Journal of Neuroscience Research 81:653–665 (2005)

Estrogen Provides Neuroprotection Against Activated Microglia-Induced Dopaminergic Neuronal Injury Through Both Estrogen Receptor-α and Estrogen Receptor-β in Microglia

Xuan Liu,[1] Xiao-Lan Fan,[1] Yan Zhao,[1] Guang-Rui Luo,[1] Xu-Ping Li,[1] Rui Li,[1,2] and Wei-Dong Le[1]*

[1]Joint Laboratory of Institute of Biomedical Sciences, RuiJin Hospital, Shanghai Second Medical University, and Health Science Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences and Shanghai Second Medical University, Shanghai, People's Republic of China
[2]Department of Neurology, Shanxi Provincial People's Hospital, Xi'an, People's Republic of China

Estrogen provides neuroprotection against neurodegenerative diseases, including Parkinson's disease. Its effects may stem from interactions with neurons, astrocytes, and microglia. We demonstrate here in primary cultures of rat mesencephalic neurons that estrogen protects them from injury induced by conditioned medium obtained from lipopolysaccharide (LPS)-activated microglia. LPS-induced nitrite production and tumor necrosis factor-α up-regulation in microglia were blocked by estrogen pretreatment. Estrogen neuroprotection was related to microglial activation of estrogen receptors (ERs), insofar as the protective effect of the microglia-conditioned medium was overridden by pretreatment of microglia with the ER antagonist ICI 182,780. On the other hand, the specific ERα antagonist, MPP dihydrochloride, only partially blocked the effects of estrogen, suggesting that estrogen protection was mediated via both ERα and ERβ. LPS treatment did not change ERα mRNA levels in microglia, astrocytes, and neurons, but it up-regulated ERβ mRNA levels in microglia and astrocytes. Similarly, increased ERβ protein levels were detected in LPS-activated microglia. More interesting was that immunocytochemical analysis revealed that ERβ was localized in the cytoplasm of microglia and in the cell nucleus of astrocytes and neurons. In summary, our results support the notion that estrogen inhibits microglial activation and thus exhibits neuroprotective effects through both ERα and ERβ activation. The cytoplasm location of microglial ERβ suggests the possible involvement of nonclassical effects of estrogen on microglia. Changes in microglial ERβ expression levels may modulate such effects of estrogen.

Key words: ERβ; estrogen; neuroprotection; dopaminergic neuron

Parkinson's disease (PD) has been accepted for a long time to be more prevalent in males than in females, with a relative male to female ratio of 1.4 to 3.7. This sexual difference has recently been confirmed again by using metaanalysis to analyze the results from seven studies (Wooten et al., 2004). The reason for this gender difference is not fully understood. There is some indication that levels of estrogens or progesterones or differences in their cognate receptor levels could account for this disparity. For example, estrogen deficiency following menopause might partially explain age and gender differences in late-onset dopaminergic (DA)-related disorders (Craig et al., 2004). Consistently with this finding, postmenopausal estrogen therapy reduced the risk of PD onset (Currie et al., 2004). Many reports have indicated that estrogen has neuroprotective effects on midbrain DA neuronal function both in vitro and in vivo (for reviews see Dluzen and Horstink, 2003; Kompoliti, 2003; Sawada and Shimohama, 2003). Even though estrogen was widely used by 38% of postmenopausal American women in 1999, its use sharply declined after the publication of a report from the Woman's Health Initiative in

Contract grant sponsor: National Science Foundation of China; Contract grant number: 30370491; Contract grant sponsor: National Academy of Science, China, 100-Talent Project; Contract grant number: 2002298; Contract grant sponsor: Shanghai E-Institute.

*Correspondence to: Wei-Dong Le, PhD, Joint Laboratory of Institute of Biomedical Sciences, RuiJin Hospital and Health Science Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences and Shanghai Second Medical University, Shanghai 200025, People's Republic of China. E-mail: wdle@sibs.ac.cn

Received 5 April 2005; Revised 17 May 2005; Accepted 18 May 2005

Published online 12 July 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.20583

2002, which announced that the standard estrogen-progestin combination poses risks for breast cancer and coagulopathies (Women's Health Initiative, 2002). Furthermore, estrogen clinical trial results for PD treatment were inconclusive; in some studies estrogen appeared to display slight prodopaminergic (or antiparkinsonian) effects (Marder et al., 1998; Blanchet et al., 1999; Tsang et al., 2000), but in others estrogen showed no beneficial effect (Strijks et al., 1999). However, recent studies have suggested that selective estrogen receptor (ER) modulators (SERMs) may be alternative PD therapeutics, insofar as they also confer neuroprotective effects (Grandbois et al., 2000; Callier et al., 2001) and have less adverse effects. Some of the SERMs are tissue selective and may have mixed agonist-antagonist properties. A better understanding of the mechanisms of the neuroprotective effects of estrogen is required for further studies on SERMs.

Reported possible mechanisms of estrogen neuroprotection include direct neuronal antiapoptotic effects (Sawada et al., 2000) as well as effects on astrocytes (Dhandapani and Brann, 2002). A recent study indicated that indirect activation of insulin-like growth factor-1 receptors, which localized in both neurons and glial cells, also contributes to the neuroprotection of DA neurons (Quesada and Micevych, 2004). Since microglial activation is thought to participate in the pathogenesis of PD (for reviews see McGeer and McGeer, 2004; Teismann and Schulz, 2004), we proposed that estrogen's neuroprotective mechanisms may involve interaction with microglia, which in turn may modulate DA neuronal function. There are some reports indicating that estrogen can inhibit microglial activation (Bruce-Keller et al., 2000; Vegeto et al., 2001; Baker et al., 2004). Microglia are central nervous system (CNS)-resident cells that function in host defense. Upon CNS injury or inflammation, microglia become activated. Nitric oxide (NO) and a variety of cytokines including tumor necrosis factor-α (TNF-α) produced by activated microglia are supposedly injurious to neurons (McGeer and McGeer, 2004; Teismann and Schulz, 2004). There is no report indicating that estrogen suppresses DA neuronal injury through inhibition of activated microglial function. We evaluated this possibility by determining the effects of estrogen (17β-estradiol) on DA neuron injury induced by conditioned medium from lipopolysaccharide (LPS)-activated microglia. To determine the individual role of the two ER subtypes (i.e., ERα and ERβ) in mediating such effects, we used a nonspecific ER blocker, ICI 182,780, and a relatively selective ERα blocker, MPP dihydrochloride, in our study. Furthermore, a previous study reported that ER expression in glia could be upregulated by excitoxic injury as well as stab injury (Garcia-Ovejero et al., 2002), so we investigated whether regulation of ER gene expression in microglia, astrocytes, or neurons might contribute to the neuroprotective effects of estrogen. Moreover, the ERβ protein expression levels and its subcellular distribution were also evaluated.

MATERIALS AND METHODS

Cell Cultures and Treatments

Primary microglial cells were isolated from cultures of newborn Spraque-Dawley rat brains as described in our previous paper (Le et al., 2001). Briefly, cerebral cortices were isolated from 1-day-old Sprague Dawley rats (Animal Center, Chinese Academy of Sciences, Shanghai), stripped of the meninges, minced in Hank's buffer, dissociated by trituration, and digested in the presence of 0.2% trypsin (Sigma, St. Louis, MO) and 0.01% DNase I (Sigma) for 15 min at 37°C. Cells were resuspended in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (FCS; Life Technologies), 100 mg/ml streptomycin, and 100 IU/ml penicillin and then seeded in 75 cm^2 flasks at a density of 10^7 cells per flask. After cells grew to confluence (7–10 days), the flasks were shaken at 180 rpm for 15 hr at 37°C in an orbital shaker to loosen microglia and oligodendrocytes from the more adherent astrocytes. Floating cells were collected and allowed to adhere to a flask for 1 hr before gentle shaking to separate oligodendrocytes from the relatively more adherent microglia. The microglia attached to flasks were then collected and plated on 24-well plates at a density of 2×10^5 cells/well for further experimental treatments. Obtained primary microglia had greater than 95% purity as determined by staining with 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-lindocarbocyanine perchlorate-acetylated low-density lipoprotein (DIL-ac-LDL) labeling. DIL-ac-LDL can be taken up specifically by microglia and then degraded by lysosomal enzymes. DIL (fluorescent probe) accumulates in the intracellular membranes then can be viewed under fluorescence microscopy (Giulian and Baker, 1986). After incubation with 5 μg/ml DIL-ac-LDL at 37°C for 4 hr, microglia were rinsed three times with DMEM and then visualized via fluorescence microscopy. Microglia were cultured in DMEM with 2% FCS for 24 hr before the following treatments for another 24 hr: 1) control group, without treatment; 2) LPS group, incubated with *Escherichia coli* LPS (serotype 0.111:B4; Sigma) at a final concentration of 500 ng/ml; 3) estrogen groups, incubated with 17β-estradiol (Sigma) at a final concentration of 1 nM (E-1 group) or 10 nM (E-2 group); 4) LPS + estrogen groups, incubated with 1 nM (LPS + E-1 group) or 10 nM (LPS + E-2 group) 17β-estradiol for 1 hr before and continually present when incubated with 500 ng/ml LPS; 5) ICI group, incubated with 100 nM ICI 182,780 (Tocris Cookson Inc., Ellisville, MO); 6) LPS + ICI + E-2 group, incubated with 100 nM ICI 182,780 for 0.5 hr before with 10 nM 17β-estradiol and then, 1 hr later, incubated with 500 ng/ml LPS; 7) MPP group, incubated with 1 μM MPP dihydrochloride (Tocris Cookson Inc.); and 8) LPS + MPP + E-2 group, incubated with 1 μM MPP dihydrochloride for 0.5 hr before with 10 nM 17β-estradiol and then, 1 hr later, incubated with 500 ng/ml LPS. After treatment, the conditioned medium was collected for nitrite assay or stored at −80°C for neuron treatment. The cells were either collected to extract RNA for RT-PCR analysis or to extract protein for Western blot assay or to be stained for immunocytochemical assay.

Primary culture of astrocyte was obtained from the same mixed glial culture for microglia culture as described previ-

ously (Hoffmann et al., 1996). Briefly, after shaking to remove microglia and oligodendrocytes, the adherent cells were allowed to grow for another 3 days. Then, flasks were shaken again under the same condition to remove the remaining microglia and oligodendrocytes. Then, the remaining adherent cells were released with 0.25% trypsin and reseeded. Third-passage astrocytes were used in subsequent experiments. Astrocyte purity was >95% based on immunocytochemical staining of a specific astrocyte marker, glial fibrillary acidic protein (GFAP). Astrocytes were seeded at a density of 10^5 cells/well in a 24-well plate and subjected to the same treatment as microglia and later harvested to extract either RNA or protein or processed for immuncytochemical evaluation.

Primary culture of mesencephalic neurons from embryonic Spraque-Dawley rat mesencephalon was performed as described previously (Le et al., 2001), with some modification. Briefly, region of the mesencephalon were dissected from embryonic 14-day rat brains and then minced and treated with trypsin (0.02%) and DNase I (0.01%). After mechanical dissociation by pipetting, the cells were seeded at a density of 10^5 cells/well in 24-well poly-L-lysine-coated plates and cultured for 6 days in DMEM/F12 (1:1) supplemented with 2% B27 (Sigma). Neurons purity was about 98% based on immunocytochemical staining with a specific neuron marker, MAP-2. About 5% of the neurons were DA neurons, which exhibited tyrosine hydroxylase (TH) positivity. The following conditions were employed: 1) negative control, no treatment; 2) control, the neuron medium was replaced with the microglia medium, i.e., DMEM with 2% FCS; 3) LPS group, medium was replaced with DMEM containing 2% FCS and 500 ng/ml LPS; 4) conditioned microglia medium (CMM) groups, medium for neuron was changed to CMM (including the groups of microglia treatment) and named CMM-control, CMM-LPS, CMM-E-1, CMM-LPS + E-1, CMM-E-2, CMM-LPS + E-2, CMM-ICI, CMM-LPS + ICI + E-2, CMM-MPP, or CMM-LPS + MPP + E-2; 5) estrogen + CMM-LPS (E-1 + CMM-LPS or E-2 + CMM-LPS) groups, medium was changed to CMM-LPS, and 1 nM or 10 nM estrogen was freshly added.

Evaluation of Cytotoxicity

The number of surviving DA neurons was determined by immunostaining as described previously (Le et al., 2001). Briefly, after fixation, cultured cells were incubated with monoclonal anti-TH antibody (1:3,000; Sigma) at 4°C for 24 hr and then with fluorescein isothiocyanate (FITC)-bound anti-mouse secondary antibody (1:100; Sigma) at RT for 2 hr. Then, they were incubated with polyclonal anti-MAP-2 antibody (1:1,000; Sigma) at 4°C for 24 hr, followed by TRITC-bound anti-rabbit secondary antibody (1:50; Sigma) at RT for 2 hr. MAP-2-positive cells were reflective of neurons (including DA neuron), whereas the TH and MAP-2 double-positive cells were designated as *DA neurons*. The number of TH-positive cells in 10 randomly selected fields (each of about 1 mm^2 surface area) was determined based on the number of surviving dopaminergic neurons. Counts were performed without knowledge of the correspondence to different experimental treatments.

MTT assay was used to evaluate cell viability of microglia. Briefly, cells were cultured in 96-well plates and incubated with the reagents. At the end of the incubation, 20 μl of the dye 3,[4,5-dimethylthiazol-2-yl-]diphenyltetrazolium bromide (MTT; 5 mg/ml) was added to each well, and the plates were incubated for 3 hr at 37°C. Then, 100 μl of lysis buffer [20% sodium dodecyl sulfate (SDS) in 50% N,N-dimethylformamide, containing 0.5% (v:v) 80% acetic acid and 0.4% (v:v) 1 N HCl] was added to each well, and the optical density (proportional to the number of live cells) was assessed with a microplate reader at 570 nm. Each experiment was performed in triplicate. Results of three independent experiments were used for statistical analysis.

Nitrite Production Assay

Levels of the nitric oxide (NO) derivative nitrite were determined in the conditioned medium of microglia with the Griess reaction. A nitrite detection kit (Beyotime Biotech Inc., Jiangsu, People's Republic of China) was used according to instructions provided by the manufacturer. The samples were assayed in triplicate, and a standard curve using $NaNO_2$ was generated for each experiment for quantification. Briefly, 100 μl of medium or standard $NaNO_2$ was mixed with 100 μl of Griess reagent in a 96-well plate. After 15 min, optical density was read in a microplate reader at 540 nm with the reference filter set at 630 nm. Each experiment was performed in triplicate. Results of three independent experiments were used for statistical analysis.

RNA Extraction and PCR

RNA was extracted with Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. To eliminate any residual genomic DNA, the RNA samples were incubated with DNase I for 15 min at room temperature. Thereafter, DNase I was inactivated by the addition of 1 μl of 25 mM EDTA and heating at 65°C for 10 min. Yield of total RNA was determined by A_{260} measurement with a spectrophotometer. Aliquots of 2 μg of total cellular RNA were used for first-strand cDNA synthesis in 20 μl of reaction volume using SuperScript II Rnase H– Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. The primers used for PCR assay are listed in Table I. The primers were the same as previously reported for TNF-α (Melhus, 2001) and for ERα (Ohsako et al., 2003) and ERβ (Zschocke et al., 2002). The primer pairs for ERs (either ERα or ERβ) were located on different exons of the gene to avoid amplification of possible genomic DNA. Primers for GAPDH were used to ensure RNA equivalence among the different conditions. All primers were purchased from Sangon Biotech. Inc. (Shanghai, People's Republic of China). PCR was carried out in a 25-μl reaction volume that contained 2 μl of cDNA, 2.5 μl of 10× PCR buffer (Invitrogen), 0.75 μl of 50 mM $MgCl_2$ (Invitrogen), 0.5 μl of 10 mM dNTPs (Invitrogen), 0.5 μl of 15 pmol forward and reverse primers, and 0.5 U of DNA *Taq* polymerase (Invitrogen). PCR cycle conditions were 94°C for 30 sec, 58 or 61°C for 30 sec, and 72°C for 45 sec for 25–35 cycles, with an initial denaturation at 94°C for 5 min and a final extension of 5 min at 72°C. The PCR products were

TABLE I. Primers Used for PCR

Gene	Forward primer	Reverse primer	Product size (bp)	Cycle used	GeneBank accession No.
TNF-α	5′-AGTCTTCCAGCTGGAGAAGG-3′	5′-GCCACTACTTCAGCATCTCG-3′	318	35	AF329987
ERα	5′-TTACGAAGTGGGCATGATGA-3′	5′-ATCTTGTCCAGGACTCGGTG-3′	711	35	NM_012389
ERβ	5′-GAGGCCTCCATGATGATGTC-3′	5′-TCTCCAGCAGCAGGTCATAC-3′	610	35	AJ002602
GAPDH	5′-CCATGTTCGTCATGGGTGTGAACCA-3′	5′-GCCAGTAGAGGCAGGGATGATGTTC-3′	229	25	XM_227696

size fractionated on agarose gels and visualized with ethidium bromide. The densities of the PCR products were quantified with Quantity One software. The relative expression level of each targeted gene was calculated as the band density of the product of that gene divided by that of the GAPDH gene derived from the same cDNA.

Western Blot

Cells were washed three times with cold TBS, harvested with a cell scraper, and lysed in 10 volume of cold lysis buffer [50 mM Tris-HCl, pH 7.2, 250 mM NaCl, 0.1% NP-40, 2 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml aprotinin, 5 μg/ml leupeptin] on ice. Lysates were centrifuged, and then the supernatant protein was denatured by mixing with an equal volume of 2× sample loading buffer and then boiling at 100°C for 5 min. An aliquot (15 mg as protein) of the supernatant was loaded onto an SDS-polyacrylamide gel, separated electrophoretically, and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA). After the PVDF membrane had been incubated with 10 mM TBS with 1.0% Tween 20 and 10% dehydrated skim milk to block nonspecific protein binding, the membrane was incubated with monoclonal anti-ERβ antibody (1:1,000; clone 9.88; Sigma) overnight at 4°C. This antibody recognizes a 53-kDa protein, which is designated as *ER*β. Blots were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Sigma) for 45 min at room temperature at a 1:3,000 dilution and then resolved by enhanced chemiluminsecence (ECL; Amersham Life Sciences, Arlington Heights, IL). The densities of the Western products were quantified with Quantity One. The relative protein expression level of each targeted protein was calculated as the band density of the protein divided by that of the β-actin derived from the same protein sample.

Immunocytochemical Assay

Cells were washed three times and then fixed for 15 min at 4°C in 4% paraformaldehyde. After incubation with phosphate-buffered saline (PBS) containing 0.2% Triton X-100 for 10 min at 4°C, cells were incubated with PBS containing 5% bovine serum albumin (BSA) and 1% normal goat serum to block nonspecific protein binding. For microglia, cells were then incubated with primary antibody (anti-ERβ; 1:1,000; Sigma) overnight at 4°C, then incubated with secondary antibody (FITC-bound anti-mouse IgG; 1:100; Sigma) at RT for 2 hr, and finally nuclear DNA was stained by incubating with 100 ng/ml 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI) in PBS at RT for 3 min. For astrocytes, cells were subjected to similar procedures, but the primary antibodies used were the anti-ERβ antibody and the polyclonal anti-GFAP antibody (1:500; Sigma) and the secondary antibodies were FITC-bound anti-mouse IgG and TRITC-bound anti-rabbit IgG (1:100; Sigma). For neuron, cells were subjected to similar procedures, and the primary antibodies used were the anti-ERβ antibody and the polyclonal anti-MAP-2 antibody (1:1,000; Sigma) and the secondary antibodies were FITC-bound anti-mouse IgG and TRITC-bound anti-rabbit IgG (1:100; Sigma).

Statistical Analysis

One-way ANOVA were performed in Graphpad Prism 4. Based on the one-way analysis of variance, a posttest (Bonferroni's multiple comparison test) was conducted. Statistical significance was set at the 95% confidence limit ($P < 0.05$). Data are expressed as mean ± SEM of at least three independent experiments.

Fig. 1. Neuroprotective effects of estrogen on DA neurons against injury induced by conditioned medium of LPS-activated microglia. **A:** Immunocytochemical staining of primary cultured mesencephalon neuron. In merged figures, non-DA neurons appeared as red (stained with MAP-2) and DA neurons appeared as yellow (doubly stained with MAP-2 and TH). Compared with CMM-control (incubated with medium of microglia without treatment), the number of DA neurons decreased after incubated with medium of LPS-activated microglia for 24 hr (CMM-LPS group). Pretreatment of microglia with 10 nM estrogen for 1 hr before LPS activation (CMM-LPS + E-2) blocked the injury of LPS-activated microglia medium on DA neurons. Shown are representative images from three independent experiments. **B:** Result of counting the number of DA (TH-positive) neurons in primary cultured mesencephalic neuron. Conditioned medium of LPS-activated microglia (CMM-LPS) caused a decrease in the number of DA neurons. Pretreatment of microglia before LPS activation with 1 nM or 10 nM estrogen (CMM-LPS + E-1 or CMM-LPS + E-2) protected DA neurons from the injury. Blocking ERs with ICI almost totally blocked the neuroprotective effects of estrogen, whereas specific blocking of ERα with MPP only partially attenuated the estrogen effects. Values are expressed as percentage of control and represent the mean ± SEM of at least three separate experiments. $^{**}P < 0.01$ vs. control.

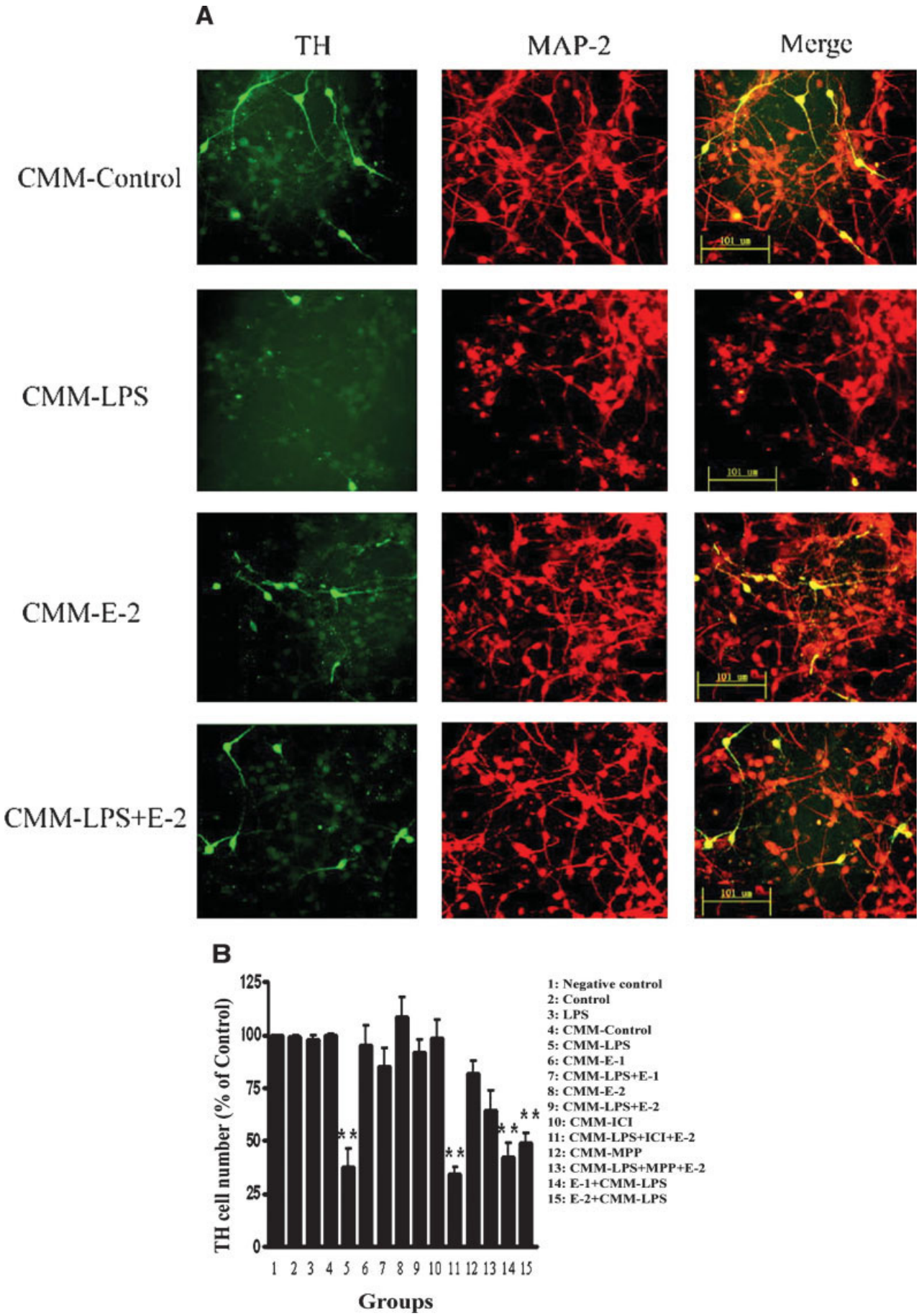
A
TH
MAP-2
Merge
CMM-Control
CMM-LPS
CMM-E-2
CMM-LPS+E-2
101 um
B
TH cell number (% of Control)
125
100
75
50
25
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Groups
1: Negative control
2: Control
3: LPS
4: CMM-Control
5: CMM-LPS
6: CMM-E-1
7: CMM-LPS+E-1
8: CMM-E-2
9: CMM-LPS+E-2
10: CMM-ICI
11: CMM-LPS+ICI+E-2
12: CMM-MPP
13: CMM-LPS+MPP+E-2
14: E-1+CMM-LPS
15: E-2+CMM-LPS

Figure 1.

RESULTS

Neuroprotection of Estrogen Against Injury Induced by CMM

The conditioned medium of LPS-activated microglia (CMM-LPS group) caused significant injury to DA neurons (Fig. 1). Figure 1A shows representative figures of MAP-2 and TH immunocytochemically stained primary mesencephalon neurons. In merged figures, non-DA neurons are MAP-2 positive (red) and DA neurons appear yellow because of double staining with MAP-2 (red) and TH (green). After exposure to conditioned medium of LPS-activated microglia for 24 hr (CMM-LPS), the neurites of DA neurons were attenuated and their numbers decreased. Pretreatment of microglia with 10 nM estrogen for 1 hr before LPS activation (CMM-LPS + E-2) inhibited injury to DA neurons following exposure to conditioned medium. Conditioned medium of nonactivated microglia treated only with 10 nM estrogen (E-2) had no effect on DA neurons. Figure 1B shows the number of TH-positive DA neurons in primary mesencephalon neuron culture after treatment. There is no difference among negative control group (without treatment), control group (neuron medium replaced with the microglia medium), and CMM-control group (conditioned medium of microglia without treatment). Also, no significant change was observed in the LPS group (500 ng/ml LPS added directly into neuron culture). In a separate study, to rule out the possibility that LPS might have direct toxic effects on mesencephalon neurons, we added LPS up to 4 μg/ml in the cultures for 24 hr, and we did not detect any morphological changes upon visual inspection and cytotoxicity assay, which is consistent with an earlier report (Le et al., 2001). Estrogen itself (CMM-E-1 and CMM-E-2 groups) had no effect on neuronal survival or growth. The conditioned medium of LPS-activated microglia decreased the number of DA neurons. Pretreatment with either 1 nM or 10 nM estrogen before LPS activation decreased DA neuronal injury equally. Furthermore, the neuroprotective effects of estrogen pretreatment may be based mainly on the effects of estrogen on microglia and not directly on the neuron. This is indicated by the fact that estrogen supplementation of the conditioned medium of LPS-activated microglia was less protective against DA neuronal loss (data not shown). Blocking ER (both ERα and ERβ) with ICI 182, 780 almost totally blocked the neuroprotective effects of estrogen. MPP dihydrochloride is a new specific ERα antagonist, which at 1 μM had no effect on ERβ (Sun et al., 2002). In the current study, 1 μM MPP dihydrochloride only partially attenuated estrogen-induced neuroprotection. This suggests that both ERα and ERβ contribute to neuroprotection. It is not possible to delineate definitively their relative contributions to neuroprotection because of the lack of availability of a specific ERβ blocker.

Inhibition of Microglial Activation by Estrogen

To test whether estrogen has inhibitory effects on LPS-induced microglial activation as a possible mechanism of neuroprotection in our cell model, we examined the morphology of microglia with low-density lipoprotein (LDL) staining (Fig. 2A), determined TNF-α mRNA levels in the microglia (Fig. 2B), and assayed nitrite production in the culture medium of microglia (Fig. 2C) in the presence or absence of estrogen. In the control group, microglia exhibited the ramified morphology typical of the resting state, which is characterized by long secondary and tertiary branched processes arising from a flattened and elongated cell body. After exposure to LPS (500 ng/ml for 24 hr), most microglial cells changed into amoeboid morphology, typical of the phagocytic and activated state. However, with estrogen (10 nM) incubation for 1 hr before LPS stimulation, the microglia showed less activation than with LPS treatment alone (Fig. 2A), which is similar to findings in previous reports (Bruce-Keller et al., 2000; Vegeto et al., 2001; Baker et al., 2004). This result indicates that estrogen can inhibit LPS-induced activation of microglia.

LPS-induced increases in the levels of TNF-α expression and nitrite production were used as indices of microglial activation. As shown in Figure 2B,C, LPS increased the expression of TNF-α and nitrite production. Pretreatment estrogen inhibited increases in TNF-α

Fig. 2. Estrogen inhibition of microglia activation induced by LPS. **A:** The LPS-induced morphological activation of microglia is prevented by pretreatment with estrogen (10 nM) for 1 hr. Microglia were stained with LDL to show the purity (more than 95%) of primary cultured microglia by comparison with the bright field photo (control). Control microglia exhibited the ramified morphology typical of the resting state, and this changed to amoeboid, activated morphology after treated with LPS (500 ng/ml) for 24 hr. Pretreatment with estrogen (10 nM) for 1 hr before LPS activation could decrease activation of microglia. Shown are representative images from three independent experiments. **B:** The LPS-induced increase of TNF-α mRNA expression in microglia is prevented by pretreatment with estrogen (10 nM) for 1 hr. The GAPDH expression level was used as internal control. The ER antagonist ICI blocked the inhibitory effects of estrogen on the TNF-α expression increase in LPS-treated microglia, whereas the specific ERα antagonist MPP can only partially block the estrogen effects. Shown are representative figures and the statistical results of three independent experiments. Values (percentage of control) are expressed as mean ± SEM. $^{**}P < 0.01$ vs. control. **C:** The LPS-induced increase of nitrite production in microglia is prevented by pretreatment with estrogen (10 nM) for 1 hr. The treatments are the same as for B. The ER antagonist ICI blocked the inhibitory effects of estrogen on nitrite production in LPS-treated microglia, whereas the specific ERα antagonist MPP can only partially block the estrogen effects. Values are expressed as percentage of LPS-treated group and represent the mean ± SEM of at least three separate experiments. $^{*}P < 0.05$ vs. LPS group. $^{**}P < 0.01$ vs. LPS group. **D:** The cell viability of microglia was not affected by treatment with estrogen (1 nM or 10 nM), ICI 182,780 (100 nM), or MPP dihydrochloride (1 μM). Values are expressed as percentage of control and represent the mean ± SEM of at least three separate experiments.

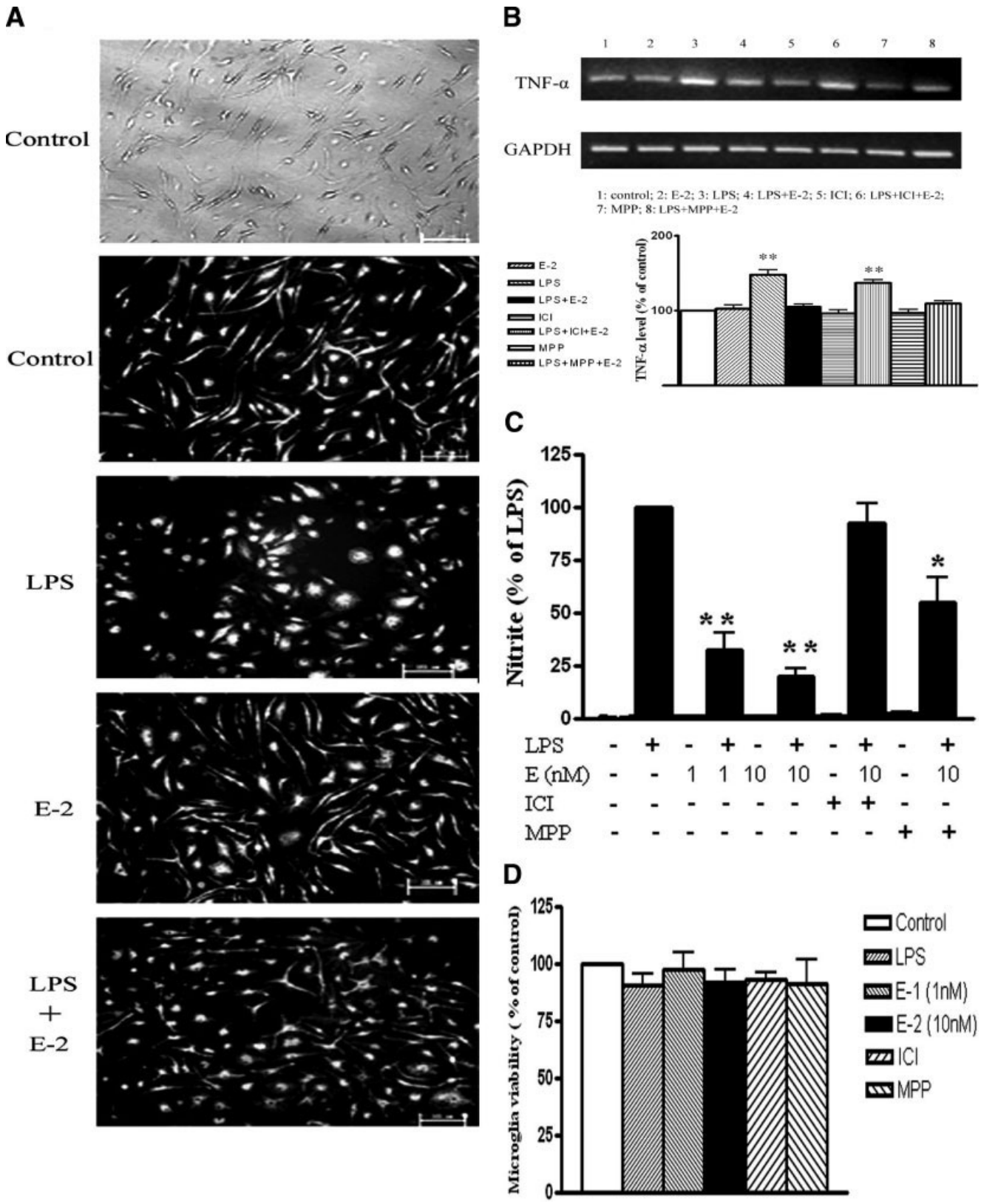
A
Control
Control
LPS
E-2
LPS
+
E-2
B
1 2 3 4 5 6 7 8
TNF-α
GAPDH
1: control; 2: E-2; 3: LPS; 4: LPS+E-2; 5: ICI; 6: LPS+ICI+E-2;
7: MPP; 8: LPS+MPP+E-2
E-2
LPS
LPS+E-2
ICI
LPS+ICI+E-2
MPP
LPS+MPP+E-2
TNF-α level (% of control)
200
100
**
**
C
Nitrite (% of LPS)
125
100
75
50
25
0
**
**
*
LPS - + - + - + - + - +
E (nM) - - 1 1 10 10 - 10 - 10
ICI - - - - - - + + - -
MPP - - - - - - - - + +
D
Microglia viability (% of control)
125
100
75
50
25
0
Control
LPS
E-1 (1nM)
E-2 (10nM)
ICI
MPP

Figure 2.

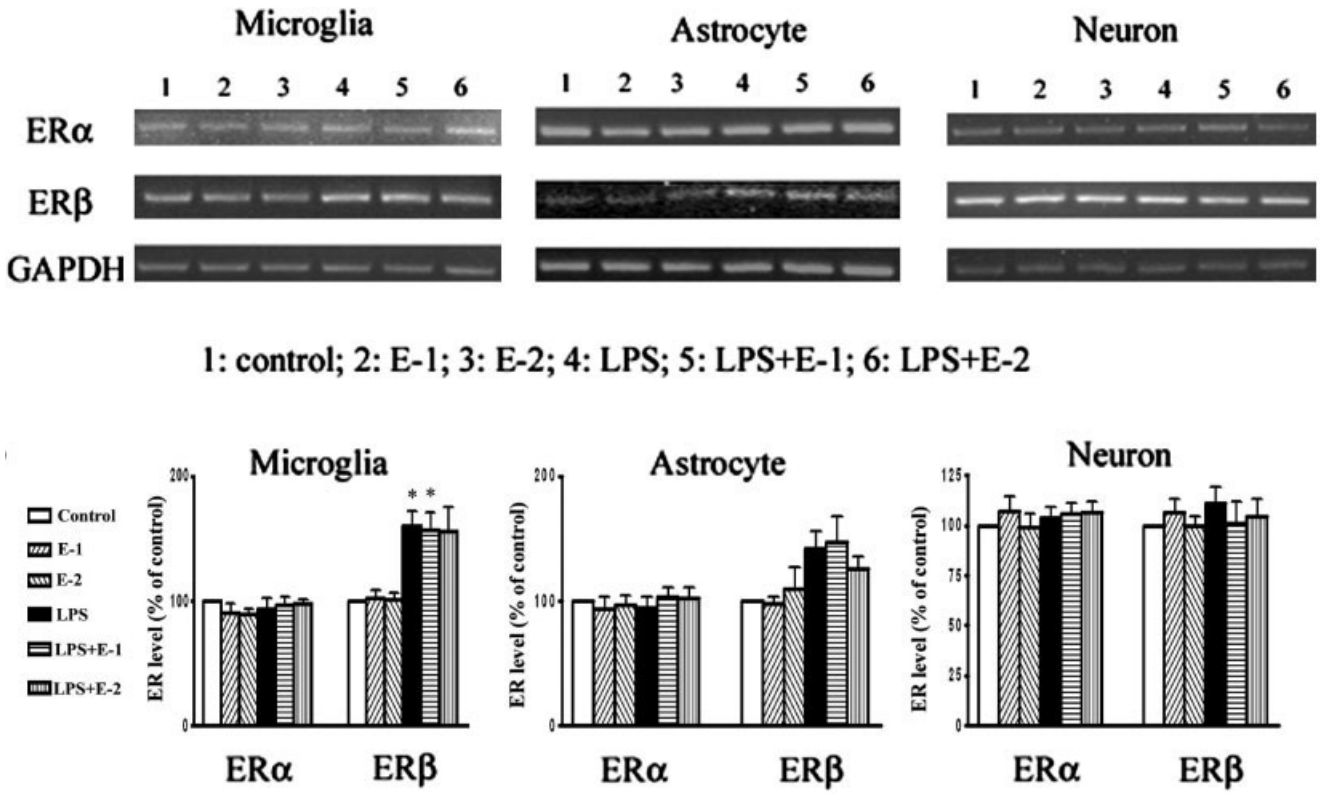

Fig. 3. Expression of ERα and ERβ mRNA in primary cultured microglia, astrocytes, and mesencephalon neurons. The expression of GAPDH was used as internal control. LPS treatment had no effect on ERα expression in all kinds of cells. For ERβ expression, LPS induced an increase in microglia. Treatment with only estrogen (1 nM or 10 nM) had no effect on either ERα or ERβ expression. Pretreatment with estrogen for 1 hr before LPS stimulation did not affect the up-regulation of ERβ in microglia and astrocyte. Shown are representative figures and the statistical result of three independent experiments. Values (percentage of control) are expressed as mean ± SEM. $*P < 0.05$ vs. control.

expression and nitrite production. The nonselective ER antagonist ICI 182, 780 blocked the inhibitory effects of estrogen on LPS-induced increases in TNF-α expression and nitrite production. The specific ERα antagonist MPP dihydrochloride only partially blocked the estrogen effects on microglial activation. Neither estrogen (1 nM or 10 nM), nor ICI 182,780, nor MPP dihydrochloride was toxic to microglia at these concentrations based on invariance of the MTT assay (Fig. 2D). Thus, estrogen inhibition of LPS-induced microglial activation is not due to effects on cell viability. Because activated microglia are believed to contribute to neuronal injury at least partially resulting from NO and TNF-α production, estrogen-induced neuroprotection may be related to declines in TNF-α expression and NO production.

mRMA Level of ER in Microglia, Astrocyte, and Neuron

By using PCR, we can detect both ERα and ERβ expression in microglia, astrocytes, and neurons (Fig. 3). In all cases, LPS had no effect on ERα expression but significantly increased the expression of ERβ in microglia and caused slight increase of ERβ in astrocytes, but not in neurons (Fig. 3). Estrogen (E-1 or E-2) had no effect on the mRNA level of either ERα or ERβ. In addition, pretreatment of estrogen before LPS stimulation (LPS + E-1 or LPS + E-2) did not totally block the increase of ERβ mRNA induced by LPS in microglia and astrocytes.

ERβ Protein Levels in Microglia, Astrocyte, and Neuron

Because LPS induced increases of ERβ gene expression in microglia, its corresponding effects on ERβ protein expression was determined by Western blot assay (Fig. 4). In microglia, an increase in ERβ protein was clearly observed after LPS activation. Estrogen (E-2) had no effect on the protein levels of ERβ, and pretreatment of estrogen before LPS stimulation (LPS + E-2) did not block, although it partially inhibited, the increase in ERβ protein induced by LPS in microglia, and no change in ERβ protein was observed in astrocytes and neurons after LPS stimulation. Estrogen (E-2) also had no effect on the protein levels of ERβ in either astrocytes or neurons.

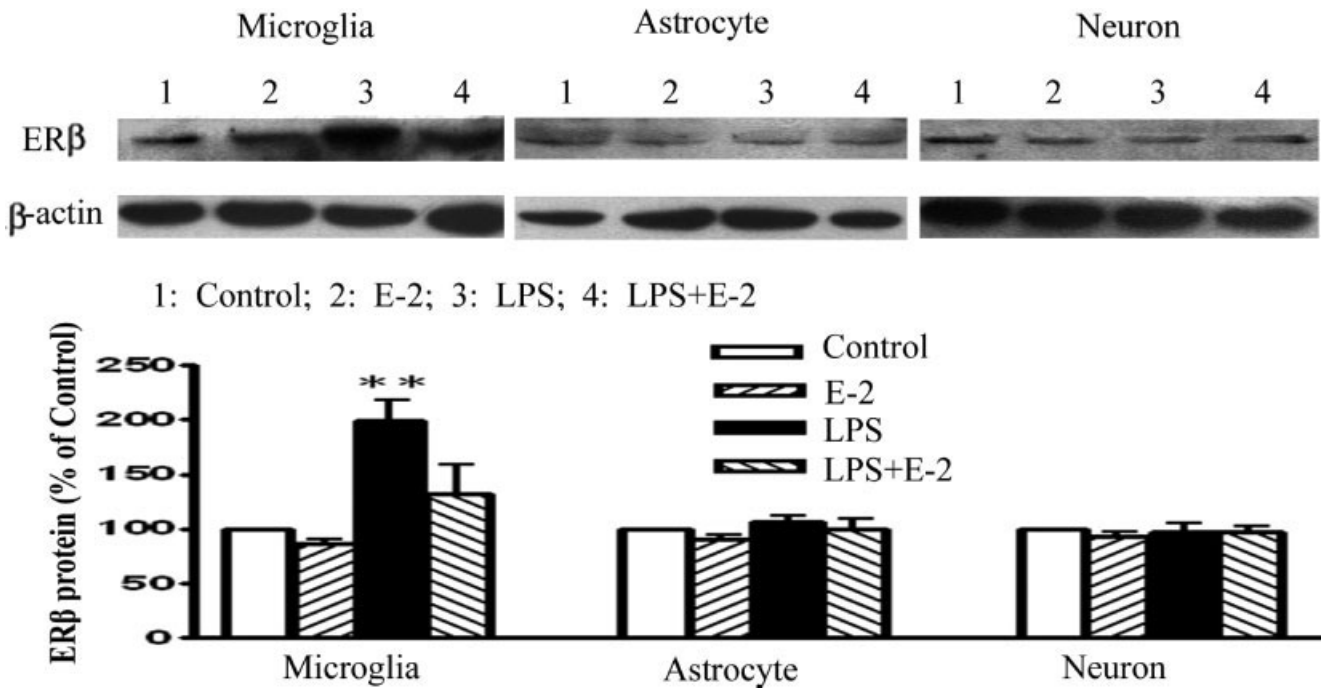

Fig. 4. Expression of ERβ protein in primary cultured microglia, astrocytes, and mesencephalon neurons. The expression of β-actin was used as internal control. LPS treatment induced an increase in microglia. Treatment with estrogen (E-2, 10 nM) had no effect on ERβ protein level. Pretreatment with estrogen for 1 hr before LPS stimulation did not affect the up-regulation of ERβ in microglia. Shown are representative figures and the statistical result of three independent experiments. Values (percentage of control) are expressed as mean ± SEM. $^{**}P < 0.01$ vs. control.

Subcellular Location of ERβ in Microglia, Astrocyte, and Neuron

Subcellular localization of ERβ in microglia (normal and activated), astrocytes, and neurons is shown in Figure 5. ERβ was expressed almost exclusively in the cytoplasm but not in the nuclei of resting microglia. This assignment is based on the immunocytochemical finding that ERβ green staining was almost completely overlayed with the LDL red signal, which marked the cytoplasm of microglia, whereas no ERβ signal was overlayed with blue nuclear DAPI staining. In LPS-activated microglia, the ERβ was also almost exclusively localized in the cytoplasm. The cytoplasmic localization of ERβ indicated that the effects of estrogen on microglia may be different from the classical nuclear ER-mediated pathway. On the contrary, ERβ was localized almost exclusively in the nuclei of astrocytes and neurons and not in the cytoplasm. To avoid the possible influence of multiple staining on ERβ detection, single immunocytochemical staining of ERβ was also conducted in the three kinds of cells (data not shown). The results of single ERβ staining were similar to the multiple staining results and clearly suggested that most of the ERβ expression is restricted to microglia cytoplasm and nuclei of astrocytes and neurons.

DISCUSSION

Evidence from post-mortem study indicated the involvement of microglial activation in PD (Rogers et al., 1988). Although it is still an open question whether microglial activation plays a role in initiating PD or occurs merely as a response to neuronal death, a positive correlation with early-life brain injuries, such as antecedent traumatic brain injury, exposure to certain viruses or other infectious agents, and late development of PD, implies that inflammation in the brain, and specifically microglial activation, plays a critical role in the early stage(s) of the pathogenesis of PD (Liu and Hong, 2003). An inflammation-mediated model of PD using continuous supranigal infusion of nanogram quantities of LPS has been established (Gao et al., 2002). LPS has no known direct toxic effects on neurons but is a powerful tool for inducing activated microglia that release a host of neurotoxic factors. The substantia nigra region of the brain is reported to be particularly susceptible to LPS-induced injury because it is rich in microglia (Kim et al., 2000). Thus, it is important to unravel mechanisms regulating microglial activation to provide insight into efficient therapeutic intervention.

The current study demonstrates that conditioned medium of LPS-activated microglia induces injury in primary rat mesencephalon DA neurons. Pretreatment of microglia before LPS activation with 1 nM or 10 nM estrogen could protect neural cells from the injury. Our result supports the hypothesis that, together with the direct effects on neurons, the neuroprotective mechanism of estrogen in DA neurons in vivo may include direct effects on microglia. In agreement with previous reports (Bruce-Keller et al., 2000; Vegeto et al., 2001;

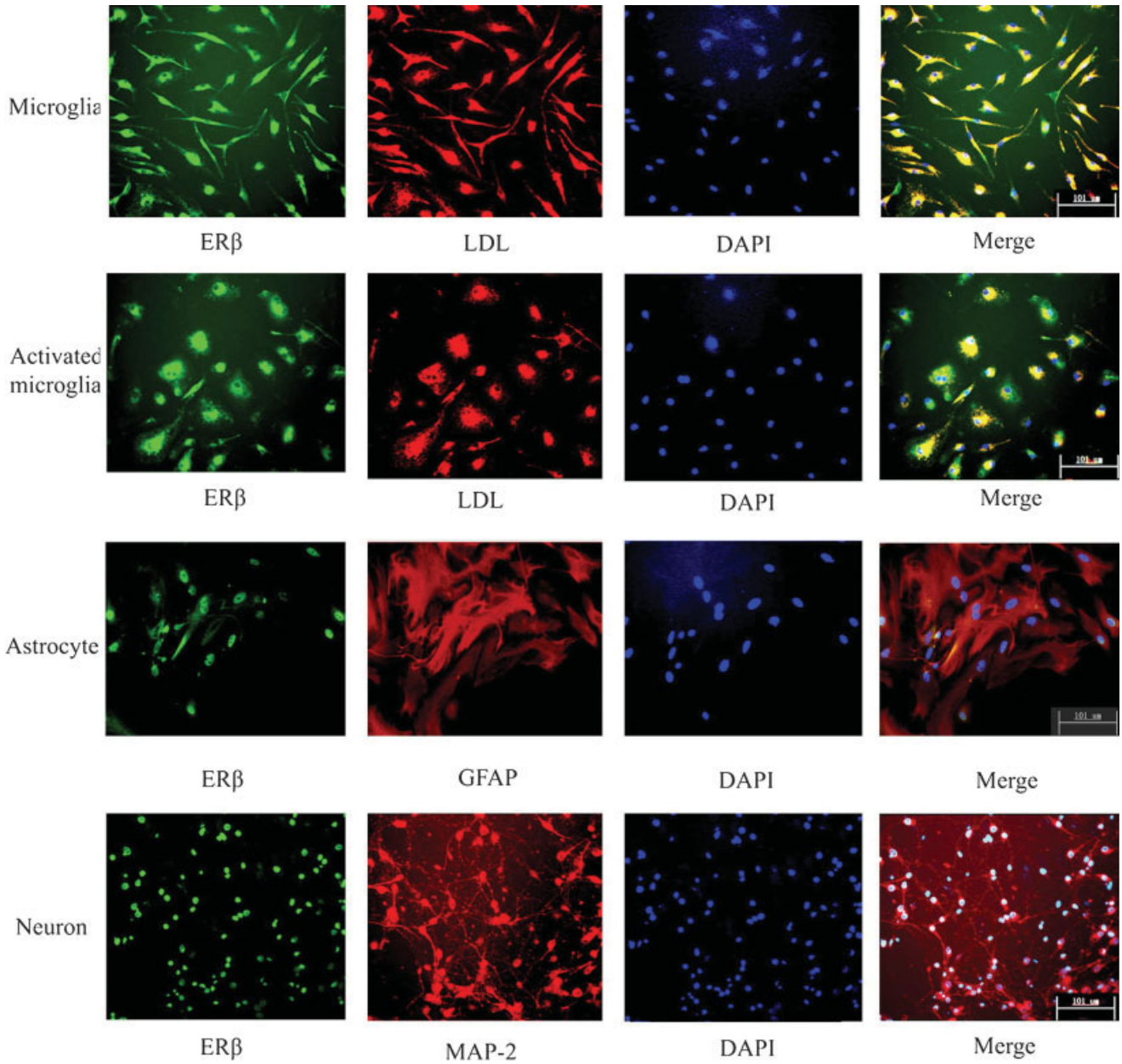

Fig. 5. Subcellular location of ERβ protein detected by immunocytochemistry in primary cultured resting microglia, LPS-activated microglia, astrocytes, and mesencephalon neurons. Microglia were stained with ERβ (green) or LDL (red), and the nuclei were counterstained with DAPI (blue). Overlay of ERβ and LDL but not DAPI indicated that the almost exclusive expression of ERβ in the cytoplasm of both resting and LPS-activated microglia. Astrocytes were stained with ERβ (green) or GFAP (red), and the nuclei were counterstained with DAPI (blue). Overlay of most of the ERβ signal and DAPI indicated the nuclear expression of ERβ in astrocytes. Neurons were stained with ER (green) or MAP-2 (red), and the nuclei were counterstained with DAPI (blue). Overlay of most of the ERβ signal and DAPI indicated the nuclear expression of ERβ in neuron. Shown are representative figures from three independent experiments.

Baker et al., 2004), in the present study estrogen inhibited the activation of microglia by LPS. The inhibition of increases in NO and TNF-α levels in activated microglia might partially contribute to the neuroprotective effect of estrogen against DA neuronal injury. Furthermore, estrogen was reported to affect in vivo both the activation of glial cells and the number of glial cells. Estrogen treatment could inhibit the activation of glial cells associated with estrogen deprivation in aged female animal (Mor et al., 1999). In female mice, accompanying the increase in age, there is a significant 20–25% increase in the numbers of microglia and astrocytes in the hippo-

campal formation (Mouton et al., 2002), although 17β-estradiol treatment significantly lowered the numbers of glial cells compared with placebo (Lei et al., 2003).

It is well known that the biological effects of estrogen are manifested through two genetically distinct estrogen receptors (ERα and ERβ) that display different expression patterns in target tissues. Mice with genetic disruptions of ERα and ERβ displayed different phenotypes, demonstrating that each receptor subtype has a distinct biological property (Couse and Korach, 1999). However, the mechanism for the difference in ERα- and ERβ-mediated biological activities is not fully clear. It has been reported that estrogen acting at the AP-1 site enhances transcription of ERα but suppresses that of ERα with the activation of ERβ (Paech et al., 1997). ERα and ERβ can interact with each other (Linberg et al., 2003). The contributions of the two ER subtypes in mediating the effects of estrogen on microglia were inconclusive. In an in vivo study in which LPS was injected into ER-null mouse brains or was systemically administered, estrogen inhibited microglial activation through the ERα subtype but not the ERβ (Vegeto et al., 2003). On the contrary, Baker et al. (2004) showed that ERβ mediated the inhibition of microglia by using BV-2 microglia thar expressed only ERβ. Previously, the lack of subtype-specific antagonists has made it difficult to define the processes that are regulated by ERα and/or ERβ. Although there is still no specific ERβ antagonist available, we were able to employ a new specific ERα antagonist, MPP dihydrochloride. The primary cultured microglia in the present study express both ERα and ERβ. The ER blocker ICI 182, 780 both blocked the estrogen-induced neuroprotective effects on neurons and inhibited its effects on microglia. The ERα-specific antagonist MPP dihydrochloride only partially blocked the effects of estrogen. Our results indicate that both ER subtypes contribute to the effects of estrogen on microglia. Furthermore, the mRNA levels of ERβ but not ERα can be up-regulated by LPS treatment in microglia. Western blot changes also paralleled variations in ERβ gene expression in microglia. The fact that the ERβ expression can be up-regulated by LPS stimulation supports the view that the role of ERβ may be more important in the activated microglia than in resting microglia. A previous report also showed that ERs on microglia and astrocytes are highly up-regulated following excitotoxic insults and stab wounds to cortical regions of rat brain (Garcia-Ovejero et al., 2002). The up-regulation of ERβ expression in microglia as well as astrocytes also indicates that the increased reactivity to estrogen after injury contributes to the effects of estrogen on glial cells.

We also detected the expression of both ER subtypes in astrocyte as well as in primary cultured mesencephalon neurons. Unlike ERα, which plays a major role in the reproductive tissues, ERβ was shown in previous reports to be very important in brain development and function, especially for the midbrain. In the ERβ knockout mice, there was degeneration of neuronal cell bodies throughout the brain, and the degeneration was particularly evident in the substantia nigra. As the mice aged, the neuronal deficit became more pronounced. A remarkable proliferation of astroglial cells was also observed (Wang et al., 2001). A recent report showed that genetic variation in the ERβ gene may influence the age of onset of PD (Westberg et al., 2004). ERβ was expressed in neuron, microglia, and astrocyte, so the role of ERβ in midbrain may include its function in all cell types. ERβ may have an important influence on neuronal survival and glial cell activation and consequently affect the development of neurodegenerative diseases such as PD.

Although estrogen receptors have been studied for many years, the exact molecular mechanism by which estrogen receptors mediate the effects of estrogen has not yet been fully clarified. In the classic model of estrogen action, the unoccupied nuclear ER resides in the nuclei of target cells in an inactive form. Binding to an agonist, such as estradiol, alters the physicochemical properties of the ER, allowing the receptor dimer to interact with specific DNA sequences (estrogen response elements) within the promoters of responsive genes. The DNA-bound ER then regulates target-gene transcription, either positively or negatively. However, it is now accepted that extranuclear ERs exist. The discovery of nonnuclear localization of ER challenged the classical mechanism of action of ERs and made the situation more complicated. A number of membrane ER and cytoplasmic ER have been reported. There is a relatively smaller plasma membrane pool than nuclear receptor pool, and signals elicited by plasma membrane ER activation initiate both posttranscriptional and transcriptional actions of estrogen (Levin, 2001; Losel and Wehling, 2003; Razandi et al., 2004; Arvanitis et al., 2004). In oligodendrocytes, ERβ was found mainly in the cytoplasm and veins (Zhang et al., 2004). In astrocytes and some kinds of neurons, ERβ was located mainly in nuclei but was also reported to be localized to the cytoplasm and plasma membrane of these cell types (McEwen, 2002; Zhang et al., 2002; Nishio et al., 2004). In a recent report using a mitochondrial marker, the ERβ colocalized almost exclusively with a mitochondrial marker in rat primary cerebral cortical and hippocampal neurons (Yang et al., 2004). In the present study, immunocytochemical staining of ERβ was located mostly in the cell nucleus of astrocyte and mesencephalon neurons but also in the cytoplasm of microglia. The localization of ERβ in primary cultured DA neuron is in agreement with a previous report (Sawada et al., 2002). The exclusive nuclear expression of ERβ in neuron and astrocyte suggests that estrogen may act through a classical genomic mechanism in these two kinds of cells. On the contrary, the cytoplasmic localization of ERβ in primary cultured microglia in the present study indicates the involvement of a nonclassical mechanism of estrogen action on microglia. As far as we know, the cytoplasmic localization of ERβ in microglia has not been reported previously. Further study is required to identify down-

stream cytoplasmic kinase cascade pathways, which will help to unravel the mechanism of estrogen's action on microglia.

In summary, the present study has demonstrated that estrogen-mediated neuroprotection against DA neuron degeneration may result from inhibition of microglial activation. Furthermore, the present study indicates that both ERα and ERβ may contribute to the estrogen-mediated microglial activation and neuroprotection. In addition, the present study shows that the inflammatory factor LPS can induce ERβ expression in microglia but not in neurons, implying that the microenvironments in the brain may play an important role in eliciting a response to estrogen. ERβ is localized in the cytoplasm of microglia, suggesting that some of the effects of estrogen on microglia may be mediated by a nonclassical mechanism.

REFERENCES

Arvanitis DN, Wang H, Bagshaw RD, Callahan JW, Boggs JM. 2004. Membrane-associated estrogen receptor and caveolin-1 are present in central nervous system myelin and oligodendrocyte plasma membranes. J Neurosci Res 75:603–613.

Baker AE, Brautigam VM, Watters JJ. 2004. Estrogen modulates microglial inflammatory mediator production via interactions with estrogen receptor beta. Endocrinology 145:5021–5032.

Blanchet PJ, Fang J, Hyland K, Arnold LA, Mouradian MM, Chase TN. 1999. Short-term effects of high-dose 17beta-estradiol in postmenopausal PD patients: a crossover study. Neurology 53:91–95.

Bruce-Keller AJ, Keeling JL, Keller JN, Huang FF, Camondola S, Mattson MP. 2000. Anti-inflammatory effects of estrogen on microglial activation. Endocrinology 141:3646–3556.

Callier S, Morissette M, Grandbois M, Pelaprat D, Di Paolo T. 2001. Neuroprotective properties of 17beta-estradiol, progesterone, and raloxifene in MPTP C57Bl/6 mice. Synapse 41:131–138.

Couse JF, Korach KS. 1999. Estrogen receptor null mice: what have we learned and where will they lead us? Endocr Rev 20:358–417.

Craig MC, Cutter WJ, Wickham H, van Amelsvoort TA, Rymer J, Whitehead M, Murphy DG. 2004. Effect of long-term estrogen therapy on dopaminergic responsively in post-menopausal women—a preliminary study. Psychoneuroendocrinology 29:1309–1316.

Currie LJ, Harrison MB, Trugman JM, Bennett JP, Wooten GF. 2004. Postmenopausal estrogen use affects risk for Parkinson disease. Arch Neurol 61:886–888.

Dhandapani KM, Brann DW. 2002. Estrogen-astrocyte interactions: implications for neuroprotection. BMC Neurosci 3:6.

Dluzen D, Horstink M. 2003. Estrogen as neuroprotectant of nigrostriatal dopaminergic system: laboratory and clinical studies. Endocrinology 21: 67–75.

Gao HM, Jiang J, Wilson B, Zhang WQ, Hong JS, Liu B. 2002. Microglial activation-mediated delayed and progressive degeneration of rat nigral dopaminergic neurons: relevance to Parkinson's disease. J Neurochem 81:1285–1297.

Garcia-Ovejero D, Veiga S, Garcia-Segura LM, Doncarlos LL. 2002. Glial expression of estrogen and androgen receptors after rat brain injury. J Comp Neurol 450:256–271.

Giulian D, Baker TJ. 1986. Characterization of ameboid microglia isolated from developing mammalian brain. J Neurosci 6:2163–2178.

Grandbois M, Morissette M, Callier S, Di Paolo T. 2000. Ovarian steroids and raloxifene prevent MPTP-induced dopamine depletion in mice. Neuroreport 11:343–346.

Hoffmann AM, Bakardjiev A, Bauer K. 1996. Carnosine-synthesis in cultures of rat glial cells is restricted to oligodendrocytes and carnosine uptake to astrocytes. Neurosci Lett 215:29–32.

Kim WG, Mohney RP, Wilson B, Jeohn GH, Liu B, Hong JS. 2000. Regional difference in susceptibility to lipopolysaccharide-induced neurotoxicity in the rat brain: role of microglia. J Neurosci 20:6309–6316.

Kompoliti K. 2003. Estrogen and Parkinson's disease. Front Biosci 8: s391–s400.

Le WD, Rowe D, Xie WJ, Ortiz I, He Y, Appel SH. 2001. Microglial activation and dopaminergic cell injury: an in vitro model relevant to Parkinson's disease. J Neurosci 21:8447–8455.

Lei DL, Long JM, Hengemihle J, O'Neill J, Manaye KF, Ingram DK, Mouton PR. 2003. Effects of estrogen and raloxifene on neuroglia number and morphology in the hippocampus of aged female mice. Neuroscience 121:659–666.

Levin ER. 2001. Cell Localization, physiology and nongenomic actions of estrogen receptors. J Appl Physiol 91:1860–1867.

Lindberg MK, Moverare S, Skrtic S, Gao H, Dahlman-Wright K, Gustafsson JA, Ohlsson C. 2003. Estrogen receptor (ER)-β reduces ERα regulated gene transcription, supporting a ''Ying Yang'' relationship between ERα and ERβ in mice. Mol Endocrinol 17:203–208.

Liu B, Hong JS. 2003. Role of microglia in inflammation-mediated neurodegenerative diseases: mechanisms and strategies for therapeutic intervention. J Pharmacol Exp Ther 304:1–7.

Losel R, Wehling M. 2003. Nongenomic actions of steroid hormones. Nat Rev Mol Cell Biol 4:46–56.

Marder K, Tang MX, Alfaro B, Mejia H, Cote L, Jacobs D, Stern Y, Sano M, Mayeux R. 1998. Postmenopausal estrogen use and Parkinson's disease with and without dementia. Neurology 50:1141–1143.

McEwen B. 2002. Estrogen actions throughout the brain. Rec Prog Horm Res 57:357–384.

McGeer PL, McGeer EG. 2004. Inflammation and neurodegeneration in Parkinson's disease. Parkinsonism Rel Disord 10:S3–S7.

Melhus A. 2001. Effects of amoxicillin on the expression of cytokines during experimental acute otitis media caused by non-typeable Haemophilus influenzae. J Antimicrob Chemother 48:397–402.

Mor G, Nilsen J, Horvath T, Bechmann I, Brown S, Garcia-Segura LM, Naftolin F. 1999. Estrogen and microglia: a regulatory system that affects the brain. J Neurobiol 40:484–496.

Mouton PR, Long JM, Lei D-L, Howard V, Jucker M, Calhoun ME, Ingram DK. 2002. Age and gender effects on microglia and astrocyte number in brains of mice. Brain Res 956:30–36.

Nishio M, Kuroki Y, Watanabe Y. 2004. Subcellular localization of estrogen receptor-β in mouse hippocampus. Neurosci Lett 355:109–112.

Ohsako S, Kubota K, Kurosawa S, Takeda K, Qing W, Ishimura R, Tohyama C. 2003. Alterations of gene expression in adult male rat testis and pituitary shortly after subacute administration of the antiandrogen flutamide. J Reprod Dev 49:275–290.

Paech K, Webb P, Kuiper GG, Nilsson S, Gustafsson J, Kushner PJ, Scanlan TS. 1997. Differential ligand activation of estrogen receptors ERα and ERβ at AP1 sites. Science 277:1508–1510.

Quesada A, Micevych PE. 2004. Estrogen interacts with the IGF-1 system to protect nigrostriatal dopamine and maintain motoric behavior after 6-hydroxdopamine lesions. J Neurosci Res 75:107–116.

Razandi M, Pedram A, Merchenthaler I, Greene GL, Levin ER. 2004. Plasma membrane estrogen receptors exist and functions as dimers. Mol Endocrinol 18:2854–2865.

Rogers J, Luber-Narod J, Styren SD, Civin WH. 1988. Expression of immune system-associated antigens by cells of the human central nervous system: relationship to the pathology of Alzheimer's disease. Neurobiol Aging 9:339–349.

Sawada H, Shimohama S. 2003. Estrogens and Parkinson disease: novel approach for neuroprotection. Endocrinology 21:77–79.

Sawada H, Ibi M, Kihara T, Urushitani M, Honda K, Nakanishi M, Akaike A, Shimohama S. 2000. Mechanisms of antiapoptotic effects of estrogens in nigral dopaminergic neurons. FASEB J 14:1202–1214.

Sawada H, Ibi M, Kihara T, Honda K, Nakamizo T, Kanki R, Nakanishi M, Sakka N, Akaike A, Shimohama S. 2002. Estradiol protects dopaminergic neurons in a MPP^+ Parkinson's disease model. Neuropharmacology 42:1056–1064.

Strijks E, Kremer JA, Horstink MW. 1999. Effects of female sex steroids on Parkinson's disease in postmenopausal women. Clin Neuropharmacol 22:93–97.

Sun J, Huang YR, Harrington WR, Sheng S, Katzenellenbogen JA, Katzenellenbogen BS. 2002. Antagonists selective for estrogen receptor alpha. Endocrinology 143:941–947.

Teismann P, Schulz JB. 2004. Cellular pathology of Parkinson's disease: astrocytes, microglia and inflammation. Cell Tissue Res 318:149–161.

Tsang KL, Ho SL, Lo SK. 2000. Estrogen improves motor disability in parkinsonian postmenopausal women with motor fluctuations. Neurology 54:2292–2298.

Vegeto E, Bonincontro C, Pollio G, Sala A, Viappiani S, Nardi F, Brusadelli A, Viviani B, Ciana P, Maggi A. 2001. Estrogen prevents the lipopolysaccharide-induced inflammatory response in microglia. J Neurosci 21:1809–1818.

Vegeto E, Belcredito S, Etteri S, Ghisletti S, Brusadelli A, Meda C, Krust A, Dupont S, Ciana P, Chambon P, Maggi A. 2003. Estrogen receptor-a mediates the brain antiinflammatory activity of estradiol. Proc Natl Acad Sci U S A 100:9614–9619.

Wang L, Andersson S, Warner M, Gustafsson JÅ. 2001. Morphological abnormalities in the brains of estrogen receptor-β knockout mice. Proc Natl Acad Sci U S A 98:2792–2796.

Westberg L, Hakansson A, Melke J, Shahabi HN, Nilsson S, Buervenich S, Carmine A, Ahlberg J, Grundell MB, Schulhof B, Klingborg K, Holmberg B, Sydow O, Olson L, Johnels EB, Eriksson E, Nissbrandt H. 2004. Association between the estrogen receptor beta gene and age of onset of Parkinson's disease. Psychoneuroendocrinology 29:993–998.

Women's Health Initiative. 2002. Risks and benefits of estrogen plus progestin in healthy postmenopausal women. JAMA 288:1–29.

Wooten GF, Currie LJ, Bovbjerg VE, Lee JK, Patrie J. 2004. Are men at greater risk for Parkinson's disease than women? J Neurol Neurosurg Psychiatry 75:637–639.

Yang SH, Liu R, Perez EJ, Wen Y, Stevens SM, Valencia T, Brun-Zinkernage AM, Prokai L, Will Y, Dykens J, Koulen P, Simpkins JW. 2004. Mitochondrial localization of estrogen receptor beta. Proc Natl Acad Sci U S A 101:4130–4135.

Zhang JQ, Cai WQ, Zhou de S, Su BY. 2002. Distribution and differences of estrogen receptor β immunoreactivity in the brain of adult male and female rats. Brain Res 935:73–80.

Zhang Z, Cerghet M, Mullins C, Williamson M, Bessert D, Skoff R. 2004. Comparison of in vivo and in vitro subcellular localization of estrogen receptors alpha and beta in oligodendrocytes. J Neurochem 89:674–684.

Zschocke J, Manthey D, Bayatti N, van der Burg B, Goodenough S, Behl C. 2002. Estrogen receptor alpha-mediated silencing of caveolin gene expression in neuronal cells. J Biol Chem 277:38772–38780.