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The Endogenous Estrogen Status Regulates Microglia Reactivity in Animal Models of Neuroinflammation

Elisabetta Vegeto, Silvia Belcredito, Serena Ghisletti, Clara Meda, Sabrina Etteri, and Adriana Maggi

Center of Excellence on Neurodegenerative Diseases and Department of Pharmacological Sciences, University of Milan, 20133 Milan, Italy

It has been previously demonstrated that 17β-estradiol (E_2) inhibits the response of microglia, the resident brain macrophages, to acute injuries in specific brain regions. We here show that the effect of E_2 in acute brain inflammation is widespread and that the hormone reduces the expression of inflammatory mediators, such as monocyte chemoattractant protein-1, macrophage inflammatory protein-2, and TNF-α, induced by lipopolysaccharide, demonstrating that microglia are a direct target of estrogen action in brain. Using the APP23 mice, an animal model of Alzheimer's disease reproducing chronic neuroinflammation, we demonstrate that ovary ablation increases microglia activation at β-amyloid (Aβ) deposits and facilitates the progression of these cells toward a highly reactive state. Long-term administration of E_2 reverts the effects of ovariectomy and decreases microglia reactivity compared with control animals. In this animal model, these events do not correlate with a reduced number of Aβ deposits. Finally, we show that E_2 inhibits Aβ-induced expression of scavenger receptor-A in macrophage cells, providing a mechanism for the effect of E_2 on Aβ signaling observed in the APP23 mice. Altogether, our observations reveal a substantial involvement of endogenous estrogen in neuroinflammatory processes and provide novel mechanisms for hormone action in the brain. (*Endocrinology* 147: 2263–2272, 2006)

IT IS WELL ESTABLISHED that 17β-estradiol (E_2) protects neurons against a vast variety of toxic insults and induces the expression in these cells of growth factors and their receptors as well as structural components and proteins involved in apoptosis in a receptor-dependent manner (1, 2). The neuronal expression of estrogen receptors (ERs) has been shown to vary among brain regions, with low levels being detected in areas such as the hippocampus, cortex, and substantia nigra (3, 4). Recently, it has been proposed that E_2 exerts neuroprotection not only by directly targeting neurons but also by inhibiting the brain inflammatory reaction (5). In fact, several lines of evidence highlighted the role of E_2 as a regulatory agent of acute brain inflammation. Studies using animal models of acute central nervous system inflammation provided *in vivo* evidence for the inhibitory activity of E_2 on the immediate inflammatory response of the brain (6–9). Hormone action in inflammation is believed to be mediated by the activation of the endogenous ERα (10–12), a ligand-dependent transcriptional factor expressed in several cell types including monocyte-macrophage cells (13). In cell-based assays, E_2 was shown to inhibit the metabolic activation of monocytes-macrophages induced by inflammatory stimuli by inhibiting the expression of inflammatory mediators, such as the inducible form of nitric oxide (iNOS) and TNF-α (14–16). However, it is still unknown whether expression of inflammatory molecules is regulated by E_2 also *in vivo* and whether the antiinflammatory activity of E_2 is present in brain areas with low neuronal expression of ERs. In addition, despite the debated role of E_2 as a protective drug against brain degenerative disorders associated with the menopause (17), little is known on the activity of E_2 on chronic brain inflammation.

Activation of microglia cells, the resident macrophage cells of the brain, is a hallmark for neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease, and amyotrophic lateral sclerosis. In these disorders, microglia activation appears at the early phases of disease development (18, 19). Increased microglia reactivity has been detected in postmortem histological studies of AD patients (20, 21), and the use of nonsteroidal antiinflammatory drugs (NSAID) has been associated with a reduced incidence of AD (22, 23). On the basis of these observations it has been proposed that microglia activation contributes to the onset and progression of the degenerative process in neurons. However, a direct demonstration of the functional role of microglia activation in chronic neurodegeneration is still lacking, and whether activation of microglia is beneficial or detrimental to brain remains a debated issue (24, 25).

The aim of this study was to assess the modalities of estrogen action in brain inflammation and the effect of hormone withdrawal/replacement on microglia reactivity in chronic neuroinflammatory pathologies affecting the brain. Our results point to the widespread distribution of E_2 antiinflammatory action in the murine brain. To study the effect of E_2 on chronic microglia reactivity, we used the APP23 transgenic mice, which express the human amyloid precursor protein (APP) with a mutation reported in familial AD in

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Abbreviations: Aβ, β-Amyloid; AD, Alzheimer's disease; APP, amyloid precursor protein; E_2, 17β-estradiol; ER, estrogen receptor; GAPDH, glyceraldehyde phosphodehydrogenase; HS, horse serum; HI, hypertrophic inflammatory; I, inflammatory; icv, intracerebroventricular; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein 1; MIP-2, macrophage inflammatory protein 2; NI, noninflammatory; NSAID, nonsteroidal antiinflammatory drug; ovx, ovariectomized; SR-A, scavenger receptor-A.

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neurons (26). These transgenic mice represent a suitable model to study the chronic neuroinflammatory process, because microglia cells display the characteristic activated morphology and immunoreactive phenotype induced by amyloid deposits, similarly to AD pathogenesis (27). The results show that long-term hormone deprivation exacerbates microglia reactivity at amyloid deposits, an event that is prevented by E_2 replacement.

Materials and Methods

Animals

The study was conducted according the guidelines of the Institutional Animal Care Committee of the University of Milan.

Hormone and lipopolysaccharide (LPS) treatments

Female Sprague Dawley rats or C57/B6 mice (Charles River Breeding Laboratories, Oggiono, Italy) were ovariectomized (ovx) at 6 wk of age. For acute hormonal treatments, 3 wk after ovx, animals were injected sc with vehicle (purified corn oil) or 50 μg/kg E_2 (Sigma, Milan, Italy) 6 h before the intracerebroventricular (icv) injection of *Escherichia coli* LPS (serotype 0.111:B4 from Sigma) (for rats, 10 μg LPS in 3 μl of saline; for mice, 1 μg LPS in 1 μl) in the third cerebral ventricle according to specific stereotaxic coordinates (for rats, bregma, −4 mm; lateral, 1 mm; depth, 5 mm; for mice, bregma, −0.25 mm; lateral, 1 mm; depth, 2.25 mm) and using a Hamilton syringe rotated on the coronal plane of about 3° from the orthogonal position, as previously described (10). Chronic estrogen replacement settings were as follows: E_2 was administered for 6 wk by implanting pellets (Innovative Research of America, Sarasota, FL) releasing for 21 d 0.025 mg/d for rats and 0.010 mg/d for mice of placebo or E_2. Pellets were replaced at the end of the third week. No alteration in animal behavior or body temperature and no acute-phase proinflammatory proteins in plasma were detected after LPS icv injection (data not shown). Effect of estrogen withdrawal or replacement was evaluated in each animal using the uterotrophic assay, a standard test for measuring estrogen activity (data not shown). The blood levels of E_2 achieved by pellet release were 20–35 pg/ml, as measured in a subset of mice/rats (data not shown). Experiments were repeated at least two times; each experimental group consisted of at least five animals.

APP23 mice treatment

APP23 mice overexpress human APP_{751} with the familial Swedish AD double mutation at positions 670/671 under the control of neuronal Thy.1 promoter element (26). Heterozygous APP23 mice underwent ovx or sham surgery at 5 months of age and immediately implanted sc with 90-d pellets (Innovative Research of America) releasing 0.01 mg/d of either placebo (for the ovx and sham groups) or E_2 for the replacement group. Pellets were replaced every 80th day. Animals were killed with an overdose of pentobarbital at 10 and 14 months of age and transcardially perfused with 4% paraformaldehyde. Before perfusion, uteri were weighted to sample the effect of ovx and E_2 replacement. Each experimental group consisted of at least six animals.

Immunohistochemistry

Animals were killed under deep anesthesia and transcardially perfused with 4% paraformaldehyde. Brains were removed and postfixed in 4% paraformaldehyde, cryoprotected, snap-frozen in liquid nitrogen, and stored at −80 C until analyzed. Using a cryostat (Microm, Walldorf, Germany) 30-μm thickness sections were collected: for rat, coronal sections were obtained from level 1, bregma +1.2 mm; level 2, bregma −3 mm; level 3, bregma −5.7 mm; for APP23 mice, hemibrain sagittal sections from bregma to lateral margin 1, +1.65 mm; margin 2, +2.6 mm; margin 3, +3.2 mm. The term margin was used to distinguish the levels analyzed in sagittal sections. Three free-floating sections, 120 μm distant, were analyzed for each level by immunohistochemistry; for rat, the rat-specific antibody ED-1 was used to detect activated microglia, whereas the horseradish peroxidase-conjugated isolectin-B4 (Sigma) was used as a macrophage-specific marker. The distinction between resting and activated microglia was based on morphological analysis; for the APP23 mice, the mouse antibody Mac-1 (Serotec, Oxford, UK) was used to specifically stain activated microglia cells. Before the immunological assay, sections were incubated in 0.05 M NH_4Cl in PBS for 20 min at room temperature to saturate aldehyde residues, washed in PBS, incubated for 5 min in 1% H_2O_2 in PBS at room temperature to inhibit endogenous peroxidases, and washed three times with PBS.

ED-1 staining. Sections were incubated with 10% horse serum (HS) in blocking solution (0.1% Triton X-100 and 3% BSA in PBS) for 1 h at room temperature and then with ED-1 antibody (5 μg/ml in PBS with 1% BSA) overnight at room temperature.

Isolectin-B4 staining. After incubation for 30 min at room temperature with bivalent cations (0.1 mM $MgCl_2$, 0.1 mM $MnCl_2$, 0.1 mM $CaCl_2$, 0.1% Triton X-100 in PBS), horseradish peroxidase-conjugated isolectin-B4 was added at the final concentration of 20 μg/ml in PBS with 0.1% Triton X-100 and left overnight at 4 C.

Mac-1 staining. Mac-1 antibody was used at the final concentration of 2 μg/ml in PBS with 10% HS overnight at 4 C.

After PBS washes, secondary biotinylated antibodies were used after ED-1 and Mac-1 reactions in PBS with 1% HS for 1 h at room temperature. Staining was obtained after incubation with the avidin-biotin-horseradish peroxidase complex (ABC kit from Vector Laboratories, Burlingame, CA) for 1 h at room temperature and the 3,3′-diaminobenzidine substrate (Sigma), as suggested by the manufacturers.

To identify fibrillar amyloid deposits, Congo Red staining was performed on Mac-1-immunostained sections according to standard protocols and counterstained with hematoxylin. Dehydrated sections mounted on slides were observed using a Zeiss Axioskop microscope (Zeiss, Milan, Italy) at magnification ×1000 and analyzed with a color-video image analysis system linked to the microscope.

Quantitative analysis

Brain areas were identified according to the rat and mouse brain atlas of Franklin and Paxinos. ED-1 and isolectin-B4-positive cells were counted using a counting frame area of 0.75 mm^2 in three sections of each brain level. Counting was done twice by two different researchers working blind. Results are the average of the observations made. Activated microglia were recognized by the presence of immunoreactive cell bodies of increased size emanating shorter and thicker cytoplasmic protrusions; in case of a phenotype not clearly definable, especially in the APP23 mice, assignment of cells to the activated state was done by the presence of cytoplasmic extensions without ramifications that were lower than 5 and with a thickness higher than 3 μm. Macroanatomical evaluation of the brain from different groups in the icv LPS experiment did not show any variation in tissue size or blood perfusion, and homogenous immunoreactivity was detected throughout all histological sections. This seems to exclude that tissue shrinkage or expansion occurred as a consequence of our experimental conditions; if this were the case, variation in brain or cell volume seems to have similarly affected all experimental groups. In the APP23 mice, fibrillary amyloid load was quantified by counting the number of Congo Red-positive plaques ranging from 20–100 μm diameter. The number of Congo Red-positive plaques surrounded by Mac-1-immunoreactive microglia were counted and subdivided into inflammatory (I) or hypertrophic inflammatory (HI) plaques on the basis of microglia morphological appearance. All three levels of APP23 brain were analyzed. Data on the APP23 mice refer only to margin 1; margins 2 and 3, although showing a lower number of plaques, had a similar inflammatory profile as that of level 1.

Cell culture

RAW 264.7 cells were purchased from American Type Culture Collection (Manassas, VA) and grown in DMEM plus 10% FBS (American Type Culture Collection) supplemented with 2 g/liter sodium carbonate, 0.11 g/liter sodium pyruvate, 5 ml/liter of a 10,000-IU streptomycin and penicillin mix, under a humidified 5% CO_2/95% air atmosphere at 37 C. For the experiments, cells were starved in medium without phenol red and without serum for 4 h; 1 nM E_2 was added 30 min before an overnight treatment with 1 μg/ml LPS or 1 μM β-amyloid (Aβ).

RT-PCR

RNA preparation. RAW 264.7 cells were harvested and resuspended in TRIzol Reagent (Invitrogen, Milan, Italy). RNA was isolated according to the manufacturer's instructions.

cDNA preparation. One microgram of RNA was used for cDNA preparation using the Moloney murine leukemia virus reverse transcriptase (Promega, Milan, Italy) as previously described (15). Control reactions without addition of the enzyme were performed for each sample.

PCR

PCRs were performed using 1 μl cDNA and 0.4 U DynaZyme DNA polymerase (Finnzymes Oy, Espoo, Finland). The following primers (from MWG Biotech, Ebersberg, Germany) were used to amplify the following mouse genes: monocyte chemoattractant protein 1 (MCP-1), *a*-5′ AGCCAACTCTCACTGAAG3′ and *b*-5′TGGAAAAGGTAGTGGATG3′; macrophage inflammatory protein 2 (MIP-2), *a*-5′ TGGCCAGTGAACTGCGCTG3′ and *b*-5′CCAGGTCAGTTAGCCTTG3′; TNF-α, *a*-5′ ATGAGCACAGAAAGCATGATCC3′ and *b*-5′CCAAAGTAGACCTGCCCGGACTC3′; scavenger receptor-A (SR-A), *a*-5′ ATGACAGAGAATCAGAGG3′ and *b*-5′CCCTCTGTCTCCCTTTTC-3′. The OD of the bands separated by agarose gel electrophoresis and relative to each amplification product was evaluated; standard internal controls were run in each gel and the OD units evaluated and used to normalize the values from different gels. Amplification of the constitutively expressed enzyme glyceraldehyde phosphodehydrogenase (GAPDH) was performed in parallel to assess for RT-PCR efficiency with the primers *a*-5′ ATGACCCCTTCATTGACC3′ and *b*-5′TGCTTCACCACCTTCTTG3′. PCR were performed as follows: for TNF-α, MIP-2, and GAPDH at 95 C for 30 sec, then 30 cycles at 94 C for 1 min, 60 C for 45 sec, and 72 C for 2 min; for MCP-1 and SR-A, the annealing temperature was adjusted at 55 and 47 C, respectively. PCR were performed on a PerkinElmer Thermal Cycler 480 (PerkinElmer, Milan, Italy).

Western blotting

RAW 264.7 cells were treated, washed with ice-cold PBS, and harvested in ice-cold hypotonic lysis buffer [50 mM HEPES (pH 7.4), 1 mM $MgCl_2$, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 1 mM Na_3VO_4, 5 mM NaF, 1 mM dithiothreitol, 0.1% Triton X-100). After 5 min incubation, cells were centrifuged at 24,000 $\times$ *g* for 15 min. The supernatant was collected as the cytosolic fraction. Protein content was determined with the Bradford assay (Pierce Chemical, Rockford, IL). Proteins (10 μg) were separated on a 7.5% SDS-polyacrylamide gel and transferred to a Hybond ECL nitrocellulose membrane (Amersham, Little Chalfont, UK). The membrane was blocked for 45 min with 5% skim milk in TBS-T [20 mM Tris (pH 7.5), 150 mM NaCl, 0.2% Tween 20]. Rat polyclonal antibody for mouse SR-A 2F8 clone (Serotec) was applied at 1:250 dilution at 4 C overnight. After extensive washing in TBS-T, the secondary horseradish peroxidase-conjugated antibody (Chemicon, Milan, Italy) was applied at 1:2000 dilution for 60 min; detection was performed using an ECL kit (Amersham).

Statistical analysis

Values are given as mean $\pm$ SEM for Figs. 1–3 and $\pm$ SD for Figs. 4–6. *P* values were calculated with ANOVA analysis followed by the Bonferroni test.

Results

Estrogen-induced decrease in brain macrophage reactivity

Neurons of the cerebral cortex are primarily and largely affected by pathological conditions, such as ischemia and AD, which show a strong neuroinflammatory reaction that progresses in parallel with the neuropathological lesions. To understand the extent to which E_2 hinders the reactivity of microglia cells in brain regions, such as the cortex and hippocampus, where neuronal expression of ERs is very low, we induced brain inflammation by the icv injection of LPS in ovx rats treated with E_2 or vehicle 6 h before the endotoxin and evaluated the spatial distribution of E_2 activity at different levels of the brain. Notably, macrophage activation was reduced by E_2 by 60–90% in the hippocampus and cerebral cortex at all levels analyzed, as shown in Fig. 1. E_2 action extended with similar potency also to other noncortical regions and at all levels; in areas such as the cingulate cortex and caudatus putamen in level 2 and the rhinal cortex in level 1, E_2 was able to inhibit LPS activity only by 20%. In general, brain regions from levels 2 and 3 showed higher microglia reactivity toward the mechanical damage, as in the case of the hippocampus, cortex, or thalamic nuclei (Fig. 1); in all cases, LPS injection resulted in an average 3-fold increase in ED-1-positive cells. The reason for the different reactivity of microglia in these regions is not yet clear; however, the higher number and susceptibility described for microglia cells in these brain areas (28) as well as their proximity to the injection site could account for the different reactivity observed in our study. Thus, the data demonstrate that E_2 is a potent inhibitor of microglia reactivity in several regions of the brain, including the cerebral cortex, hippocampus and noncortical areas.

Because the effect reported in Fig. 1 was observed after 24 h of LPS injection, we then asked whether E_2 maintained its inhibitory activity for a longer period of time. Animals were analyzed 1, 3, 5, and 7 d after LPS injection, and brain macrophages were stained with isolectin-B4; this macrophage-specific marker allowed us to distinguish between resting and activated microglia cells on the basis of their morphological appearance. In addition to microglia cells, we also scored the number of round-shaped cells reminiscent of infiltrated monocytes. It has been suggested that the appearance of round-shaped cells in brain parenchyma after 24 h of LPS icv administration corresponds to monocytes recruitment (29, 30), although isolectin-B4 staining does not clearly allow one to distinguish between monocytes and highly reactive microglia, which display an ameboid-like morphology. Cells were counted in the CA1, and results are shown in Fig. 2. LPS induced a 6-, 44-, and 13-fold increase in the number of activated microglia cells after 1, 3, and 5 d, respectively, whereas no effect could be observed after 7 d (Fig. 2A); at any time point analyzed, E_2 treatment strongly limited microglia activation resulting in 70, 80, and 33% inhibition of LPS activity after 1, 3, and 5 d, respectively. In according with these data, E_2 also blocked the increase in monocyte-like cells. In fact, after 1 d of LPS injection, the number of round-shaped cells reached an average of 326 $\pm$ 22 cells/mm^2, which decreased to 61 $\pm$ 23 cells/mm^2 after 3 d and returned to control levels after 5 d. At all time points, E_2 decreased the number of monocyte-like cells, which was reduced by 84 and 54% after 1 and 3 d (Fig. 2B). Thus, these data showed that also after longer periods of time after LPS administration, activation of brain macrophages does not occur when E_2 is injected before the endotoxin, thus indicating that in E_2-treated animals the absence of inflammatory signs is not because of a delay in the response to LPS.

Interestingly, the antiinflammatory effect of E_2 was observed not only when hormone was administered a few hours before LPS but also when a physiological, nanomolar concentration of E_2 was delivered to the animals for a pro-

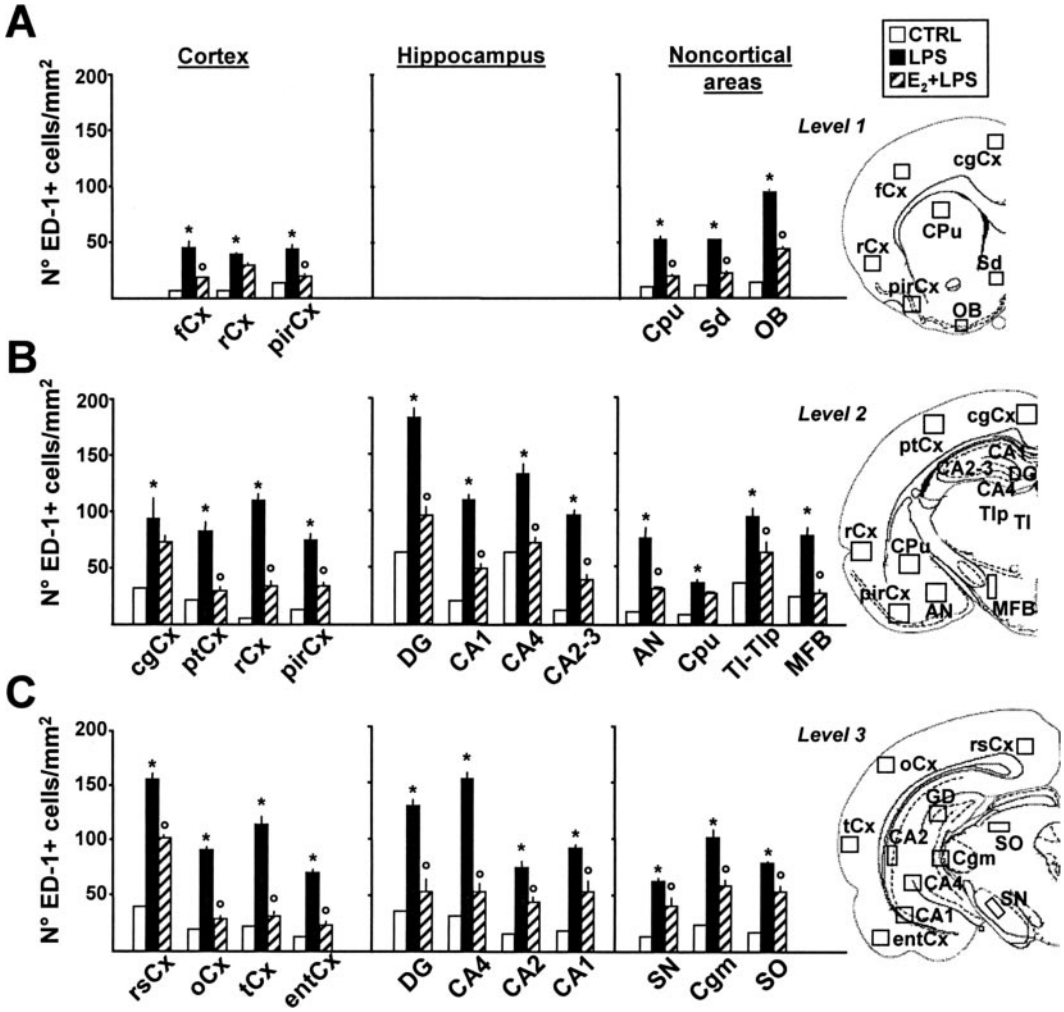

FIG. 1. Effect of estrogen on acute inflammatory response in brain. Animals were icv injected with vehicle (*white bars*), LPS (*black bars*), or LPS preceded by a 6-h E_2 treatment (*dashed bars*); after 24 h, brain coronal sections were analyzed for the presence of reactive microglia by immunohistochemistry using the ED-1 antibody. Immunopositive cells were counted in various regions of the central nervous system at level 1, 1.2 mm (A); level 2, −3 mm (B); and level 3, −5.7 mm (C) from bregma. Data are the mean ± SEM; n = 5. *, $P < 0.01$ *vs.* control (CTRL); °, $P < 0.01$ *vs.* LPS. Cortical areas included the following: fCX, frontal cortex; rhCx, rhinal cortex; pirCx, piriform cortex; cgCx, cingulate cortex; ptCx, parietal cortex; rsCx, retrospinal cortex; oCx, occipital cortex; tCx, temporal cortex; entCx entorhinal cortex. Hippocampal areas included the following: DG, dentate gyrus; CA, CA region of the hippocampus. Noncortical areas included the following: Cpu, Caudatus Putamen; Sd, dorsal septal nuclei; OB, olfactory bulb; AN, amygdaloid nuclei; TlTlp, thalamic nuclei; MFB, medial forbrain bundle; SN, substantia nigra; Cgm, geniculate medial nuclei; SO, superior culliculus.

longed period of time before the endotoxin. Figure 3 shows that a 6-wk treatment with physiological doses of E_2 strongly limited microglia activation, resulting in 40 and 80% inhibition, and reduced the increase of monocyte-like cells by 40 and 67% compared with LPS alone 1 and 3 d after endotoxin injection. In repeated experiments using 3-wk-old and 6-wk-old ovx animals, we observed that a longer time of hormone deprivation increases brain macrophage reactivity toward both the mechanical injury and LPS (data not shown).

Altogether, these data show that the antiinflammatory activity of E_2 is widespread in the rodent brain and that physiological levels of hormone strongly limit the inflammatory response of the brain. These results thus supported a study in which the antiinflammatory activity of E_2 could be tested in an animal model of chronic neuroinflammation.

Estrogen replacement delays activation of microglia cells in the APP23 mice

Neurodegenerative pathologies are associated with a chronic inflammatory reaction, which increases gradually in parallel with disease progression. To test whether E_2 was able to limit the inflammatory response associated with neurodegenerative insults, we used the APP23 mice, a mouse model of AD (26), in which amyloid deposits have been shown to stimulate activation of surrounding microglia cells (31). Five-month-old female APP23 mice were either ovx or sham operated; immediately after surgery, animals were implanted with pellets chronically releasing physiological doses of E_2 or placebo. Animals were killed at 10 and 14 months of age; this age was chosen because it corresponds to

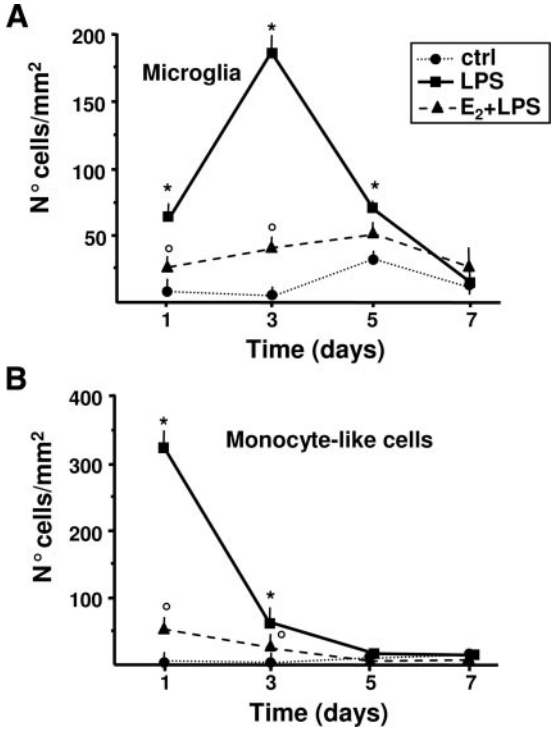

FIG. 2. Acute E_2 administration hinders brain macrophage reactivity induced by LPS. A, Activation of microglia cells; B, infiltration of monocyte-like cells in the CA1 region of animals injected icv with vehicle (●, control), LPS (■), or LPS preceded by a 6-h E_2 treatment (▲). Brain sections were analyzed by immunohistochemistry using the isolectin-B4 1, 3, 5, and 7 d after the icv injections. Activated microglia and monocyte-like cells were scored on the basis of their morphological appearance. Data are the mean ± SEM; n = 5. *, $P < 0.05$ *vs.* control; °, $P < 0.05$ *vs.* LPS.

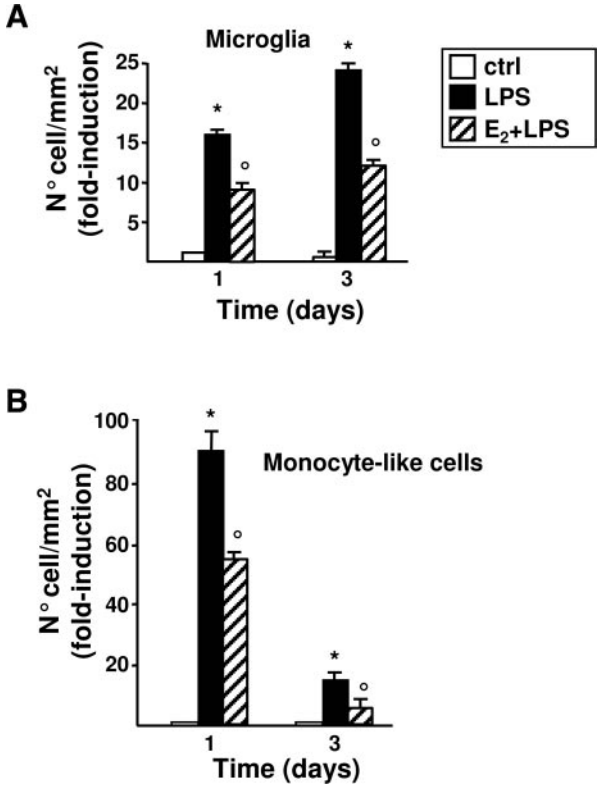

FIG. 3. A chronic treatment with E_2 is effective in reducing brain macrophage reactivity. A, Activation of microglia cells; B, infiltration of monocyte-like cells in the CA1 region of animals injected icv with vehicle (*white bar*, control) or LPS (*black bar*) or pretreated for 6 h with E_2 (*dashed bar*). Brain sections were analyzed by immunohistochemistry using the isolectin-B4 1 and 3 d after the icv injections. Activated microglia and monocyte-like cells were scored on the basis of their morphological appearance. Values are expressed as fold induction with respect to control animals. Data are the mean ± SEM; n = 5. *, $P < 0.05$ *vs.* control; °, $P < 0.05$ *vs.* LPS.

an early stage of disease development, in which amyloid deposits and reactive microglia are already observed. Brain sections were analyzed by immunohistochemistry using Mac-1 antibody to recognize activated microglia cells and stained with Congo Red to visualize fibrillar amyloid deposits. The analysis of brain samples revealed the presence of three kinds of congophilic plaques: 1) Congo Red-positive amyloid deposits devoid of activated microglia cells, named noninflammatory (NI) plaques (shown in panel a of Fig. 4A); 2) amyloid plaques surrounded by Mac-1-positive, reactive microglia cells, named inflammatory (I) plaques (shown in panel b of Fig. 4A); and 3) plaques surrounded by microglia cells with a more advanced degree of morphological activation, defined as hypertrophic inflammatory (HI) plaques (shown in panel c of Fig. 4A). In fact, microglia cells associated with HI plaques show an increased cell body size with shorter and thicker cytoplasmic processes (see *insets* in Fig. 4A). We noticed that HI plaques could be observed only in the motor cortex of 14-month-old mice, suggesting that the inflammatory reaction was indeed progressing within the time frame analyzed in our study. We thus counted the number of NI, I, or HI plaques in the sensory cortex and motor cortex, the brain regions that show relevant plaque formation and microglia reactivity at these early stages of disease progression in APP23 mice. Ten-month-old animals were analyzed first. Sham-operated mice revealed an average 22% of plaques bearing activated microglia (I plaques) in the sensory cortex and 10% in the motor cortex (see Fig. 4B). The number of I plaques increased significantly in the brain of ovx mice, reaching an average of 58% in the two cortices; on the contrary, E_2 replacement resulted in a sharp decrease of I plaques compared with the ovx group, and interestingly, E_2 reduced the number of I plaques below the level observed in control animals, leading to an average 3% of I plaques in both brain areas (Fig. 4B). Thus, these results indicate that ovx increases microglia reactivity induced by amyloid deposition; chronic replacement with E_2 is able to reduce the neuroinflammatory signs associated with the APP genetic defect and to prevent the effect caused by ovx.

We then analyzed 14-month-old mice. In the sensory cortex, the percentages of I plaques among the experimental groups were similar to those observed in 10-month-old animals, although E_2 was less efficacious and resulted in a number of I plaques similar to that observed in sham-operated animals (Fig. 4C). The HI plaques number reflected a similar pattern of regulation compared with I plaques, suggesting that hormone is able to regulate the degree of activation of microglia cells. This observation was further confirmed by analyzing the data from the motor cortex. In fact,

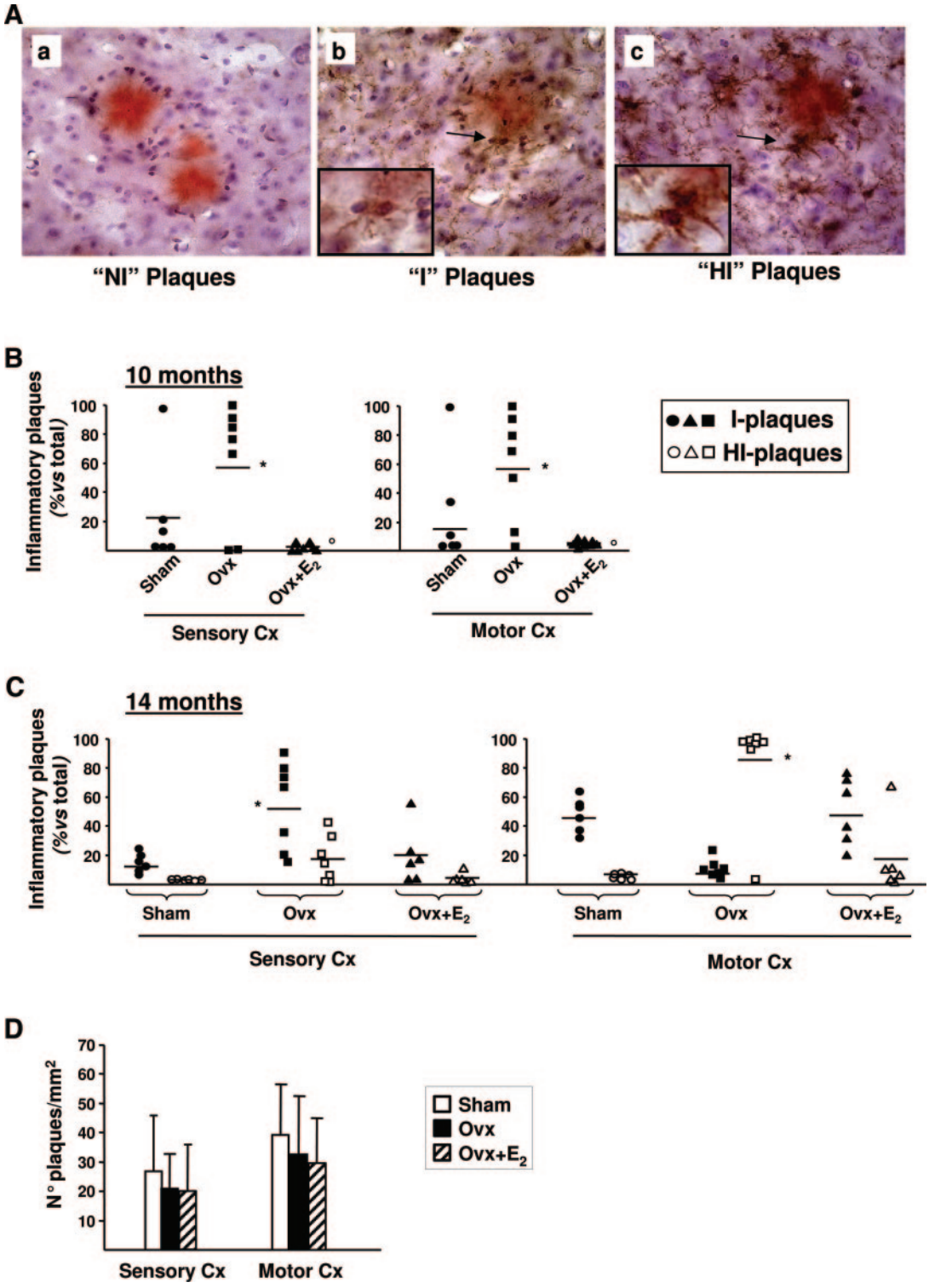

FIG. 4. Ablation of circulating estrogens or replacement with E_2 influences the brain inflammatory phenotype in APP23 mice. A, representative Congo Red-stained amyloid deposits showing three different kinds of plaques: a, NI plaques surrounded by Mac-1-negative cells; b, I plaque associated with Mac-1-positive reactive microglia; and c, HI plaque showing highly reactive microglia cells. The number of Congo Red-positive I (*black symbols*) and HI plaques (*white symbols*) were counted in the sensory and motor cortex (Cx) of 10-month-old (B) or 14-month-old (C) animals. Data represent the percentage of I or HI plaques *vs.* total plaque number in sham (● and ○), ovx (■ and □), and E_2-replaced APP23 mice (▲ and △). *Bar*, mean value for the treatment group; *, $P < 0.05$ *vs.* sham. D, Brain amyloid deposition was analyzed by counting the number of Congo Red-positive plaques in the sensory and motor cortex of sham (*white bar*), ovx (*black bar*), and E_2-replaced APP23 mice (*dashed bar*) at 10 months of age. For 10-month-old animals, n = 6 sham, n = 7 ovx, and n = 6 ovx + E_2; for 14-month-old mice, n = 6 sham, n = 7 ovx, and n = 6 ovx + E_2.

control APP23 mice showed an increased percentage of I plaques compared with that observed at 10 months of age, reaching an average of 46%, whereas HI plaques were about 5% of all plaques (see Fig. 4C). Remarkably, hypertrophic microglia was the most frequent phenotype observed in the ovx group, whereas HI plaques were almost absent in E_2-replaced mice, and the I plaques number was similar to that observed in control mice (see Fig. 4C). Thus, these data showed that E_2 levels clearly affect the state and degree of microglia activation also in 14-month-old APP23 mice.

Because it is known that inflammatory cells play a key role in Aβ processing and deposition, we asked whether the hormonal status might influence the amount of Aβ deposition in the APP23 mice. As shown in Fig. 4D, the number of Aβ deposits and the size of these plaques (data not shown) did not vary significantly among the experimental groups, thus showing that the hormonal levels do not modify amyloid deposition in this experimental model of AD.

In conclusion, these results show that the degree of activation of microglia cells increases in parallel with the progression of the pathology and that hormone deprivation facilitates this process and leads to the earlier appearance of highly reactive microglia at amyloid deposition sites. Physiological levels of hormone delay the appearance of activated microglia and prevent the effect induced by ovx; this different neuroinflammatory phenotype does not correlate with a modification in Aβ deposition.

Estrogen prevents induction of inflammatory mediators in the brain

To demonstrate that microglia cells are a direct target of E_2 action *in vivo*, we analyzed the expression levels of inflammatory markers, such as MCP-1, MIP-2, and TNF-α, known to be involved in the immediate response of the brain to inflammatory stimuli and to participate in the early events that lead to chronic neurodegenerative disorders. We analyzed the mRNA levels of MIP-2, MCP-1, and TNF-α in the cortex and hippocampus of mice under different hormonal status after the icv injection of LPS. This endotoxin induces a 5- to 7-fold induction of MCP-1, MIP-2, and TNF-α mRNA levels with respect to control levels (see Fig. 5), whereas the chronic administration of E_2 results in a significant reduction of the mRNA of these inflammatory markers. Thus, these data show that E_2 prevents the burst of cytokines and chemokines induced by inflammatory stimuli, suggesting that E_2 is able to inhibit the induction of inflammatory mediators also *in vivo*.

Estrogen inhibits the induction of Aβ-binding proteins

It is well known that SR-A is primarily responsible for initiating the process of cell activation induced by Aβ in microglia cells; in fact, SR-A has been shown to interact with Aβ fibrils and activate membrane-associated signaling pathways (32). SR-A is highly expressed in microglia cells from aged APP23 mice (31), and it is used as a marker for microglia activation induced by diverse signals, such as Aβ or LPS. Because we observed that E_2 regulates microglia activation in APP23 mice, we postulated that inhibition of SR-A expression induced by Aβ may underlie the inhibitory effect of

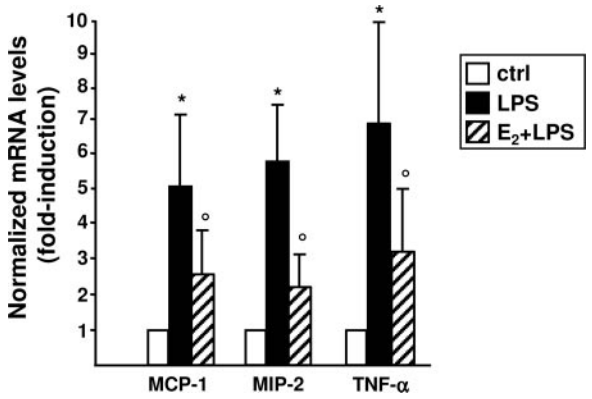

FIG. 5. E_2 limits the expression of inflammatory mediators induced by LPS. Expression of MCP-1, MIP-2, and TNF-α was analyzed in mice injected icv with vehicle (control, *white bars*), LPS (*black bars*), or LPS preceded by a 6-h E_2 treatment (*dashed bars*). After 3 h, the contralateral hippocampus and cerebral cortex were removed and mRNA levels for the inflammatory markers analyzed by RT-PCR using specific primers. Control, n = 8; ovx, n = 12; ovx + E_2, n = 12. Data are the mean ± SD; *, $P < 0.05$ *vs.* control; °, $P < 0.05$ *vs.* LPS.

E_2 observed on microglia activation. To directly evaluate the effect of Aβ and E_2 on SR-A expression, we used macrophage cells in culture. As shown in Fig. 6A, treatment with Aβ

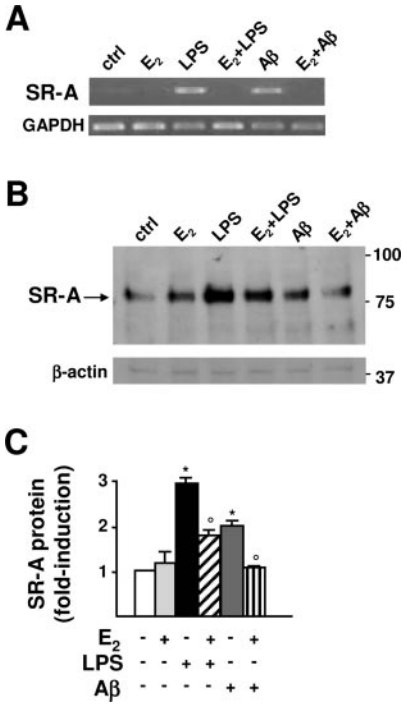

FIG. 6. E_2 limits the expression of SR-A induced by Aβ. Expression of SR-A was analyzed in macrophage cells treated with Aβ and LPS in the presence or absence of a pretreatment with E_2, as indicated. A, Agarose gel bands from RT-PCR of SR-A and the housekeeping gene GAPDH; B, representative Western blot analysis of macrophage cells treated as in A, revealing the presence of an 80-kDa protein corresponding to the glycosylated form of SR-A. Protein loading was verified using an antibody against β-actin. Experiments were repeated at lest three times. C, The OD of the bands appearing in Western blot assays was evaluated. Data are calculated as fold induction with respect to untreated cells and are the mean ± SD of three different experiments. *, $P < 0.05$ *vs.* control; °, $P < 0.05$ *vs.* LPS.

strongly increased SR-A mRNA levels, and this effect was significantly prevented by the addition of a physiological concentration of E_2. A similar expression profile was also observed using LPS, further confirming that E_2 prevents SR-A expression induced by inflammatory agents (Fig. 6A). Concordantly, when we analyzed SR-A expression at the protein level, we observed that induction of SR-A mediated by Aβ or LPS was strongly reduced by E_2 treatment (Fig. 6B). Thus, these data suggest that induction of SR-A expression by Aβ is inhibited by E_2 in macrophage cells, providing a potential mechanism for E_2 inhibitory activity on microglia responsiveness observed in the APP23 mouse model.

Discussion

The present study shows the wide distribution of E_2 action in brain inflammatory cells and provides the first experimental evidence for the key role played by this hormone against chronic neuroinflammatory stimuli. Using the APP23 mice, an animal model of chronic neuroinflammation, removal of endogenous ovarian hormones results in a robust increase in microglia reactivity, and replacement with E_2 delays this neuroinflammatory process and prevents the effect induced by ovx. These data thus extend our knowledge on the role of E_2 in brain and sustain the hypothesis of the hormone as a key regulator of the inflammatory process in the central nervous system.

The widespread effect of E_2 on microglia reactivity in brain

Our previous work described the effect of E_2 on acute inflammation in selected brain areas (10). To analyze in more detail the responsiveness of brain macrophages to E_2, several cerebral regions at different brain levels were analyzed in the present study. We observe that E_2 is a general inhibitor of microglia activation in brain, because its activity extends with similar potency in all regions analyzed. Our present observation may be particularly relevant considering some areas, such as the cerebral cortex, where E_2 has been shown to limit neuronal cell death induced by hypoxia (33), despite the fact that expression of ERs in cortical neurons is extremely low (3, 4). Our data suggest that the antiinflammatory activity of E_2 may play a key role in the neuroprotective effect of hormone in these regions. We also show that long-term replacement with E_2, administered to maintain a constant physiological concentration of the hormone, is as active as an acute administration in inhibiting microglia activation. These data imply that physiological levels of circulating E_2 modulate the intensity of the inflammatory response to acute brain injuries.

Expression of ERs in microglia cells in brain has not been demonstrated yet. The small size of microglia cells as well as the low abundance of ERs hinders receptor immunodetection in microglia; nevertheless, microglia cells in culture were shown to express ERs (15). Considering that ERs are expressed and activated by E_2 also in astrocytes and that this interaction is certainly involved in the neuroprotective action of E_2 (34, 35), it is possible that astrocytes influence some of the effects observed on microglia. However, it has been shown that in brain parenchyma, the LPS receptor, Toll-like receptor-4, is expressed in microglial cells and not in astrocytes (36); thus, the inhibitory activity of E_2 on the induction of inflammatory mediators observed shortly after LPS administration (Fig. 5) leads us to assume that indeed microglia cells are primary targets for estrogen in brain.

In conclusion, our observations further sustain the hypothesis that the endogenous E_2 status regulates brain physiology through a concerted action on diverse cell types and molecular targets (2).

E_2 and chronic inflammation

A progressive inflammatory phenotype is reproduced in the APP23 mice model, as indicated by the increased state of microglia activation observed in aging APP23 mice. We chose to analyze animals of 10 and 14 months of age, with the intention to examine the effect of estrogen on the initial phases of microglia activation at amyloid deposits. Our results show that hormone deprivation accelerates the appearance of highly reactive microglia and that this event is reversed by E_2 administration; this suggests that E_2 has a physiological role in the susceptibility of the innate immune system of the brain toward chronic inflammatory stimuli. Thus, it can be hypothesized that changes in hormonal levels, such as those occurring in women at menopause, may impact on brain innate immunity and modify the susceptibility toward chronic inflammatory stimuli, whereas a continuous administration of exogenous hormone reduces this reactivity.

It has been previously shown that the chronic inflammatory response in bone is regulated by endogenous E_2, because ovx causes an increase of macrophage activity (37). Although the functional outcome of the inhibitory activity of the hormone on bone macrophages is clear, because E_2 administration prevents bone loss, the final effect of E_2 action in brain inflammation has been poorly analyzed and is still a matter of controversy. In the present study, we show that E_2 levels, although affecting microglia reactivity, do not modify disease progression, as shown by the lack of alteration in Aβ deposition. The reason for this lack of effect is still unknown. However, it is possible that the animal model used does not allow a regulatory event, such as microglia reactivity, to affect the mutated APP processing. Moreover, it has been shown that the oxidative damage induced by Aβ overexpression might compromise the beneficial activity of estrogen (38), suggesting that the cumulative oxidative damage occurring in the APP23 mice may hinder E_2 activity on APP processing. Finally, it is possible that a different time of onset and duration of hormone withdrawal/replacement may modify the final effect of E_2. In fact, although our results are in line with previous work (39, 40), a study by Zheng *et al.* (41) did show a modification of Aβ levels by E_2 in Tg2576 animals under different experimental conditions. We do not rule out the hypothesis that E_2, through its activity on microglia, might exert a protective effect in other diseases. For instance, it is important to mention the role of inflammation on neuronal damage after induction of experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis (42–44), and the beneficial antiinflammatory effects of E_2 administration, which abolishes neuron loss induced by cytokines (45) and prevents tissue damage stimulated by the

neuroinflammatory reaction in experimental autoimmune encephalomyelitis (8, 11, 12, 46–48).

The molecular mechanism of estrogen action in brain inflammation

SR-A is expressed by microglia cells under inflammatory conditions and acts to recognize extracellular Aβ and activate specific intracellular signaling pathways (49). The demonstration that E_2 inhibits SR-A induction in macrophage cells suggests that E_2 is able to reduce the reactivity of microglia toward Aβ by inhibiting the expression of molecules, such as SR-A (this study) or CD-14 (50), that mediate Aβ signaling (32, 34). The identification of SR-A as a target of estrogen antiinflammatory action might provide a novel marker for the identification of candidate drugs that, by modifying the neuroinflammatory process, might be of benefit for delaying neuroinflammatory diseases.

We recently demonstrated a specific mechanism for E_2 action in inflammatory cells, showing that hormone acts to inhibit the intracellular transport of nuclear factor-κB, a family of transcription factors that stimulate inflammatory gene transcription (51). This mechanism of action is not shared by other antiinflammatory drugs, such as the NSAIDs. NSAID administration was shown to decrease microglia activation and reduce Aβ deposition in animal models of AD (52, 53). These drugs were shown to act on microglia and APP processing by targeting specific molecules, including cyclooxygenase, peroxisome-proliferating activator receptor-γ, and γ-secretase (54–56). Thus, it is plausible that drugs acting on the inflammatory pathway elicit different outcomes on brain diseases depending on the molecular mechanism of action. The identification of the intracellular effectors of E_2 action will help in identifying new targets for the control of brain inflammation.

Conclusions

Recent clinical trials using hormone replacement therapy in AD reported conflicting results on the therapeutic potential of these drugs (57–59). Yet the menopause is considered as a risk factor for AD. Our data show that withdrawal of ovarian hormones induces a higher neuroinflammatory reaction, whereas continuous administration of E_2 prevents this effect, thus revealing a substantial involvement of endogenous E_2 to the inflammatory process in the brain of female rodents.

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Address all correspondence and requests for reprints to: Elisabetta Vegeto, Center of Excellence on Neurodegenerative Diseases and Department of Pharmacological Sciences, University of Milan, Via Balzaretti 9, 20133 Milan, Italy. E-mail: elisabetta.vegeto@unimi.it.

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References

1. **Wise PM** 2002 Estrogens and neuroprotection. Trends Endocrinol Metab 13: 229–230
2. **Maggi A, Ciana P, Belcredito S, Vegeto E** 2004 Estrogens in the nervous system: mechanisms and nonreproductive functions. Annu Rev Physiol 66: 291–313
3. **Shughrue PJ, Lane MV, Merchenthaler I** 1997 Comparative distribution of estrogen receptor-α and -β mRNA in the rat central nervous system. J Comp Neurol 388:507–525
4. **Mitra SW, Hoskin E, Yudkovitz J, Pear L, Wilkinson HA, Hayashi S, Pfaff DW, Ogawa S, Rohrer SP, Schaeffer JM, McEwen BS, Alves SE** 2003 Immunolocalization of estrogen receptor β in the mouse brain: comparison with estrogen receptor α. Endocrinology 144:2055–2067
5. **Vegeto E, Ciana P, Maggi A** 2002 Estrogen and inflammation: hormone generous action spreads to the brain. Mol Psychiatry 7:236–238
6. **Jansson L, Holmdahl R** 1998 Estrogen-mediated immunosuppression in autoimmune diseases. Inflamm Res 47:290–301
7. **Santizo RA, Anderson S, Ye S, Koenig HM, Pelligrino DA** 2000 Effects of estrogen on leukocyte adhesion after transient forebrain ischemia. Stroke 31: 2231–2235
8. **Ito A, Bebo Jr BF, Matejuk A, Zamora A, Silverman M, Fyfe-Johnson A, Offner H** 2001 Estrogen treatment down-regulates TNF-α production and reduces the severity of experimental autoimmune encephalomyelitis in cytokine knockout mice. J Immunol 167:542–552
9. **Matsuda J, Vanier MT, Saito Y, Suzuki K, Suzuki K** 2001 Dramatic phenotypic improvement during pregnancy in a genetic leukodystrophy: estrogen appears to be a critical factor. Hum Mol Genet 10:2709–2715
10. **Vegeto E, Belcredito S, Etteri S, Ghisletti S, Brusadelli A, Meda C, Krust A, Dupont S, Ciana P, Chambon P, Maggi A** 2003 Receptor-α mediates the brain antiinflammatory activity of estradiol. Proc Natl Acad Sci USA 100:9614–9619
11. **Garidou L, Laffont S, Douin-Echinard V, Coureau C, Krust A, Chambon P, Guery JC** 2004 Estrogen receptor α signalling in inflammatory leukocytes is dispensable for 17β-estradiol-mediated inhibition of experimental autoimmune encephalomyelitis. J Immunol 173:2435–2442
12. **Polanczyk MJ, Jones RE, Subramanian S, Afentoulis M, Rich C, Zakroczymski M, Cooke P, Vandenbark AA, Offner H** 2004 T lymphocytes do not directly mediate the protective effect of estrogen on experimental autoimmune encephalomyelitis. Am J Pathol 165:2069–2077
13. **Vegeto E, Pollio G, Pellicciari C, Maggi A** 1999 Estrogen and progesterone induction of survival of monoblastoid cells undergoing TNF-α-induced apoptosis. FASEB J 13:793–803
14. **Bruce-Keller AJ, Keeling JL, Keller JN, Huang FF, Camondola S, Mattson MP** 2000 Antiinflammatory effects of estrogen on microglial activation. Endocrinology 141:3646–3656
15. **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
16. **Hayashi T, Yamada K, Esaki T, Muto E, Chaudhuri G, Iguchi A** 1998 Physiological concentrations of 17β-estradiol inhibit the synthesis of nitric oxide synthase in macrophages via a receptor-mediated system. J Cardiovasc Pharmacol 31:292–298
17. **Turgeon JL, McDonnell DP, Martin KA, Wise PM** 2004 Hormone therapy: physiological complexity belies therapeutic simplicity. Science 304:1269–1273
18. **Gonzalez-Scarano F, Baltuch G** 1999 Microglia as mediators of inflammatory and degenerative diseases. Annu Rev Neurosci 22:219–240
19. **Kalaria RN** 1999 Microglia and Alzheimer's disease. Curr Opin Hematol 6:15–24
20. **McGeer PL, McGeer EG** 1995 The inflammatory response system of brain: implications for therapy of Alzheimer and other neurodegenerative diseases. Brain Res Brain Res Rev 21:195–218
21. **Cagnin A, Brooks DJ, Kennedy AM, Gunn RN, Myers R, Turkheimer FE, Jones T, Banati RB** 2001 In-vivo measurement of activated microglia in dementia. Lancet 358:461–467
22. **Rich JB, Rasmusson DX, Folstein MF, Carson KA, Kawas C, Brandt J** 1995 Nonsteroidal anti-inflammatory drugs in Alzheimer's disease. Neurology 45: 51–55
23. **Stewart WF, Kawas C, Corrada M, Metter EJ** 1997 Risk of Alzheimer's disease and duration of NSAID use. Neurology 48:626–632
24. **Depino AM, Earl C, Kaczmarczyk E, Ferrari C, Besedovsky H, del Rey A, Pitossi FJ, Oertel WH** 2003 Microglial activation with atypical proinflammatory cytokine expression in a rat model of Parkinson's disease. Eur J Neurosci 18:2731–2742
25. **Minghetti L** 2005 Role of inflammation in neurodegenerative diseases. Curr Opin Neurol 18:315–321
26. **Sturchler-Pierrat C, Abramowski D, Duke M, Wiederhold KH, Mistl C, Rothacher S, Ledermann B, Burki K, Frey P, Paganetti PA, Waridel C, Cal-**

houn ME, Jucker M, Probst A, Staufenbiel M, Sommer B 1997 Two amyloid precursor protein transgenic mouse models with Alzheimer disease-like pathology. Proc Natl Acad Sci USA 94:13287–13292
27. **Stalder M, Phinney A, Probst A, Sommer B, Staufenbiel M, Jucker M** 1999 Association of microglia with amyloid plaques in brains of APP23 transgenic mice. Am J Pathol 154:1673–1684
28. **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
29. **Riva-Depaty I, Fardeau C, Mariani J, Bouchaud C, Delhaye-Bouchaud N** 1994 Contribution of peripheral macrophages and microglia to the cellular reaction after mechanical or neurotoxin-induced lesions to the rat brain. Exp Neurol 128:77–87
30. **Montero-Menei CN, Sindji L, Garcion E, Mege M, Couez D, Gamelin E, Darcy F** 1996 Early events of the inflammatory reaction induced in rat brain by lipopolysaccharide intracerebral injection: relative contribution of peripheral monocytes and activated microglia. Brain Res 724:55–66
31. **Bornemann KD, Wiederhold KH, Pauli C, Ermini F, Stalder M, Schnell L, Sommer B, Jucker M, Staufenbiel M** 2001 Aβ-induced inflammatory processes in microglia cells of APP23 transgenic mice. Am J Pathol 158:63–73
32. **El Khoury J, Hickman SE, Thomas CA, Cao L, Silverstein SC, Loike JD** 1996 Scavenger receptor-mediated adhesion of microglia to β-amyloid fibrils. Nature 382:716–719
33. **Rau SW, Dubal DB, Bottner M, Gerhold LM, Wise PM** 2003 Estradiol attenuates programmed cell death after stroke-like injury. J Neurosci 23:11420–11426
34. **Azcoitia I, Sierra A, Garcia-Segura LM** 1999 Localization of estrogen receptor β-immunoreactivity in astrocytes of the adult rat brain. Glia 26.260–267
35. **Azcoitia I, Garcia-Ovejero D, Chowen JA, Garcia-Segura LM** 2001 Astroglia play a key role in the neuroprotective actions of estrogen. Prog Brain Res 132:469–478
36. **Chakravarty S, Herkenham M** 2005 Toll-like receptor 4 on nonhematopoietic cells sustains CNS inflammation during endotoxemia, independent of systemic cytokines. J Neurosci 25:1788–1796
37. **Cuzzocrea S, Mazzon E, Dugo L, Genovese T, Di Paola R, Ruggeri Z, Vegeto E, Caputi AP, Van De Loo FA, Puzzolo D, Maggi A** 2003 Inducible nitric oxide synthase mediates bone loss in ovariectomized mice. Endocrinology 144:1098–1107
38. **Coma M, Guix FX, Uribesalgo I, Espuna G, Sole M, Andreu D, Munoz FJ** 2005 Lack of oestrogen protection in amyloid-mediated endothelial damage due to protein nitrotyrosination. Brain 128:1613–1621
39. **Heikkinen T, Kalesnykas G, Rissanen A, Tapiola T, Iivonen S, Wang J, Chaudhuri J, Tanila H, Miettinen R, Puolivali J** 2004 Estrogen treatment improves spatial learning in APP + PS1 mice but does not affect β amyloid accumulation and plaque formation. Exp Neurol 187:105–117
40. **Green PS, Bales K, Paul S, Bu G** 2005 Estrogen therapy fails to alter amyloid deposition in the PDAPP model of Alzheimer's disease. Endocrinology 146:2774–2781
41. **Zheng H, Xu H, Uljon SN, Gross R, Hardy K, Gaynor J, Lafrancois J, Simpkins J, Refolo LM, Petanceska S, Wang R, Duff K** 2002 Modulation of Aβ peptides by estrogen in mouse models. J Neurochem 80:191–196
42. **Diestel A, Aktas O, Hackel D, Hake I, Meier S, Raine CS, Nitsch R, Zipp F, Ullrich O** 2003 Activation of microglial poly(ADP-ribose)-polymerase-1 by cholesterol breakdown products during neuroinflammation: a link between demyelination and neuronal damage. J Exp Med 198:1729–1740
43. **Hobom M, Storch MK, Weissert R, Maier K, Radhakrishnan A, Kramer B, Bahr M, Diem R** 2004 Mechanisms and time course of neuronal degeneration in experimental autoimmune encephalomyelitis. Brain Pathol 14:148–157
44. **Aktas O, Smorodchenko A, Brocke S, Infante-Duarte C, Topphoff US, Vogt J, Prozorovski T, Meier S, Osmanova V, Pohl E, Bechmann I, Nitsch R, Zipp F** 2005 Neuronal damage in autoimmune neuroinflammation mediated by the death ligand TRAIL. Neuron 46:421–432
45. **Bebo Jr BF, Fyfe-Johnson A, Adlard K, Beam AG, Vandenbark AA, Offner H** 2001 Low-dose estrogen therapy ameliorates experimental autoimmune encephalomyelitis in two different inbred mouse strains. J Immunol 166:2080–2089
46. **Koski CL, Hila S, Hoffman GE** 2004 Regulation of cytokine-induced neuron death by ovarian hormones: involvement of antiapoptotic protein expression and c-JUN N-terminal kinase-mediated proapoptotic signaling. Endocrinology 145:95–103
47. **Hoffman GE, Le WW, Murphy AZ, Koski CL** 2001 Divergent effects of ovarian steroids on neuronal survival during experimental allergic encephalitis in Lewis rats. Exp Neurol 171:272–284
48. **Matejuk A, Adlard K, Zamora A, Silverman M, Vandenbark AA, Offner H** 2001 17β-Estradiol inhibits cytokine, chemokine, and chemokine receptor mRNA expression in the central nervous system of female mice with experimental autoimmune encephalomyelitis. J Neurosci Res 65:529–542
49. **Fassbender K, Walter S, Kuhl S, Landmann R, Ishii K, Bertsch T, Stalder AK, Muehlhauser F, Liu Y, Ulmer AJ, Rivest S, Lentschat A, Gulbins E, Jucker M, Staufenbiel M, Brechtel K, Walter J, Multhaup G, Penke B, Adachi Y, Hartmann T, Beyreuther K** 2004 The LPS receptor (CD14) links innate immunity with Alzheimer's disease. FASEB J 18:203–205
50. **Vegeto E, Ghisletti S, Meda C, Etteri S, Belcredito S, Maggi A** 2004 Regulation of the lipopolysaccharide signal transduction pathway by 17β-estradiol in macrophage cells. J Steroid Biochem Mol Biol 91:59–66
51. **Ghisletti S, Meda C, Maggi A, Vegeto E** 2005 17β-Estradiol inhibits inflammatory gene expression by controlling NF-κB intracellular localization. Mol Cell Biol 25:2957–2968
52. **Yan Q, Zhang J, Liu H, Babu-Khan S, Vassar R, Biere AL, Citron M, Landreth G** 2003 Anti-inflammatory drug therapy alters β-amyloid processing and deposition in an animal model of Alzheimer's disease. J Neurosci 23:7504–7519
53. **Lim GP, Yang F, Chu T, Chen P, Beech W, Teter B, Tran T, Ubeda O, Ashe KH, Frautschy SA, Cole GM** 2000 Ibuprofen suppresses plaque pathology and inflammation in a mouse model for Alzheimer's disease. J Neurosci 20:5709–5714
54. **Weggen S, Eriksen JL, Das P, Sagi SA, Wang R, Pietrzik CU, Findlay KA, Smith TE, Murphy MP, Bulter T, Kang DE, Marquez-Sterling N, Golde TE, Koo EH** 2001 A subset of NSAIDs lower amyloidogenic Aβ42 independently of cyclooxygenase activity. Nature 414:212–216
55. **Eriksen JL, Sagi SA, Smith TE, Weggen S, Das P, McLendon DC, Ozols VV, Jessing KW, Zavitz KH, Koo EH, Golde TE** 2003 NSAIDs and enantiomers of flurbiprofen target γ-secretase and lower Aβ42 in vivo. J Clin Invest 112:440–449
56. **Takahashi Y, Hayashi I, Tominari Y, Rikimaru K, Morohashi Y, Kan T, Natsugari H, Fukuyama T, Tomita T, Iwatsubo T** 2003 Sulindac sulfide is a noncompetitive γ-secretase inhibitor that preferentially reduces Aβ42 generation. J Biol Chem 278:18664–18670
57. **Anderson GL, Limacher M, Assaf AR, Bassford T, Beresford SA, Black H, Bonds D, Brunner R, Brzyski R, Caan B, Chlebowski R, Curb D, Gass M, Hays J, Heiss G, Hendrix S, Howard BV, Hsia J, Hubbell A, Jackson R, Johnson KC, Judd H, Kotchen JM, Kuller L, LaCroix AZ, Lane D, Langer RD, Lasser N, Lewis CE, Manson J, Margolis K, Ockene J, O'Sullivan MJ, Phillips L, Prentice RL, Ritenbaugh C, Robbins J, Rossouw JE, Sarto G, Stefanick ML, Van Horn L, Wactawski-Wende J, Wallace R, Wassertheil-Smoller S; Women's Health Initiative Steering Committee** 2004 Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the Women's Health Initiative randomized controlled trial. JAMA 291:1701–1712
58. **Sherwin BB** 2005 Estrogen and memory in women: how can we reconcile the findings? Horm Behav 47:371–375
59. **Wise PM, Dubal DB, Rau SW, Brown CM, Suzuki S** 2005 Are estrogens protective or risk factors in brain injury and neurodegeneration? Reevaluation after the Women's Health Initiative. Endocr Rev 26:308–312

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