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Selective Estrogen Receptor Modulators Decrease Reactive Astrogliosis in the Injured Brain: Effects of Aging and Prolonged Depletion of Ovarian Hormones

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After brain injury, astrocytes acquire a reactive phenotype characterized by a series of morphological and molecular modifications, including the expression of the cytoskeletal protein vimentin. Previous studies have shown that estradiol down-regulates reactive astrogliosis. In this study we assessed whether raloxifene and tamoxifen, two selective estrogen receptor modulators, have effects similar to estradiol in astrocytes. We also assessed whether aging and the timing of estrogenic therapy after ovariectomy influence the action of the estrogenic compounds. Four groups of animals were studied: 1) young rats, ovariectomized at 2 months of age; 2) middle-aged rats, ovariectomized at 8 months of age; 3) aged rats, ovariectomized at 18 months of age; and 4) aged rats, ovariectomized at 2 months and sham operated at 18 months of age. Fifteen days after ovariectomy or sham surgery, animals received a stab wound brain injury and the treatment with the estrogenic compounds. The number of vimentin-immunoreactive astrocytes after injury was significantly higher in the hippocampus of aged rats after a long-term ovariectomy compared with aged animals after a short-term ovariectomy and middle-aged rats. In addition, reactive astrocytes were more numerous in the two groups of aged animals than in young animals. Despite these differences, the estrogenic compounds reduced reactive astrogliosis in all animal groups. These findings indicate that estradiol, raloxifene, and tamoxifen are potential candidates for the control of astrogliosis in young and older individuals and after a prolonged depletion of ovarian hormones. (*Endocrinology* 150: 5010–5015, 2009)

Reactive astrogliosis is a complex phenomenon that includes a mixture of positive and negative responses for neuronal survival and regeneration. Reactive astroglia maintain the integrity of the blood-brain barrier and may contribute to protect the damaged neural tissue but may also prevent axonal regeneration and increase local inflammation (1–4). The identification of factors that regulate reactive astrogliosis is of practical interest for the development of therapeutic strategies to reduce neural damage and promote regeneration after central nervous system injuries and decrease neuronal death in neurodegenerative disorders. The activation of astrocytes is modulated by local factors and substances transported by the systemic circulation, such as the hormones secreted by the gonads and adrenals, including the ovarian hormone estradiol (5). Control of astrogliosis may be one of the mechanisms involved in the neuroprotective effects of estradiol. The hormone down-regulates both astroglial proliferation and the morphological activation of astrocytes after stab wound brain lesions (6–8), after excitotoxin-induced neurodegeneration (9), in an experimental model of Parkinson's disease (10), and after lesion of the cholinergic basal forebrain (11).

Estrogen receptors (ERs) are candidate targets for possible neuroprotective and antiinflammatory therapies based on estrogen actions. The transcriptional activity of ERs is regulated by their association with transcriptional

Abbreviations: ER, Estrogen receptor; GFAP, glial fibrillary acidic protein; SERM, selective ER modulator.

cofactors that have a tissue or cell-specific expression (12). This association depends on the three-dimensional conformation of the ERs (13), which is modified by different ER ligands (13–16). Therefore, several ER ligands, known as selective ER modulators (SERMs), are able to exert tissue- and cell-specific induction or repression of the activity of ERs, acting as ER agonists in some tissues and as antagonists in others. SERMs may therefore represent an alternative to estradiol for the treatment or the prevention of neurodegenerative disorders because they may have estrogenic neuroprotective actions similar to estradiol in the brain and at the same time an absence of estrogenic actions, or even antiestrogenic effects, in other tissues, thus avoiding the peripheral risks associated with estrogen therapy.

Previous studies have shown that two SERMs that are in clinical use in humans, raloxifene and tamoxifen, are able to reduce the activation of microglia induced by the bacterial endotoxin lipopolysaccharide, *in vitro* and *in vivo* (17, 18). In this study we assessed the effects of 17β-estradiol, tamoxifen, and raloxifene on the number of reactive astrocytes after a stab wound in the hippocampus of ovariectomized rats. In addition, a long period of ovarian hormone deprivation in women increases the risk of neurological disorders, cognitive impairment, or dementia (19), and it is suspected that aging, the timing after menopause, or both, affect the outcome of estrogen therapy in postmenopausal women (20, 21). In agreement with this possibility, studies in rodents have shown that the neuroprotective actions of estradiol are impaired in reproductively senescent females (22) and after a prolonged period of ovarian hormone deprivation (23). Therefore, in the present study, we also assessed whether aging and the duration of ovarian hormone depletion before estrogen therapies influence the ability of the estrogenic compounds to reduce reactive astrogliosis.

Materials and Methods

Animals and experimental treatments

Wistar albino female rats from the Complutense University animal colony were kept on a 12-h light, 12-h dark schedule and received food and water *ad libitum*. Animals were handled in accordance with the guidelines published in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the principles presented in the Guidelines for the Use of Animals in Neuroscience Research by the Society for Neuroscience and following the European Union guidelines (Council Directive 86/609/EEC). Experimental procedures were approved by our institutional animal use and care committee. Special care was taken to minimize suffering and to reduce the number of animals used to the minimum required for statistical accuracy.

Experimental design

In this study we assessed the effect of estradiol, raloxifene, and tamoxifen on reactive astroglia in ovariectomized rats. To determine whether the effects of estrogenic compounds are altered by aging, we studied three groups of animals: 1) young rats ovariectomized at 2 months of age; 2) middle-aged rats ovariectomized at 8 months of age, and 3) old rats ovariectomized at 18 months of age. All animals received brain injury 15 d after ovariectomy. In addition, to determine whether the effects of estrogenic compounds in aged animals are altered by the timing of estrogenic therapy after ovariectomy, another group of rats was ovariectomized at 2 months of age (*i.e.* at the same age as the young animals) and sham ovariectomized at 18 months of age (*i.e.* at the same age as the first group of aged animals was ovariectomized) 15 d before brain injury. Thus, both groups of aged rats were of the same age, 18.5 months, when they received the brain injury and the treatment with the estrogenic compounds. Because Wistar female rats in our colony still maintain relatively high estrogen levels in plasma at 18 months (68 ± 9 pg/ml^{-1}), the first group of aged rats was deprived of ovarian hormones for only 15 d before brain injury. In contrast, the second group of aged rats was deprived of ovarian hormones for 16.5 months before brain injury.

Brain injury

Animals were bilaterally ovariectomized under halothane anesthesia (Fluothane; AstraZeneca Farmacéutica, Madrid, Spain). For brain surgery, animals were anesthetized with halothane and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). An incision of the scalp was made and the cranium exposed. Then a unilateral opening of the skull was made with a dental drill. A solid stainless steel cannula, with a 0.45-mm outer diameter, was used to make a longitudinal stab wound in the left hemisphere. The cannula was positioned at 2 mm lateral to the midline in young rats, at 2.4 mm lateral to the midline in middle-aged and aged rats, and at 2 mm posterior to bregma in all age groups and introduced into the brain until the tip reached a depth of 5.5 mm. Then the cannula was displaced caudally 3 mm (bregma −5 mm) and finally removed from the brain. Bleeding was inhibited by compression with a gel-foam sponge. The scalp wound was sutured with surgical silk.

Treatments with estrogenic compounds

Animals received one sc injection of 17β-estradiol (E2758; Sigma-Aldrich, St. Louis, MO; 100 μg/kg), raloxifene (R1402; Sigma-Aldrich; 1 mg/kg), or tamoxifen (T5648; Sigma-Aldrich; 1 mg/kg) immediately after injury, a second injection 24 h later, and a third injection 48 h later. Thus, the estrogenic compounds were administered during the period of glial activation (24). The doses of estradiol and estrogenic compounds were selected on the basis of previous studies. This dose of estradiol is known to stimulate neuroprotective signaling cascades and exert neuroprotection in the hippocampus (25, 26). These effects may be at least in part due to the high levels of the hormone achieved shortly after the injections. The doses of raloxifene and tamoxifen were previously shown to be neuroprotective in a model of excitotoxin-induced neurodegeneration *in vivo* (9) and to reduce microglial activation in a model of brain inflammation (18).

Tissue fixation and immunohistochemistry

One week after brain injury, animals were deeply anesthetized with pentobarbital (100 mg/kg; Normon Veterinary Division, Madrid, Spain) and perfused through the left cardiac ventricle, first with 50 ml saline solution (0.9% NaCl) and then with 250 ml fixative solution [4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4)]. Brains were removed and immersed overnight at 4 C in the same fixative solution and then rinsed with phosphate buffer. Coronal sections of the brain, 50 μm thick, were obtained using a Vibratome (VT 1000 S; Leica Microsystems, Wetzlar, Germany).

Immunohistochemistry was carried out on free-floating sections under moderate shaking. All washes and incubations were done in 0.1 M phosphate buffer (pH 7.4) containing 0.3% BSA and 0.3% Triton X-100. The endogenous peroxidase activity was quenched for 10 min at room temperature in a solution of 3% hydrogen peroxide in 30% methanol. After several washes in buffer, sections were incubated overnight at 4 C with a monoclonal antibody for vimentin (diluted 1:500, clone V9; Dako, Barcelona, Spain), a marker of reactive astrocytes (9), or a monoclonal antibody for glial fibrillary acidic protein (GFAP; diluted 1:1000; clone GA5; Sigma-Aldrich), a marker of reactive and resting astroglia. Sections were then rinsed in buffer and incubated for 2 h at room temperature with biotinylated goat antimouse IgG (diluted 1:300; Pierce, Rockford, IL). After several washes in buffer, sections were incubated for 90 min at room temperature with avidin-biotin-peroxidase complex (diluted 1:250, ImmunoPure ABC peroxidase staining kit; Pierce). The reaction product was revealed by incubating the sections with 2 μg/ml 3,3'-diaminobenzidine (Sigma-Aldrich) and 0.01% hydrogen peroxide in 0.1 M phosphate buffer. Then sections were dehydrated, mounted on gelatinized slides, coverslipped, and examined with a Leica DMRB-E microscope.

Morphometric analysis

Only brains that showed a complete lesion from the dorsal to the ventral limit of the dorsal hippocampus were selected for morphometry. The number of vimentin and GFAP immunoreactive astrocytes was assessed in CA1 and within a distance of 350 μm from the lateral border of the wound. The number of immunoreactive cells was estimated by the optical disector method (27) using total section thickness for disector height (28) and a counting frame of 55 $\times$ 55 μm. A total of 78 counting frames were assessed per animal. Section thickness was measured using a digital length gauge device (Heidenhain-Metro MT 12/ND221; Traunreut, Germany) attached to the stage of a Leitz microscope. Cell nuclei from immunoreactive cells that came into focus while focusing down through the disector height were counted. All counts were performed on coded sections. The volume of CA1 was estimated using the point-counting method of Weibel (29). Because no significant differences in this parameter were observed among the different experimental groups, the changes in the number of immunoreactive cells per unit volume with the optical disector method are assumed to reflect changes in the total number of immunoreactive cells.

The n used for statistical analysis was the number of animals (n = 4–6). Because the Levene's test revealed nonhomogeneity of variances, data were analyzed using the nonparametric Kruskal-Wallis test followed by the Mann-Whitney test for paired comparisons, with $P < 0.05$ considered to be significant.
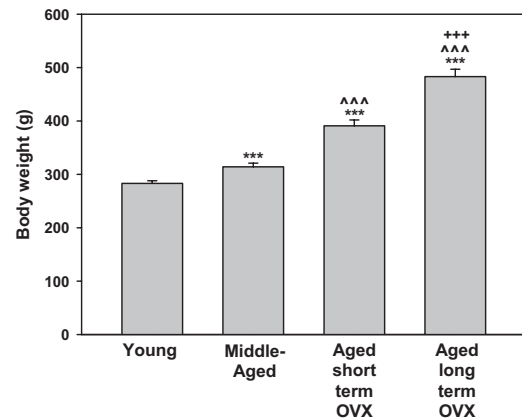

FIG. 1. Body weight (grams) of the animals at the time of brain injury. Young rats, n = 21; middle-aged rats, n = 23; aged rats after short-term ovariectomy (OVX), n = 17; and aged rats after long-term OVX, n = 19. Data are represented as means ± SEM. Significant differences: ***, $P < 0.001$ *vs.* young values; ^^^, $P < 0.001$ *vs.* middle-aged values; +++, $P < 0.01$ *vs.* aged rats with short-term OVX.

Results

Body weights of the animals at the time of brain surgery are represented in Fig. 1. Middle-aged and aged animals had a significantly higher body weight compared with young animals. Aged animals had a significantly higher body weight than middle-aged animals. Aged animals that had a prolonged depletion of ovarian hormones had a higher body weight than aged animals with shorter term (15 d) depletion.

The qualitative inspection of the sections immunostained for vimentin, to detect reactive astrocytes, revealed a prominent glial scar along the borders of the wound in all animal groups. However, animals treated with estradiol, raloxifene, or tamoxifen showed a glial scar with a
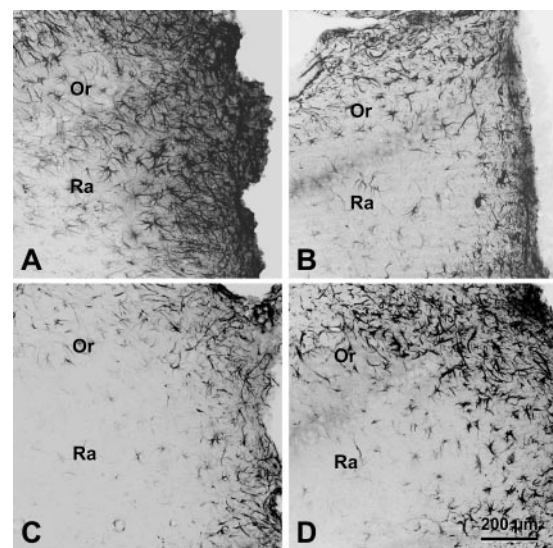

FIG. 2. Panoramic view of the border of the lesion in CA1 showing representative examples of vimentin immunoreactivity in young rats after the administration of vehicle (A), estradiol (B), raloxifene (C), and tamoxifen (D). All figures are at the same magnification. Or, Stratum oriens; Ra, stratum radiatum.
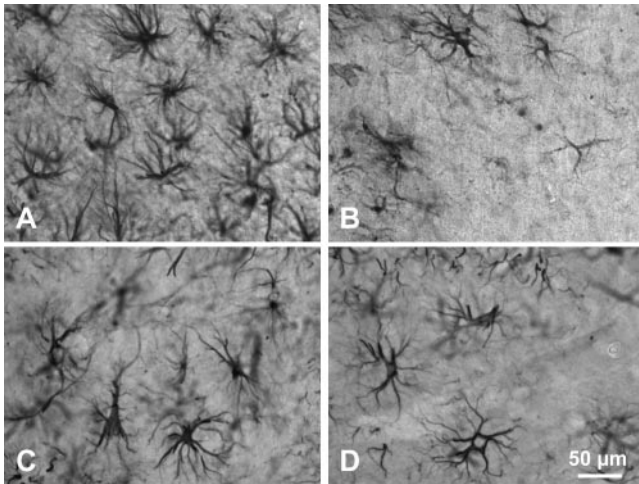
FIG. 3. High magnification of vimentin immunoreactive astrocytes in the CA1 stratum radiatum at a distance of approximately 100–200 μm from the lateral border of the wound. The panels illustrate representative examples from aged rats ovariectomized 15 d before the brain injury after the administration of vehicle (A), estradiol (B), raloxifene (C), and tamoxifen (D). All figures are at the same magnification.

lower cellular density compared with control animals (Figs. 2 and 3). Similar qualitative differences were observed in young, middle-aged, and aged animals.

The morphometric analysis with the optical disector method confirmed the qualitative observations (Fig. 4).
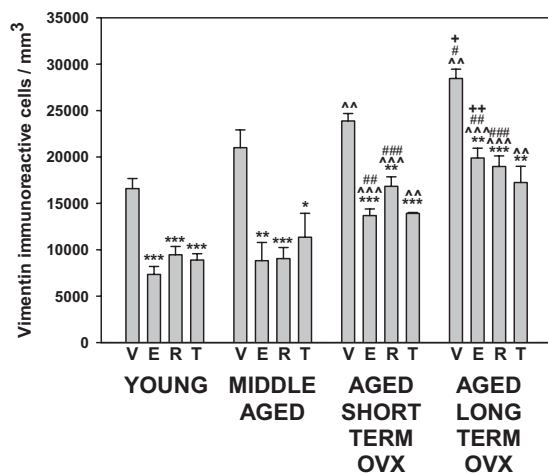

FIG. 4. Number of vimentin immunoreactive astrocytes per cubic millimeter within a distance of 350 μm from the lateral border of the wound in animals injected with vehicle (V), estradiol (E), raloxifene (R), or tamoxifen (T) in young rats (stab wound performed 15 d after ovariectomy; V, n = 5; E, n = 5; R, n = 6; T, n = 5); middle aged rats (stab wound performed 15 d after ovariectomy; V, n = 6; E, n = 6; R, n = 5; T, n = 6); aged rats after short-term ovariectomy (OVX) (stab wound performed 15 d after ovariectomy; V, n = 5; E, n = 4; R, n = 4; T, n = 4), and aged rats after long-term OVX (stab wound performed 16 months and 15 d after OVX; V, n = 4; E, n = 4; R, n = 6; T, n = 5). Data are represented as means $\pm$ SEM. Significant differences: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* vehicle values, within age groups; $^{\wedge\wedge}$, $P < 0.01$; $^{\wedge\wedge\wedge}$, $P < 0.001$ *vs.* young rats, within the same treatment; #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ *vs.* middle aged rats, within the same treatment; +, $P < 0.01$; ++, $P < 0.05$ *vs.* aged rats with short-term OVX, within the same treatment.

The administration of estradiol, raloxifene, or tamoxifen resulted in a significant decrease in the number of reactive astrocytes in the border of the wound. The decrease in the number of reactive astrocytes in response to each of the estrogenic compounds was detected in young, middle-aged, and aged animals (Fig. 4).

In addition, the morphometric analysis revealed that both the age and the duration of ovarian hormone depletion affected the number of reactive astrocytes in animals injected with vehicle (Fig. 4). Thus, a prolonged depletion of ovarian hormones increased the number of reactive astrocytes in aged rats compared with those aged rats that had a short-term hormone depletion prior injury. The number of reactive astrocytes was also higher in aged rats with a prolonged depletion of ovarian hormones than in middle-aged rats and was higher in both groups of aged animals than in young animals (Fig. 4).

Significant differences between the experimental groups were also detected in the number of reactive astrocytes after the treatment with estrogenic compounds. Reactive astrocytes were less numerous in young animals than in either group of aged animals after the treatment with estradiol, raloxifene, or tamoxifen and less numerous in middle-aged animals than in either group of aged animals after the treatment with estradiol and raloxifene (Fig. 4). Furthermore, there were more reactive astrocytes after estradiol treatment in aged animals with prolonged depletion of ovarian hormones than in those treated after short-term hormone depletion (Fig. 4).

To exclude the possibility of an effect of estrogenic compounds on the expression of vimentin, rather than on the number of reactive astrocytes, we assessed the number of GFAP immunoreactive cells in middle-aged animals. As shown in Fig. 5, both estradiol, raloxifene, and tamoxifen resulted in a significant reduction in the number of GFAP immunoreactive cells.
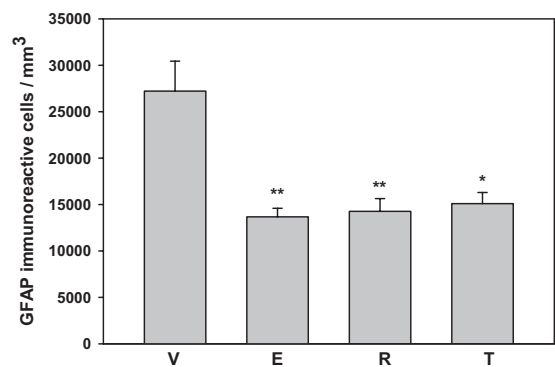

FIG. 5. Number of GFAP immunoreactive astrocytes per cubic millimeter within a distance of 350 μm from the lateral border of the wound in middle-aged rats injected with vehicle (V; n = 6), estradiol (E; n = 6), raloxifene (R; n = 5), or tamoxifen (T; n = 6). Data are represented as means $\pm$ SEM. Significant differences: *, $P < 0.05$; **, $P < 0.01$ *vs.* vehicle values.

Discussion

Previous studies have shown that the systemic administration of estradiol to ovariectomized female rats significantly decreases reactive gliosis after a stab wound in the hippocampus (6). Our present findings, using vimentin to label reactive astrocytes and GFAP to label resting and reactive astrocytes, confirm these previous observations. In addition, we have observed that two SERMs currently used in the clinic, raloxifene, and tamoxifen are also able to reduce reactive astroglia in the borders of the wound.

Although tamoxifen has been shown to act as an antagonist for several effects of estradiol on neurons (30–33), our findings suggest that this SERM acts as an estrogen agonist in reactive astrocytes. Different expression of transcriptional coregulators in neurons and astrocytes (12) or the activation by SERMs of estrogen response element-independent transcriptional mechanisms in astrocytes (4) may explain the cell-specific actions of tamoxifen.

We observed that aging increases the number of reactive astrocytes after brain injury in female rats. This parallels previous observations in male rats, in which the gliotic response after stab wound lesions (34), excitotoxic damage (35), intracerebral hemorrhage (36), and cerebral ischemia (37), increases with aging. In addition, we found that prolonged depletion of ovarian hormones also contributes to increased reactive astrogliosis after brain injury. This is particularly relevant when considering neurodegenerative diseases in women after menopause when aging is associated with loss of ovarian hormones.

Despite the differences in gliosis after injury, estradiol, raloxifene, and tamoxifen were able to significantly reduce the number of reactive astrocytes in all age groups. In addition, the three estrogenic compounds decreased astrogliosis in aged rats after a prolonged depletion of ovarian hormones. Estradiol, raloxifene, and tamoxifen produced a similar reduction in the number of reactive astrocytes in young and middle-aged animals, which is in agreement with the results of a recent study showing that estradiol retains its effects on neuronal survival in the hippocampus of middle-aged female rats in a global ischemia model (38). However, the number of reactive astrocytes in estradiol and raloxifene-treated animals was higher in aged animals compared with young and middle-aged rats. A prolonged depletion of ovarian hormones also resulted in a higher number of astrocytes remaining reactive after estradiol treatment in aged animals. The increased basal level of astrogliosis with aging and after prolonged hormonal depletion may contribute to these differences. In addition, a previous study in an animal model of stroke indicated that prolonged depletion of ovarian hormones disrupts both peripheral and central antiinflammatory actions of estradiol (23). Therefore, it is possible that the long-term loss of ovarian hormones results in an altered estrogenic regulation of both central and peripheral cytokines and may decrease the protective effects of estrogens in the aged rats.

In addition to aging and a prolonged depletion of ovarian secretions, body weight at the time of injury is also an important parameter to take in consideration. Body weight may affect the pharmacokinetics of estrogenic compounds and may affect reactive gliosis because higher body fat *per se* is associated with inflammation (39). Therefore, the observed differences in body weight between the experimental groups may contribute to the differences in astrogliosis after brain injury and after the treatments with the estrogenic compounds.

In conclusion, estradiol and SERMs have a potential therapeutic value in controlling astrogliosis after traumatic brain injury, and these may remain beneficial, even in aged animals that have experienced a prolonged depletion of ovarian hormones. The actions of the estrogenic compounds on astrocytes may in turn reduce neural damage by decreasing brain inflammation (4). However, the exact functional consequences of the reduction of astrogliosis in the hippocampus after a stab wound injury needs to be assessed because reactive astrocytes release substances, including estradiol (40, 41), that promote neuronal survival and remyelination (42). Therefore, further studies should determine whether the antigliotic effects of estradiol and SERMs are associated with less neuronal loss or better functional recovery in this model. In addition, the antigliotic effect of estradiol and SERMs on aged females may be particularly relevant for chronic neurodegenerative diseases associated with aging, in which it is suspected that a dysfunction of glial cells may contribute to increased neural damage (43, 44).

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