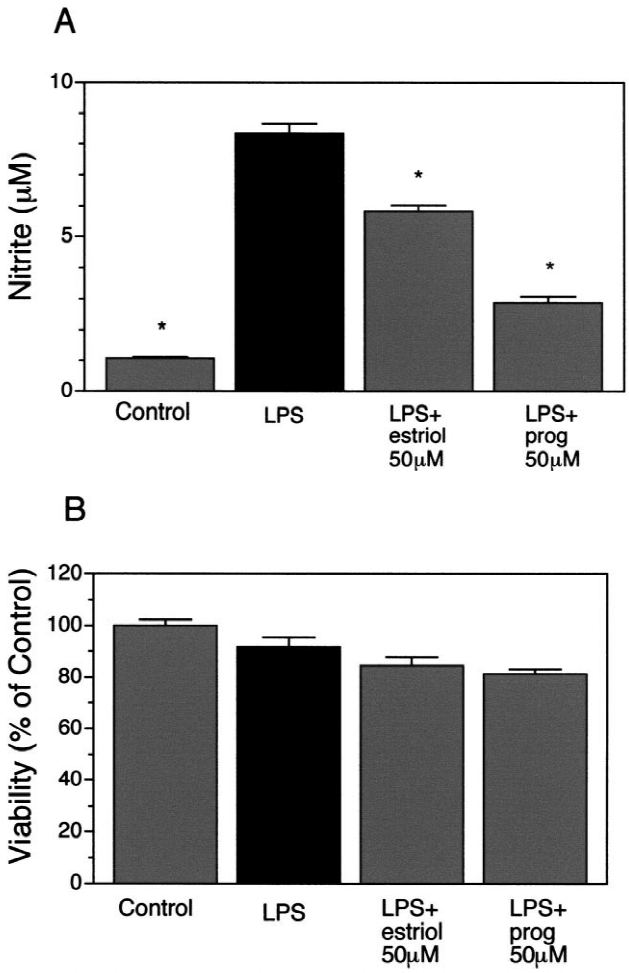


Fig. 2. Female sex steroids inhibits LPS induction of nitrite in primary microglia. Cells were pre-treated for 1 h with the steroid hormones estriol or progesterone (50 μM). LPS (0.5 μg/ml) was added as indicated and 24 h later the concentration of nitrite in the culture medium was determined (A). Cell viability was determined by MTT assay (B). Values represent the mean±s.e.m. for quadruplicate cultures. *$P<0.001$ vs. LPS treated cultures.

sex steroids may modulate multiple sclerosis, in part, through effects on cytokine-stimulated microglial cell activation.

### *3.5. Effect of female sex steroids upon TNF-α production by microglial cells*

TNF-α, like NO, is produced by activated microglia. This inflammatory cytokine can be toxic to a variety of cells. Previously, female sex steroids were demonstrated to inhibit TNF-α production by macrophages (Shanker et al., 1994; Deshpande et al., 1997; Miller and Hunt, 1998). The present studies indicated that LPS induced the production of TNF-α by N9 microglial cells. The estrogenic steroids estriol and β-estradiol, as well as progesterone, inhibited TNF-α elicited from these cells, as determined by ELISA analyses (Fig. 7A). A combination of estriol and progesterone at concentrations typical of serum levels at late-pregnancy also inhibited TNF-α production by N9 microglial cells (Fig. 7B). The female sex steroids did not affect the viability of these microglial cells (data not shown). To summarize, these data collectively indicate that female sex steroids strongly inhibit production of NO and TNF-α, molecules potentially toxic to oligodendrocytes, by microglial cells.

## 4. Discussion

Multiple sclerosis is a demyelinating disease characterized by infiltration of mononuclear cells, principally T-cells and macrophages, into the CNS. The etiology of multiple sclerosis is unknown. However, it is widely believed that T-cells reactive to self myelin antigens contribute to pathogenesis in genetically susceptible individuals, perhaps through mechanisms involving molecular mimicry to an as yet undetermined pathogen. In addition to peripheral immune cells, resident CNS cells including microglia are believed to be important mediators of multiple sclerosis.

Previous studies suggested that sex hormones play an important role in multiple sclerosis. For example, the disease is more common in women than men (Duquette and Girard, 1993). Multiple sclerosis symptoms are altered during pregnancy (Confavreux et al., 1998) and during the menstrual cycle (Pozzilli et al., 1999). Female rodents are also more susceptible to development of EAE (Voskuhl et al., 1996) and the female sex hormones estriol and to a lesser extent β-estradiol, decreased the severity of EAE (Jansson et al., 1994; Kim et al., 1999). Female sex hormones may mediate disease, at least in part, through effects upon T-cell function. For example, in vitro studies indicate that a variety of estrogens and progesterone stimulate the release of primarily anti-inflammatory cytokines from T-cells (Piccinni et al., 1995; Gilmore et al., 1997; Correale et al., 1998). This suggests that these sex hormones shift T-cell populations toward a Th2 phenotype.

Activated microglia are associated with a wide variety of neuroimmunological and neurodegenerative diseases including multiple sclerosis, Alzheimer's disease, Parkinson's disease, AIDS encephalopathy and stroke (McGeer et al., 1988; Sriram and Rodriguez, 1997; Gonzalez-Scarano and Baltuch, 1999; McGeer and McGeer, 1999). Upon activation, microglia produce substances including NO and TNF-α. These molecules can be toxic to cells including neurons and oligodendrocytes (Perry and Gordon, 1997; Raine, 1997), thus potentially contributing to the development of these diseases. The levels of NO and TNF-α are elevated in EAE and multiple sclerosis and inhibition of the synthesis of these molecules blocks development of EAE (Ruddle et al., 1990; Selmaj et al., 1991; Zhou et al., 1996; Ding et al., 1998).

The present studies indicated that supraphysiologic

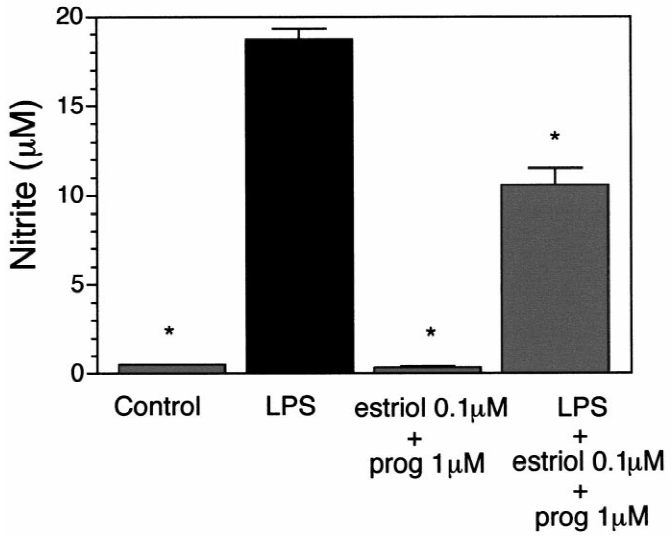


Fig. 3. Pregnancy levels of female sex steroids inhibit nitrite production in microglial cells. N9 cells were pre-treated for 1 h with estriol (100 nM) and progesterone (1 μM), concentrations present in serum at late pregnancy. LPS (0.5 μg/ml) was added as indicated and 24 h later the concentration of nitrite in the culture medium was determined. Values represent the mean±s.e.m. for quadruplicate cultures. *$P<0.001$ vs. LPS treated cultures.

concentrations of estrogens and progesterone, as well as physiologic concentrations of these hormones consistent with late pregnancy, inhibit NO and TNF-α production by microglial cells. The studies suggest that estrogen/progesterone inhibition of microglia activation may decrease the severity of multiple sclerosis symptoms during pregnancy. The fact that these female sex hormones inhibit microglial activation in response to IFN-γ and TNF-α, further supports the relevance of these findings to multiple sclerosis.

Multiple sclerosis occurs more frequently in females than in males. In addition, activated microglia are believed to contribute to the pathogenesis of multiple sclerosis. Thus, our preliminary data indicating that female sex steroids inhibit activation of microglia appear paradoxical. If female sex steroids inhibit microglial activation, why do women who produce these hormones exhibit increased prevalence of multiple sclerosis? The biphasic effect of estrogen upon immune function may contribute toward an explanation of this paradox. For example, low concentrations of estrogen may activate microglia, while high concentrations consistent with pregnancy may inhibit activation of these cells. This biphasic effect of sex steroids may explain, for example, the paradox that female mice normally express increased levels of NO relative to males (Ding et al., 1997), yet the present studies indicate that elevated concentrations of estriol and progesterone consistent with late pregnancy inhibit nitrite production by microglial cells.

Women are more likely to develop a Th1 response to an

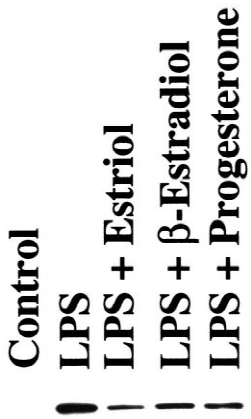


Fig. 4. Female sex steroids inhibit iNOS protein levels in microglial cells – Western analyses. Cells were pre-treated for 1 h with the indicated steroid hormones (50 μM), then LPS (2 μg/ml) was added as indicated and 16 h later cellular proteins were prepared for Western blot analyses.

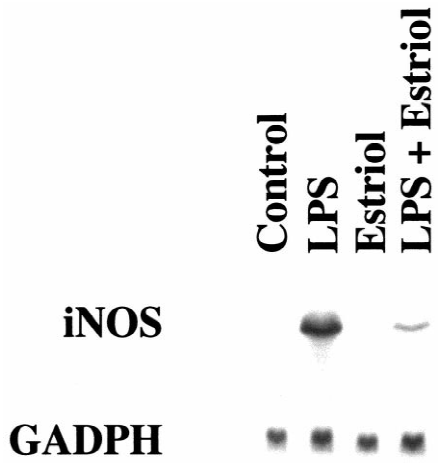


Fig. 5. Female sex steroids inhibit iNOS RNA in microglial cells – Northern analyses. Cells were pre-treated with estriol (50 μM) as indicated and then LPS (2 μg/ml) was added as indicated to culture media. Following 6 h treatment, RNA was isolated and iNOS and G3PDH (control) levels determined by Northern-blot analyses.

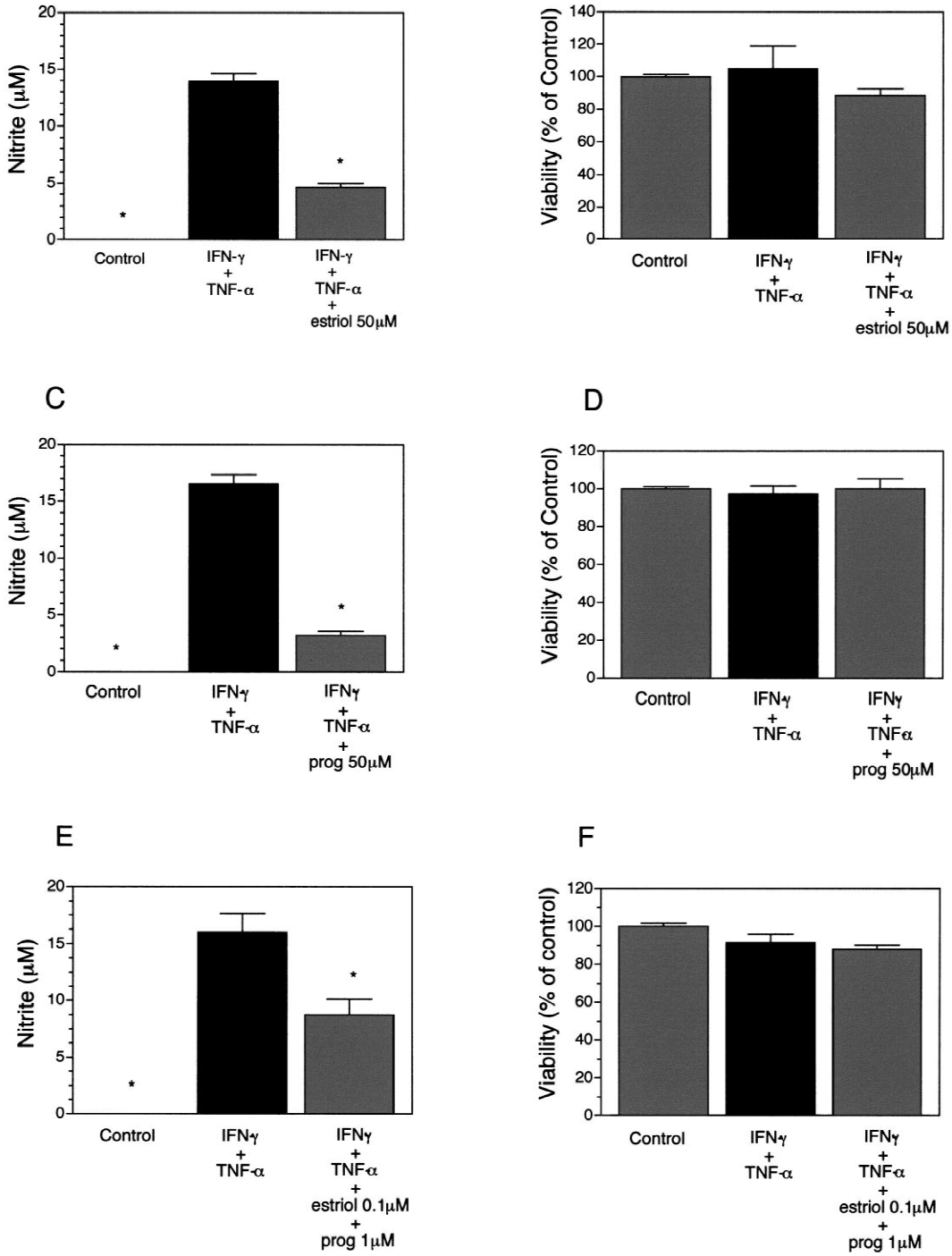


Fig. 6. Female sex steroids inhibit IFN-γ plus TNF-α induction of nitrite in microglial cells. N9 cells were pre-treated for 1 h with the steroid hormones estriol (50 μM); progesterone (50 μM); or a combination of estriol (100 nM) and progesterone (1 μM). LPS (0.5 μg/ml) was added as indicated and 24 h later the concentration of nitrite in the culture medium was determined (A, C, E). Cell viability was determined by MTT assay (B, D, F). Values represent the mean±s.e.m. for quadruplicate cultures. *$P<0.001$ vs. IFN-γ and TNF-α treated cultures.

antigen or infectious agent than men, except during pregnancy where women exhibit a pronounced Th2 response. This suggests that women may be more susceptible to autoimmune diseases including MS due to an increased Th1 response which is characteristic of many of these diseases. However, during pregnancy high estrogen and progesterone levels may contribute to a Th2 response in women and decreased disease severity. This model is consistent with the observation that SLE, an autoimmune disease characterized by a strong Th2 response, does not improve during pregnancy. It is possible that female sex steroids may play at least as important a role in modulating

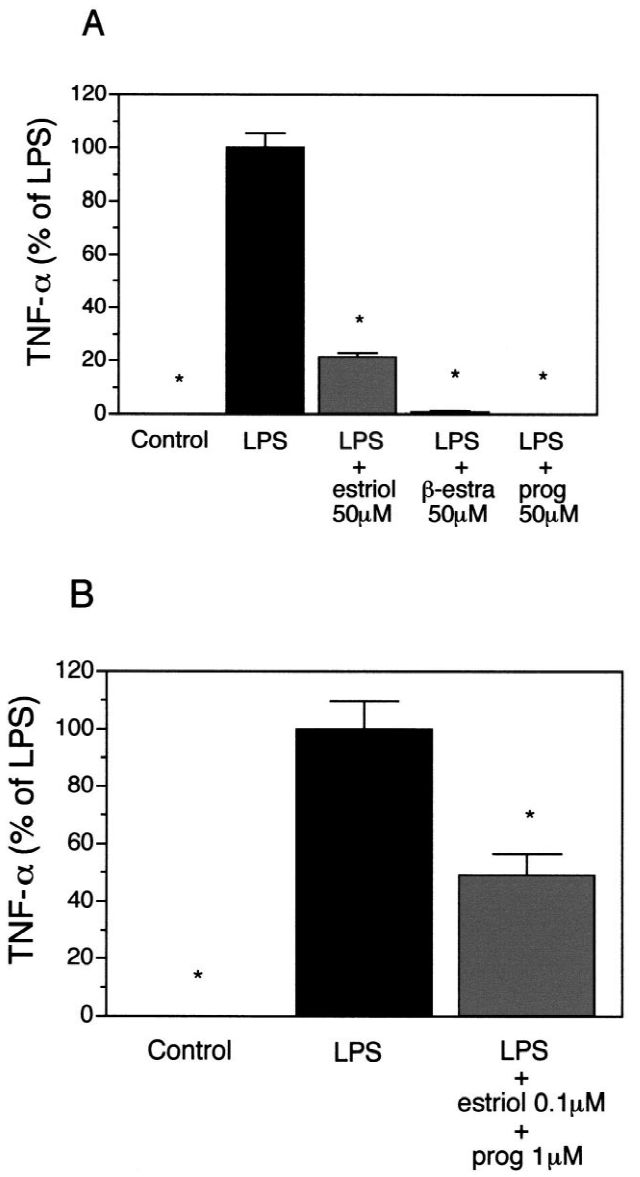


Fig. 7. Female sex steroids inhibit TNF-α protein production by microglial cells – ELISA analyses. N9 cells were pre-treated for 1 h with the steroid hormones estriol; β-estradiol; or progesterone (each at 50 μM) (A) or a combination of estriol (100 nM) and progesterone (1 μM) (B), then LPS (2 μg) was added as indicated and 24 h later TNF-α levels were determined in culture media by ELISA. Values are expressed relative to cultures treated with LPS alone (100%). Values represent the mean±s.e.m. for quadruplicate cultures. *$P<0.001$ vs. LPS treated cultures.

existing MS, as influencing disease susceptibility. Relatively high levels of these hormones have been shown to promote the development of a Th2 response and, as we show here, inhibit microglial cell activation, both of which may contribute to improvement of MS symptoms.

We have not investigated the effects of male sex hormones including testosterone on microglial activation. Previously, testosterone was shown to inhibit the development of EAE and induce a Th2 bias in myelin basic protein-specific T-cells (Dalal et al., 1997). This suggests that testosterone, like female sex hormones, may suppress immune reactivity. Although multiple sclerosis is more common in women, the disease likely progresses more rapidly in men (Wynn et al., 1990). At least one study suggests that male multiple sclerosis patients have reduced serum concentrations of testosterone (Poser et al., 1979). Sex steroid effects upon immune reactivity are dependent upon the concentration of the hormones. In addition, serum levels of sex hormones change with age. Thus, both female and male sex steroids likely modulate multiple sclerosis through complex mechanisms, which have not been completely elucidated.

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