

Estrogen Enhances Uptake of Amyloid β -Protein by Microglia Derived from the Human Cortex

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Abstract: In recent years, inflammatory mechanisms have been increasingly appreciated as important steps in the pathology of Alzheimer's disease (AD). There are two pathological defects in AD: chronic inflammation and impaired clearance of amyloid β -peptide ($A\beta$). In the periphery, estrogen both increases macrophage phagocytosis and has antiinflammatory effects. If estrogen had a similar effect in the CNS, it could reverse inflammatory defects in AD. Although microglia are a key component of the immune system and help clear $A\beta$ deposits in the AD brain, little is known about the effects of estrogen on CNS microglia. Therefore, we sought to determine the relationship between estrogen treatment and internalization of $A\beta$ by microglia by quantifying the internalization of aggregated $A\beta$ by human cortical microglia. $A\beta$ uptake was found to be dose- and time-dependent in cultured microglia. Increased $A\beta$ uptake was observed at 1.5 and 24 h after addition of aggregated $A\beta$ (50, 100, or 1,000 nM $A\beta$), and this uptake was enhanced by pretreatment with estrogen. The expression of estrogen receptor (ER) β (ER- β) was also up-regulated by estrogen treatment. Cells co-treated with ICI 182,780, an ER antagonist, showed significantly reduced internalization of $A\beta$ in cultured microglia. These results indicate that microglia express an ER- β but that the effect of estrogen on enhancing clearance of $A\beta$ may be related to the receptor-independent action of estrogen or to nonclassical ER effects of estrogen. Thus, stimulation of the ER might contribute to the therapeutic action of estrogen in the treatment of AD.

Key Words: Estrogen—Receptors—Phagocytosis—Amyloid protein—Microglia—Alzheimer's disease. *J. Neurochem.* **75**, 1447–1454 (2000).

Alzheimer's disease (AD) is a progressive neurodegenerative disease. The pathological hallmarks of AD include extracellular plaques containing amyloid β -peptide ($A\beta$), neurofibrillary tangles, dystrophic neurites, activated microglia, reactive astrocytes, and neuronal loss (Rogers et al., 1996). In vitro evidence indicates that insoluble $A\beta$ is neurotoxic (Pike et al., 1991; Pike, 1999). Although the mechanisms of elevated cellular $A\beta$ production and deposition are under investigation, studying the normal clearance of insoluble $A\beta$ from the extracellular compartment may be equally significant in describing the biochemical pathogenesis of AD.

Studies have shown that resting microglial cells can be activated by $A\beta$ in brain (Weldon et al., 1997; Kalara, 1999). These activated microglia migrate and surround the region of compact $A\beta$ deposits (Wegiel and Wisniewski, 1990), and $A\beta$ stimulates phagocytosis of microglia (Kopeck and Carroll, 1998). Substantial evidence suggests that activated microglial cells help remove and internalize $A\beta$ deposits (Frautschy et al., 1992; Ard et al., 1996; Paresce et al., 1997; Chung et al., 1999). Furthermore, ultrastructurally amyloid fibrils have been found in the microglial cytoplasm in amyloid precursor protein transgenic mice, suggesting atypical phagocytosis (Stalder et al., 1999). In the animal brain, phagocytosis of insoluble $A\beta$ by microglial cells starts soon after $A\beta$ injection and lasts for days (Weldon et al., 1997). In the AD cerebral cortex, antibodies to $A\beta$ directed to the C but not the N terminus detected $A\beta_{1-40}$ and $A\beta_{1-42}$ immunoreactive granules within microglia and astrocytes that were not associated with complement activation (Akiyama et al., 1999). The concentration of $A\beta_{1-42}$, the major component of the diffuse plaques seen in the brain of AD patients, was significantly lower in CSF than in plasma in AD patients, whereas levels of other fragments of $A\beta$ were no different between CSF and plasma (Tamaoka, 1998). These data suggest that increased clearance of $A\beta$ in CSF, or decreased secretion of $A\beta$ into the CSF, may be due to increased deposition in neuropil in patients with AD.

Collectively, it seems that the clearance of $A\beta$ in AD brain is an active mechanism and that microglia play a key role in this processing. However, the mechanisms of microglial uptake of $A\beta$ are still not fully understood. Enhancing $A\beta$ clearance by vaccination (Schenk et al., 1999) and by targeting various receptors has been sug-

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Abbreviations used: $A\beta$, amyloid β -peptide; AD, Alzheimer's disease; ER, estrogen receptor; fluo- $A\beta$, fluorescein-labeled β -amyloid $_{1-42}$; fluo-*E. coli*, fluorescein-labeled *Escherichia coli* K-12 BioParticles; HBSS, Hanks' balanced salt solution.

gested (Christie et al., 1996; El Khoury et al., 1996; Paresce et al., 1996).

AD is one of the most common types of dementia among the aged population, and some studies have suggested that women are much more likely to develop late-onset AD than age-matched men (Molsa et al., 1982). Menopause, a well-established AD risk factor, produces a rapid state of hypoestrogenism in women, and estrogen replacement therapy reduces the risk of developing AD (Paganini-Hill and Henderson, 1994; Birge, 1997; Kawas et al., 1997; McEwen et al., 1997; Genazzani and Gambacciani, 1999). However, it is unclear what role estrogen plays in the CNS and how its action relates to AD. Many studies have demonstrated that estrogen influences the CNS: It has direct neuroprotective effects (Arimatsu and Hatanaka, 1986; Singer et al., 1996; Xu et al., 1999), it can alter astrocytic function (Dodel et al., 1999; Garcia-Segura et al., 1999), and it acts as an antioxidant (Inestrosa et al., 1998). Estrogen can also affect the inflammatory system. Furthermore, Mor et al. (1999) found that microglia express estrogen receptor (ER) β (ER- β).

In contrast to the sparse studies done on steroids and microglia, much is known about macrophage regulation by steroids, which are similar in function and in antigenic recognition to microglia. In general, estrogens have both immunostimulatory and antiinflammatory properties on macrophages or macrophage-like cells (Cutolo et al., 1993). The potent ability of estrogen to stimulate the complement protein C3 and its mRNA has been studied predominantly in uterine macrophages in relation to estrus (Brown et al., 1990). Activated ERs have been shown to bind directly to the estrogen-responsive sequences within the C3 promoter (Fan et al., 1996). In fact, physiological concentrations of estrogen have been shown to stimulate phagocytosis of murine macrophages (Chao et al., 1996). However, the effects of estrogen on microglia from the CNS have not been reported, nor have data supporting estrogen action on A β clearance in the brain of AD patients been reported. This study was designed to investigate the effect of estrogen on A β clearance in the brain and uses human cultured microglial cells from aged human brains.

MATERIALS AND METHODS

Isolation and characterization of microglia

Microglial cells obtained from brain tissues were prepared and isolated according to a previously described method (Lue et al., 1996). In brief, frontal cortex samples were removed at autopsy under aseptic conditions and then quickly immersed in ice-cold Hanks' balanced salt solution (HBSS; Irvine Scientific, U.S.A.). Enzymatic dissociation was processed as described elsewhere (Kim et al., 1983). Samples were washed with several changes of HBSS, after which all visible connective tissues and blood vessels were removed. The tissues were minced, incubated with a Ca²⁺- and Mg²⁺-free HBSS containing 2.5% trypsin (Life Technologies, U.S.A.) and 2 mg/ml DNase I (Amersham, U.S.A.), and then incubated in a shaking water bath at 37°C for 30 min and 150 rpm. After the incubation, 2 ml

of fetal bovine serum was added to stop the enzymatic digestion. The digested sample was triturated and centrifuged at 1,500 rpm for 30 min. The pellets were resuspended in 40 ml of HBSS and filtered through mesh with 130-, 100-, and 200- μ m pores. The filtrate was subsequently spun at 15,000 rpm in 100% Percoll (Sigma) for 30 min at 3°C. The viable cell layer (middle layer) was transferred to 50-ml centrifuge tubes and washed twice with HBSS. A third wash was performed in growth medium consisting of Dulbecco's modified Eagle's medium (Life Technologies) with high glucose (25 mM), 2% HEPES, 1% sodium pyruvate, 10% fetal bovine serum, and 0.1% gentamicin (Sigma). After the third wash, cells were resuspended in growth medium and plated in 24-well plates (Corning, U.S.A.) coated with poly-D-lysine (Sigma) at a density of $\sim 1.0 \times 10^6$ cells per well. Cells were incubated with the growth medium at 37°C in 5% CO₂ for 18–24 h, when microglial cells adhered to the cultureware surfaces. The nonadherent astrocytes were removed. The microglial cells were then cultured in the growth medium with weekly medium change, and cells were used 2 weeks after the initial plating for studies.

Experimental treatment of cultures

Estrogen (17 β -estradiol; catalogue no. E8875; Sigma) was freshly prepared by dissolving in ethanol (50 mg/ml) and diluted with water before use. Microglial cultures were treated with estrogen at day 1 in vitro for 48 h. After a 48-h estrogen exposure, cells were treated with fluorescein-labeled β -amyloid_{1–42} (fluo-A β ; NEN, U.S.A.) or control medium. The β -amyloid exposure was maintained for 24 h, after which cells were processed for either immunofluorescence phagocytosis assay or immunocytochemistry. To determine the optimal dosage and duration of treatment, curves for dose-effect and time course relationship for estrogen and fluo-A β uptake in microglia were also performed.

Because many of the effects of estrogen on cellular function are mediated by the activation of ERs, we examined A β uptake in both the presence and absence of the ER antagonist ICI 162,780 (Tocris, U.S.A.), which inhibits both the α and β isoforms of ERs. Cell number was counted by hemacytometry to monitor the effect of A β on cell survival.

Immunofluorescence phagocytosis assay

Microglial phagocytic activity was quantified by measuring the fluorescence of fluo-A β or fluorescein-labeled *Escherichia coli* K-12 BioParticles (fluo-*E. coli*; Molecular Probes, U.S.A.) that were internalized. Cells were cultured in 24-well plates. To determine general phagocytic activity after treatment with various concentrations of estrogen, cells were incubated with fluo-*E. coli* for 2 h at room temperature. Trypan blue was added immediately after removing the fluo-*E. coli* from cells to quench the extracellular probe. The wells of the plate were read by a Wallac (U.S.A.) Victor2 1420 multilabel counter using excitation at 480 nm and emission at 520 nm. Data were analyzed by Wallac Explore. For measuring the effect of estrogen on internalization of A β , the fluo-A β was dissolved in dimethyl sulfoxide, diluted in serum-free culture medium, and allowed to aggregate for 1 h at room temperature. The aggregated A β was vortex-mixed and sonicated before being added to microglial cultures. Microglia were pretreated with estrogen or vehicle for 48 h before fluo-A β administration. The conditioned medium and cell lysate were quantified by the Wallac Victor2 1420 multilabel counter.

Immunocytochemistry

Immunocytochemistry of microglial cells was performed with monoclonal ER- α and polyclonal ER- β (Santa Cruz, U.S.A.). Cells were fixed with 4% paraformaldehyde for 15 min at room temperature, washed with phosphate-buffered saline three times, and then blocked with blocking buffer (2% bovine serum albumin, 2% goat serum, and 0.05% Triton X-100 in phosphate-buffered saline) for 30 min at room temperature. Cells were incubated with primary antibody for 3 days at 4°C. Then cells were rinsed with phosphate-buffered saline three times and incubated with secondary antibodies (Santa Cruz) for 1 h at room temperature. To verify the internalization of A β by microglia, two sets of cells were incubated with unlabeled A β_{1-42} (Bachem, U.S.A.). Before blotting the cells with the anti-A β antibody (1:500 dilution, N-terminal 16–24; Senetek, U.S.A.), one set of cells was permeabilized with 0.1% Triton X-100, and the other was treated without it. Fluorescence microscopy (Axiovert 100; Zeiss, Germany) with 25 \times and 100 \times objectives was used to observe a large number of cells per field.

RESULTS

Microglial uptake of aggregated A β

To examine the uptake of aggregated A β in microglia, we administered fluo-A β to human primary microglial cultures. Fluorescence was observed in microglia, suggesting that microglia have the ability to internalize fluo-A β (Fig. 1A and B). To ensure that A β was internalized instead of sticking on the cell surface, we had also treated microglia with unlabeled A β_{1-42} and treated the cells with or without 0.1% Triton X-100 before incubation of anti-A β antibody. We found that the cells without Triton X-100 permeabilization had very little labeled anti-A β antibody (Fig. 1C and D), whereas permeabilized cells expressed heavy labeling with the antibody (Fig. 1A and B). Thus, our results suggest that the majority of aggregated A β was taken up by microglia.

Figure 2A illustrates that the internalization of aggregated A β was dose-dependent in cultured microglia. The fluorescence of fluo-A β from microglia lysate increased significantly at 24 h after fluo-A β treatment at a dose ranging from 10 to 1,000 nM (Fig. 2A). As Fig. 2B indicates, A β -induced increases in the fluorescence of cell lysate were time-dependent; an increase in fluorescence was seen at 1.5 h after incubation of A β at a dosage of 100 nM, and a much greater increase was seen after 24 and 48 h of stimulation of fluo-A β (Fig. 2B). The uptake of A β increased only after a 24- or 48-h incubation with a lower dose of A β (50 nM). The increase of fluorescence is not due to possible changes in cell density because cell numbers were not altered due to A β treatments; this indicates that A β does not improve survival of microglia as others have suggested (Chung et al., 1999).

Estrogen increases internalization of A β in microglia

To evaluate the hypothesis that estrogen modulates A β clearance in the CNS, we examined intracellular fluo-A β in microglia in the presence and absence of estrogen. Cells were treated for 24 h with 0, 10, 50, and

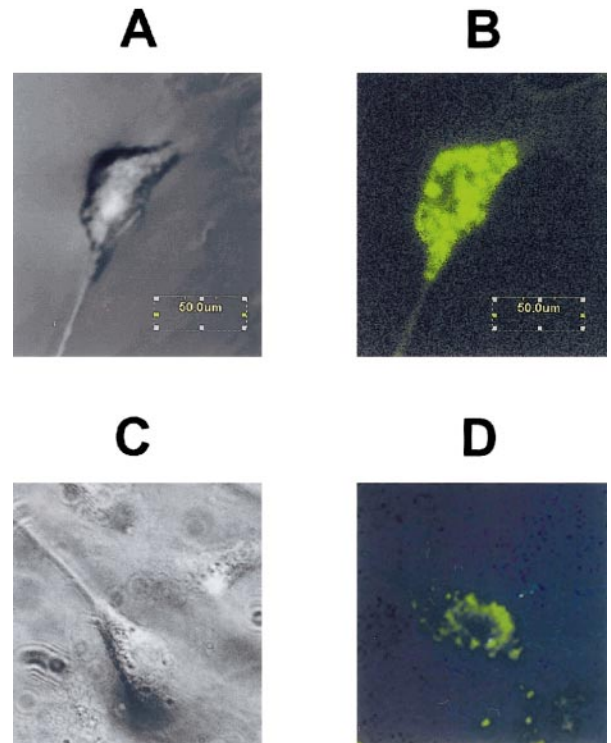


FIG. 1. Microglia takes up unlabeled A β aggregates. Two sets of microglial cells were incubated with aggregated unlabeled A β (1 μ M) at 37°C for 24 h. One set of cells was fixed by 4% paraformaldehyde with 0.1% Triton X-100 for 30 min at room temperature, whereas another set was fixed with 4% paraformaldehyde only. Both sets of cells were then labeled with the anti-A β antibody and processed for immunofluorescence. Cells incubated with A β and permeabilized with Triton X-100 are shown in **B**, and the unpermeabilized cells are shown in **D**. A phase-contrast image of the cells is shown in **A** and **C**, respectively.

100 nM estrogen, followed by various amounts of fluorescent A β (10–100 nM). In this culture system, microglia exhibited basal levels of fluo-A β uptake in the absence of estrogen (Fig. 3C and E). However, the examination of treated cells revealed that estrogen significantly increased internalization of fluo-A β (Fig. 3D and F). Estrogen-treated microglia became brightly labeled by fluo-A β , and more cells actively internalized A β .

The effects of estrogen treatments on the uptake of A β by microglia also appear to be dose- and time-dependent. At 48 h after estrogen treatment at a dose of 100 nM, the most significant enhanced internalization of A β was found (197% of A β -treated alone, 552% of untreated); in contrast, no significant alteration was observed at 1.5 h after the same dose compared with A β -treated alone (Fig. 4).

To examine the specificity of estrogen on the internalization of A β by microglia, we incubated the estrogen-treated cells with fluo-*E. coli* as a nonspecific ligand and detected the internalized fluorescence in the cells. Our data indicate that the internalization of fluo-*E. coli* in the microglia by estrogen occurs at doses similar to those observed in fluo-A β uptake (Fig. 5), suggesting that estro-

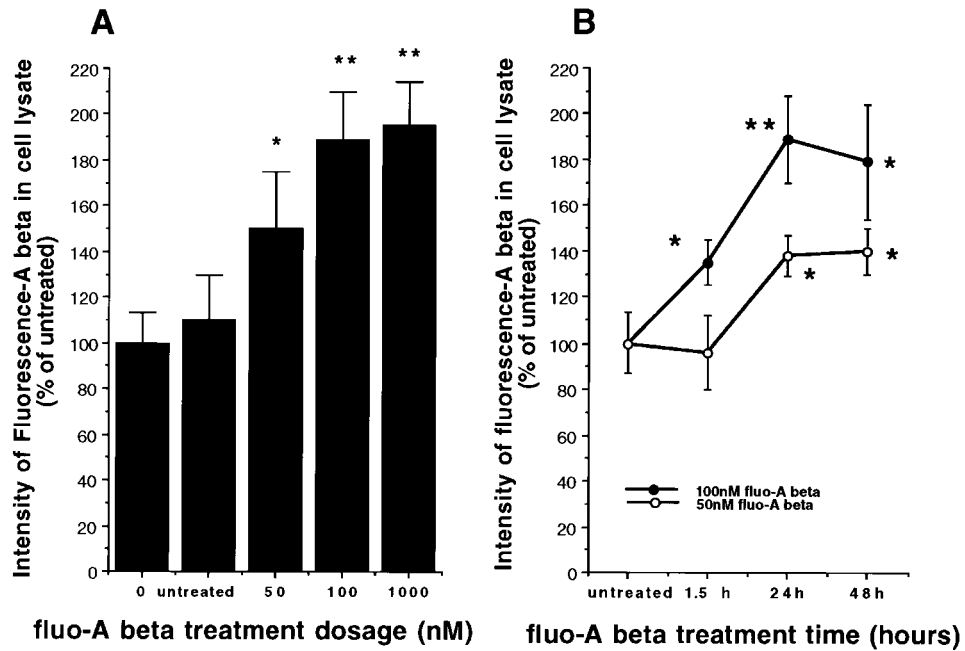


FIG. 2. Effect of aggregated fluo-Aβ on phagocytosis in microglia. **A:** Dose-effect relationship for aggregated Aβ internalization by microglia. Multiple wells of cells were incubated with various doses of fluo-Aβ at 37°C for 24 h. **B:** Time course of the effect of internalization of fluo-Aβ aggregates by microglia. Cells were incubated with fluo-Aβ (50 or 100 nM) for 1.5, 24, or 48 h. Cells were then washed and lysed to determine the intensity of fluorescence. Data are mean ± SEM (bars) values of triplicates from a representative experiment repeated three times. **p* < 0.05, ***p* < 0.01.

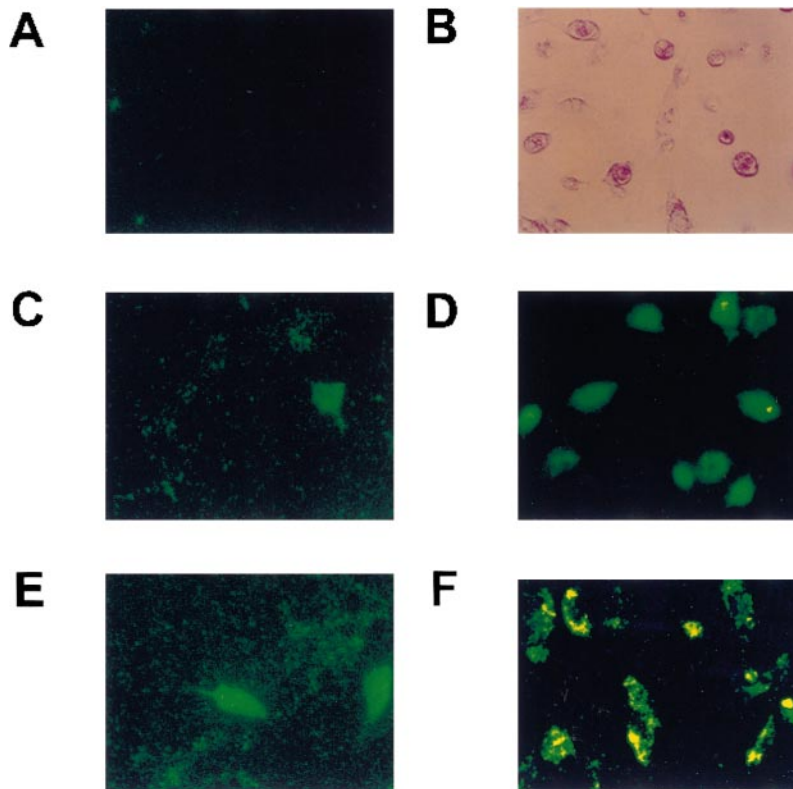


FIG. 3. Effect of estrogen treatment on fluo-Aβ uptake by microglia. Microglia were treated with estrogen (100 nM) for 48 h at 37°C. Where indicated, cells were then incubated with fluo-Aβ aggregates for 24 h. The cells were then rinsed, fixed, and observed by phase-contrast or fluorescence microscopy. Microglia were treated with **(A and B)** medium only, **(C)** 50 nM fluo-Aβ, **(D)** 100 nM estrogen plus 50 nM fluo-Aβ, **(E)** 100 nM fluo-Aβ, and **(F)** 100 nM estrogen plus 100 nM fluo-Aβ. The cells shown in A-F are from matched incubations carried out in one experiment.

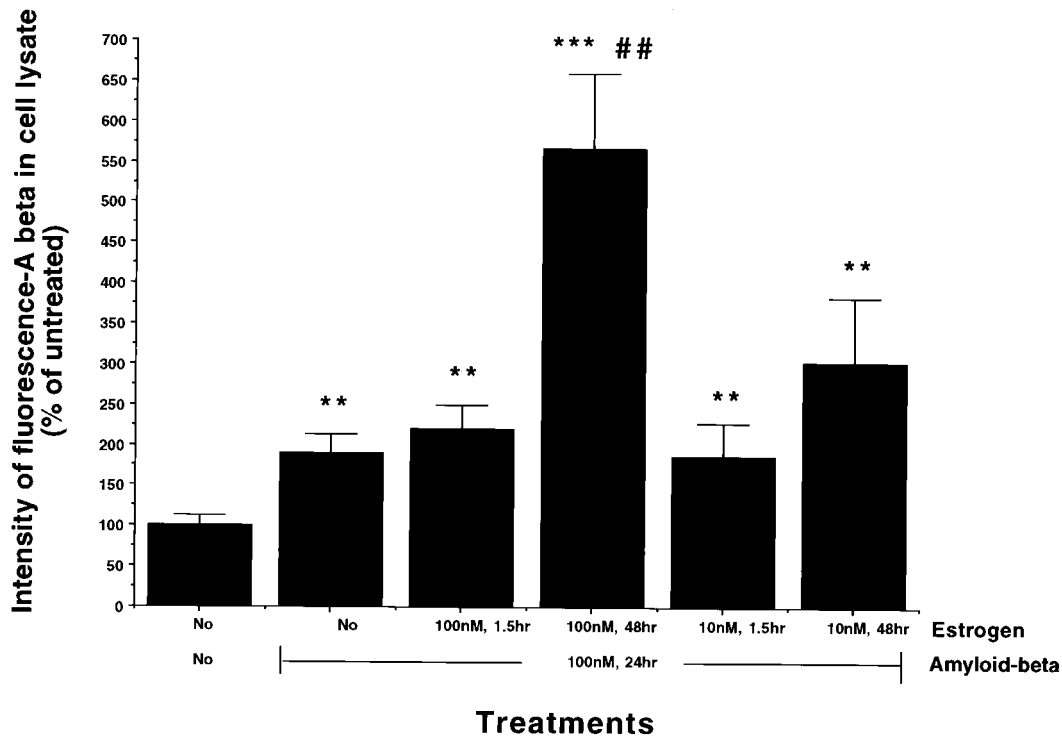


FIG. 4. Dose-effect relationship for estrogen treatment on aggregated fluo-A β uptake by microglia. Cells were treated with estrogen (10 or 100 nM) for 1.5 or 48 h at 37°C and then incubated with fluo-A β (100 nM) for another 24 h. Data are mean \pm SEM (bars) of triplicates from a representative experiment repeated three times. ** p < 0.01; *** p < 0.001 for treated versus untreated, ## p < 0.01 for estrogen plus A β treatment versus A β alone.

gen increases microglial phagocytic activity through a general uptake mechanism that might not be specific to A β .

ER antagonist reduces estrogen-stimulated A β uptake in microglia

To investigate the possible mechanisms of estrogen-induced enhancement of A β uptake in human microglia, we cotreated microglia with estrogen and with the ER antagonist ICI 182,780. Our data show that estrogen treatment increases A β uptake, and treating microglia with ICI 182,780 significantly reduced but did not abolish the enhancement of A β uptake associated with estrogen treatment (Fig. 6). Using an immunocytochemistry double-labeling technique, we detected ER- β and fluo-A β in cultured microglia. However, the cells that had strong fluo-A β labeling did not necessarily have ER- β staining. Some cells labeled with ER- β lacked fluo-A β staining. No ER- α was detected in our microglial culture (data not shown). Our data indicate that there is no clear colocalization between ER- β and uptake activity of A β . However, estrogen treatment increased the expression of ER- β in our microglial cultures. More cells expressed ER- β in estrogen-treated cells than in estrogen-untreated cells (Fig. 7).

DISCUSSION

In the AD brain, activated microglia are concentrated in the regions of amyloid deposits. It is unclear whether

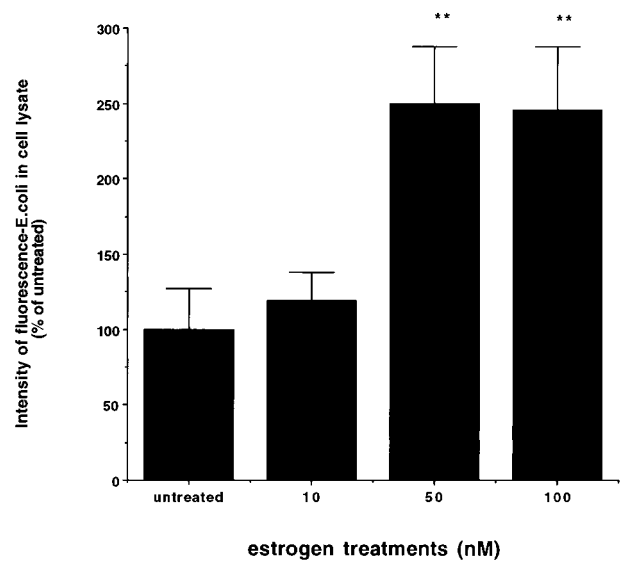


FIG. 5. Dose-effect relationship of estrogen on internalization of fluo-*E. coli* by microglia. Cells were cultured in 24-well plates, treated with estrogen for 48 h, and then incubated with fluo-*E. coli* (0.5 mg/ml) for 2 h at room temperature. Cells were then rinsed with phosphate-buffered saline, and 100 μ l of trypan blue was added to quench the extracellular fluorescence. The intensity of fluorescence of internalized fluo-*E. coli* was measured by a fluorescence microplate reader. Data are mean \pm SEM (bars) values from triplicate determinations. ** p < 0.01.

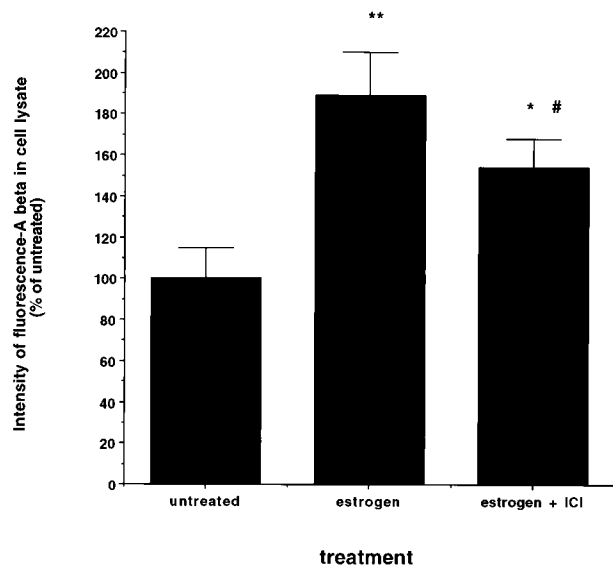


FIG. 6. ER dependency of fluo-A β uptake by microglia. Cells were treated with estrogen in the absence or presence of ICI 182,780 (10 μ M). The cells were then rinsed and lysed. The internalized fluo-A β was detected by measuring fluorescence via fluorescence microplate reader. Data are mean \pm SEM (bars) values from triplicate determinations. * p < 0.05, ** p < 0.01 compared with untreated; # p < 0.05 versus estrogen-treated only.

the activated microglia contribute to the formation of plaques or play a role in the clearance of amyloid fibrils. Recent studies demonstrated that rat microglia could degrade A β ₁₋₄₂ in cell culture (Shaffer et al., 1995; Qiu et al., 1997) and that inhibition of the degradation with protease inhibitors increased A β accumulation in a murine microglial cell line (Chu et al., 1998). Another early report also showed phagocytic properties from primary cultures of microglia from human brain (Lue et al., 1996).

Taken together, the data presented in this article show that human aged cortical microglia can internalize A β aggregates. This internalization of A β by human cortical microglia is both dose- and time-dependent (Fig. 2). Although some membrane receptors, such as scavenger and Fc, might be involved in the early phagocytic action in activated microglia (Vedeler et al., 1994; Paresce et al., 1996; El Khoury et al., 1998), the quick early action and ongoing internalization response suggest that the uptake of A β by microglia might be regulated via both membrane and nuclear mechanisms.

A significant association between estrogen replacement therapy and reduced risk of developing AD has been reported (Birge, 1997; Kawas et al., 1997; McEwen et al., 1997). However, the underlying molecular and cellular mechanism of estrogen's inhibitory actions in AD pathology remains unclear. In this study, we present several lines of evidence that estrogen significantly increases A β internalization in microglia from the human brain. First, A β internalization in microglia was enhanced by estrogen. Second, the strongest response oc-

curred 48 h after pretreatment with estrogen. This finding of phagocytic response to estrogen in human microglia is consistent with its known effects on peripheral macrophages (Chao et al., 1996). However, the estrogen dosage we used in this studies is higher than the physiological concentration, and this could be due to the possible lower activation of ERs in aged individuals as suggested (Post et al., 1999). The lower sensitivity of microglia to estrogen might be also due to the antiinflammatory effect of 10% fetal bovine serum and dimethyl sulfoxide, both of which may alter the threshold of phagocytosis. The delayed action of estrogen treatment on uptake of A β by microglia suggests that nuclear ERs might be involved.

There are two types of ERs: ER- α and ER- β . Both receptors have been identified in human peripheral mononuclear cells, lymphocytes, and the CNS. Recent studies have reported that microglial cells from the rat brain, as a target for estrogen action, express ER- β (Mor et al., 1999). Our immunocytochemistry data not only provide evidence of ER- β expression in human brain microglia, but also demonstrate an increase of ER- β expression due to estrogen treatment (Fig. 7). Furthermore, in this study, the ER antagonist ICI 182,780 was able to block partially estrogen's action on uptake of A β by microglia (Fig. 6). As Fig. 6 highlights, our data indicate that the partial blocking action of ICI 182,780 on uptake of A β by microglia may be due to the up-regulation of ER- β by estrogen treatment. However, the ICI 182,780-insensitive part of A β internalization in microglia may relate to its nonclassical ER (Norfleet et al., 1999) or interaction with other binding proteins (Keefe et al., 1991). Furthermore, our double-staining immunocytochemistry data suggested no colocalization between ER- β and uptake activity of A β in microglia (data not shown). Taken together, these findings suggest that the action of estrogen on microglia might be partially medi-

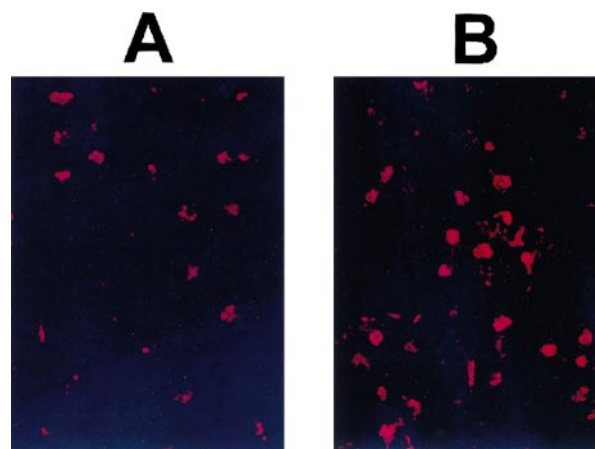


FIG. 7. Estrogen up-regulated ER- β expression in microglia. Microglia were incubated in the (A) absence or (B) presence of estrogen for 48 h. Cells were then rinsed, fixed, and permeabilized, and ER- β was detected by immunofluorescence as described in Materials and Methods.

ated by ER- β and that other mechanisms are likely involved.

Estrogen has been implicated in both the prevention and treatment of AD. In the CNS, estrogen protects neurons against A β -induced apoptosis by increasing the expression of the antiapoptotic protein Bcl-xl (Pike, 1999) in cultured hippocampal neurons and by reducing the production of A β in mouse brain (Inestrosa et al., 1998; Xu et al., 1998). Estrogen also increases cerebral blood flow, increases cerebral glucose utilization, and improves cholinergic tone (Genazzani and Gambacciani, 1999). It is interesting that it was found that estrogen increases apolipoprotein E mRNA and protein expression in rat astrocytes and microglia (Stone et al., 1997). The results of this experiment are the first to present evidence that estrogen may enhance A β clearance in the human brain by influencing microglial function.

Our study shows that estrogen not only increases the uptake of A β , but also enhances the phagocytosis of fluo-*E. coli* by microglia (Fig. 5). This suggests that the action of estrogen on the internalization of A β in microglia is mediated by phagocytosis. However, the uptake of A β occurs much less efficiently in microglia than in *E. coli*. This could be due to the expression of complement component C1q in microglia. Recent studies have shown that microglia express complement component C1q, which binds to A β and could block uptake of A β by microglia (Korotzer et al., 1995; Webster et al., 2000). However, C1q also enhances phagocytosis in general. Therefore, the expression of C1q in microglia might be responsible for the difference between phagocytosis of *E. coli* microglia and A β specific internalization.

Finally, these data not only support the trophic action of estrogen to neurons found in other studies, they also demonstrate enhancement of phagocytosis of *E. coli* and increased removal of A β by microglial phagocytosis. The mechanism responsible for the enhancement of phagocytosis of *E. coli* and uptake of A β in estrogen-treated microglia is unknown. Fc γ receptor affinity is increased in macrophages isolated from estrogen-treated animals (Friedman et al., 1985), and up-regulation of the scavenger receptor in estrogen-treated rat liver has been found to contribute to enhanced phagocytosis and A β uptake. In addition, if the mitogen-activated protein kinase pathway could be activated through ER stimulation, as other studies have suggested (Singer et al., 1999), the effects of estrogen treatment on phagocytosis could also be generated through the activation of the Ras/mitogen-activated protein kinase signal transduction pathway (Yamamori et al., 2000).

Taken together, these findings have important implications for the therapeutic use of estrogen in the treatment of AD.

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