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Effect of Estradiol on Neuronal Swedish-Mutated β-Amyloid Precursor Protein Metabolism: Reversal by Astrocytic Cells

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Alzheimer's disease is the most frequent neurodegenerative disorder in the aged population and is characterized by the deposition of the 40/42-residue amyloid β protein (Aβ), a proteolytic fragment of the β-amyloid precursor protein (APP). Recently, it has been shown that physiological doses of estradiol reduce the generation of endogenous Aβ in primary cortical neurons. Here we investigate the influence of estrogen in amyloidogenesis and sAPPα secretion in the CNS. By means of primary cortical neurons overexpressing humanized APP_{695} bearing the Swedish mutation ($hAPP_{695sw}$), we analyzed APP maturation in the absence or in the presence of estrogen. We show that estrogen at a 2 μM concentration increases the release of the neuroprotective sAPPα fragment but does not reduce the release of Aβ in primary neurons overexpressing the Swedish-mutated form of APP. Furthermore, neurons cocultured with astrocytic cells or grown with astrocytes conditioned media do not exhibit the estrogen-induced increase in sAPPα secretion. Altogether, our data indicate that astrocytes interfere with estrogen in the regulation of sAPPα secretion, probably via secreted factor(s).

Key Words: **Alzheimer's disease; neurons; astrocytes; estrogen; transgenic mice; βAPP; Aβ; sAPPα.**

Tremendous efforts have been made during the past years to better understand the molecular mechanisms responsible for Alzheimer's disease (AD), the most common form of dementia in the aged population. The major histopathological hallmark of the disease in the central nervous system is the senile plaque, composed mainly of β-amyloid (Aβ) aggregates (1). This 4-kDa peptide is generated from a larger type I transmembrane precursor, the β-amyloid precursor protein (APP) by two proteases named β- and γ-secretase, whereas a third activity, α-secretase precludes the formation of Aβ by cleavage in the middle of its sequence and release of a soluble ectodomain fragment referred to as sAPPα (for review see 2 and 3). This secreted form of APP has been demonstrated to have a neuroprotective effect (4). The most common form of AD is sporadic and occurs with a late onset. Recent genetic evidences suggest apoE as a risk factor for such AD cases since the ε4 allele frequency is augmented in AD patients (5, 6). Accumulating data also suggest a possible protective role for estrogen in Alzheimer's disease (for review see 7). Several studies suggest that postmenopausal estrogen replacement therapy can prevent or delay the onset of AD (8). Cell biology approaches have demonstrated that estrogen can improve neuronal viability (9) and attenuate oxidative injury and cell death induced by AD-related insults (10). Interestingly, physiological levels of estradiol reduce the endogenous production of Aβ and increase the secretion of the nonpathological fragment sAPPα in neural and nonneural cells (11, 12). In the present study, we examined the influence of estrogen on APP maturation by primary cultured-neurons prepared from transgenic mice expressing the Swedish-mutated form of APP. We report that estrogen induces sAPPα secretion but do not reduce Aβ production and we establish that astrocytes reverse the beneficial effect produced by estrogen.

MATERIALS AND METHODS

Transgenic mice. Transgenic mice expressing a chimeric mouse/human APP_{695} bearing the Swedish mutation (K670N, M671L) driven by the mouse prion promoter were kindly provided by Dr. Borchelt (13) and maintained on the C57BL6 background. This APP isoform is highly expressed in fetal cerebral cortex and neurons (14). All animal use was according to protocols approved by the Institutional Animal Care and Use Committee of the Rockefeller University.

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Cells and treatments. Primary cultures of neurons were prepared with cerebrocortical tissue derived from embryonic day-14 $hAPP_{695sw}$-transgenic mice as previously described (15). In brief, cells were mechanically dissociated in HAM's F12 medium (Gibco, BRL), supplemented with 10% fetal calf serum (Gibco, BRL) and antibiotics (Gibco, BRL) and plated at the density of 15×10^6 cells in 100 mm dishes precoated with poly-L-lysine (10 μg/ml) (Sigma). Neurons were grown for 4 days at 37°C in a humidified atmosphere of 5% CO_2/95% air and treated with cytosine arabinofuranoside (5 μM) (Sigma) in order to prevent glial cell proliferation. These neurons were previously shown to be virtually devoid of astrocytic cells (15). For estrogen treatment, water-soluble cyclodextrin-encapsulated 17β-estradiol (Sigma, 2 μM final concentration) was added in the media immediately after plating the cells and at day 2. Primary astrocytes were obtained with cerebral hemispheres derived from wild-type FVB/N mice (Jackson Laboratories) according to the same procedure except that cells were grown for 2 weeks in the absence of cytosine arabinofuranoside. Again, astrocytes are virtually devoid of oligodendrocytes and microglia as previously established (15). To obtain astrocytes conditioned media, after 2 weeks in culture cells were washed twice with PBS and incubated with 10 ml of fresh media (F12/10% FCS) for 4 days. Media were collected and used to grow $hAPP_{695sw}$-neurons in the absence or in the presence of water-soluble 17β-estradiol (2 μM). For coculture experiments, neurons were directly plated on 2-week-old astrocytes and cultured for 4 days in the absence or in the presence of 17β-estradiol (2 μM).

Metabolic labeling, immunoprecipitation and quantification. For APP processing analysis, cells grown in 100 mm dishes were incubated for 45 min with methionine-free Dulbecco's modified eagle's medium (DMEM) (Gibco BRL) and subjected to metabolic labeling for 4 hours with 500 μCi/ml [^{35}S]methionine (NEN/Dupont) in DMEM (1.5 ml/plate). Media and cell lysates (lysis buffer: 0.5% NP40; 0.5% deoxycholate; 100 U/ml aprotinin; 20 μM pepstatin; 10 μM phosphoramidon; 2 mM AEBSF) were immunoprecipitated with 6E10 (Senetek, St Louis, MO, dilution 1/500), a monoclonal antibody specific for the amino-terminal part (residues 5–11) of human Aβ, in the presence of a rabbit anti-mouse linker antibody (ICN Pharmaceuticals Inc., dilution 1/250) and protein A-Sepharose (Pharmacia, 30 μl/sample). Samples were extensively washed, resuspended in loading buffer (Novex, San Diego, CA) and subjected to SDS-PAGE using 16% Tris/Tricine gels for Aβ, 4–12% Tris/Glycine gels for sAPPα or 4–20% Tris/Glycine gels for APP detection. All gels were dried and autoradiographed on Kodak X-Omat films. For quantification, specific bands on gels were cut according to autoradiograms, incubated overnight with 250 μl of 30% hydrogen peroxide at 56°C, and the radioactivity was measured after the addition of scintillation fluid.

Analysis of data. Statistical analyses were performed with the Prism software (Graphpad Software, San Diego, CA) using the Newman-Keuls multiple comparison test for one-way ANOVA, and unpaired *t* test for pairwise comparisons. All results are expressed as means $\pm$ SEM values, and a p value <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

In an attempt to investigate the role of estrogen in APP metabolism in conditions that mimic the *in vivo* situation, we prepared and characterized primary neuron cultures overexpressing human APP_{695sw} as a representative model of APP expression in the brain. In order to validate the conditions of immunoprecipitation of APP and its derivatives, media and lysates prepared from 4-day old neurons overexpressing the humanized APP_{695sw} transgene as well as 2-week old wild-type mouse astrocytes were metabolically labeled and immunoprecipitated with 6E10. As expected, 6E10 only detected the humanized transgene-derived APP, sAPPα and Aβ in transgenic neurons without significant background from mouse astrocytes (Fig. 1A), thereby ruling out a 6E10-mediated cross-reaction with endogenous mouse APP or its catabolites. Additionally, 6E10 was used to immunoprecipitate various quantities of labeled lysates and media prepared from hAPP-overexpressing neurons. We observed a linear dose-dependent increase in the detection of APP, sAPPα and Aβ (not shown) that indicated that our system represents a reliable way to perform quantitative analyses of APP expression and processing. Thus, we established a cell system that is likely to be an *in vitro* representation of physiological conditions found in the intact brain *in vivo*.

The effect of estrogen on APP_{695sw} metabolism by neurons was examined by treating the cells for 4 days with 2 μM of water-soluble estradiol before metabolic labeling and immunoprecipitation. First, whereas no modification of APP expression was observed, the amount of sAPPα secreted by pure trangsenic neurons was increased by 28 $\pm$ 8% in estrogen-treated cells when compared to control (Figs. 1A and 1B). This result is in good agreement with previous studies which demonstrated that estradiol could induce similar increases of sAPPα release in human breast carcinoma cells as well as in cerebrocortical neurons (11, 12). However, in contrast with the initial report, we did not detect any decrease in Aβ production (Figs. 1A and 1B). The reason for this discrepancy may be related to the fact that, in our experiments, neurons were treated for only 4 days with estrogen instead of 7–10 days in the initial report (12). Another possibility would be that estrogen is able to modulate Aβ production from wild-type APP_{695} but not APP_{695sw} since it has been shown that the Swedish mutation results in an alternative cellular pathway for Aβ production (16).

Interestingly, estrogen treatment of neurons cocultured with astrocytes prepared from wild-type mice did not exhibit any variation in sAPPα secretion (Figs. 2A and 2B). In order to determine whether this effect required cell-cell contact, we grew APP-transgenic neurons with astrocytes conditioned media instead of cocultures. Here again, we did not observe any effect of estrogen on sAPPα release (Figs. 2A and 2B). Thus, it is our hypothesis that the observed effect is mediated by astrocytic secreted factor(s). Moreover, the same experiments monitored with astrocytes derived from apoE-deficient mice or mice overexpressing human apoE2, E3 or E4 (17) lead to the same results, thereby precluding the possibility that apoE could be part of the observed effect (not shown). Thus, we speculate that astrocytes could abolish, via secreted factor(s), the protective effect of estrogen on APP_{695sw} metabolism by neurons. One possibility could be that astrocytic fac-

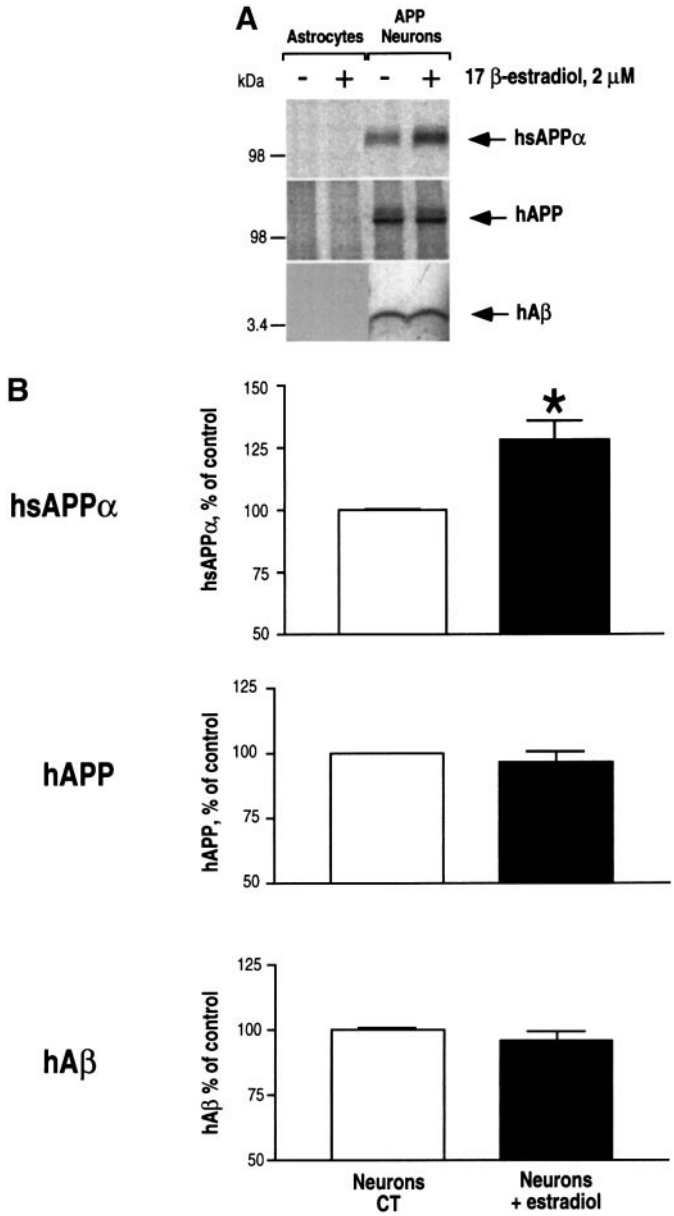

FIG. 1. Estrogen specifically increases sAPPα secretion in primary neurons overexpressing APP_{695sw}. Media and lysates prepared from 4-day-old mouse primary neurons overexpressing the Swedish-mutated form of human APP_{695} and 2-week-old wild-type astrocytes were grown in the absence (−) or in the presence (+) of 2 μM of estrogen (17β-E2), subjected to metabolic labeling, immunoprecipitated with the human-specific 6E10 antibody, and analyzed by SDS–PAGE (see Materials and Methods). (A) Autoradiography analysis of a typical experiment. (B) Bars represent the quantification analysis of APP, sAPPα, and Aβ secretion expressed as the percent of controls corresponding to untreated cells. *, $P < 0.01$ compared to untreated cells (unpaired two-tailed t test). All values represent means ± SEM of 5 to 7 independent experiments.

tor(s) diminished neuronal sAPPα secretion in the presence of estrogen via direct or indirect interaction with α-secretase. On the other hand, it is also possible that estrogen affects sAPPα uptake by astrocytes from the media. In regards to this latter hypothesis, sAPPα uptake and degradation have been shown to be mediated by an LDL receptor-related protein that also binds to apoE and is present on reactive but not resting astrocytes (18, 19). Thus, estrogen could eventually promote sAPPα uptake via this receptor family although it appeared that KPI-containing forms of APP (APP_{751} and APP_{770} but not APP_{695}) were the preferred ligands (18). However, the fact that apoE-deficient astrocytes also reverse the estrogen-induced sAPPα release raised the question whether sAPPα could bind to the LDL-related receptor in the absence of apoE. Finally, we cannot exclude the possibility that sAPPα is internalized via a yet unidentified estrogen-sensitive receptor.

Interestingly, gliosis is a common feature of afflicted brain areas in AD (20) and there exists a preferential association of reactive astroglia with plaques (21). In regard to these observations and if we assumed that cultured astrocytes are considered reactive (22), one could envision that our experiments illustrate a molec-

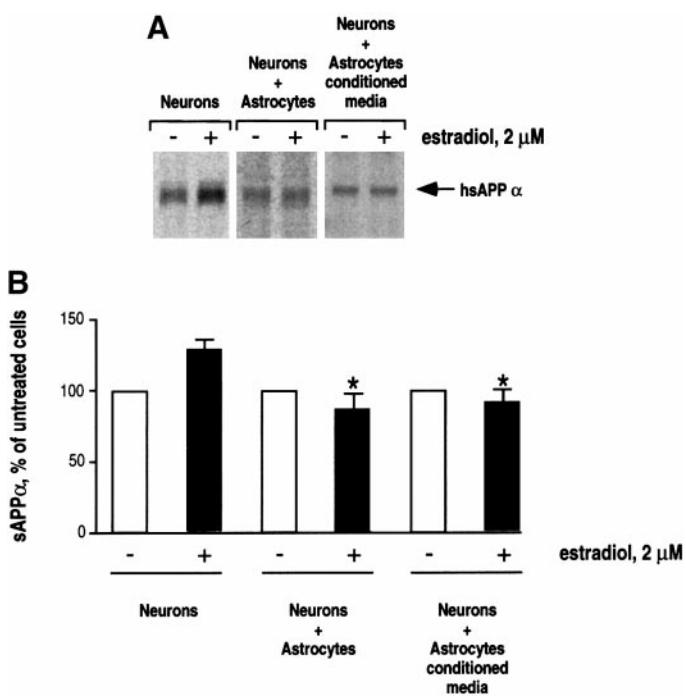

FIG. 2. Astrocytes abolish neuronal sAPPα secretion induced by estrogen. $hAPP_{695sw}$-overexpressing primary neurons were grown alone, cocultured with astrocytes, or with astocytes conditioned media for 4 days in the absence or in the presence of 2 μM of estradiol. Cells were metabolically labeled and sAPPα secreted in the conditioned media was measured by immunoprecipitation with 6E10 as described under Materials and Methods. (A) Autoradiography analysis of a representative experiment. Histograms in B represent the quantification analysis of sAPPα secretion obtained for each condition in absence (white bars) or in the presence (black bars) of 2 μM estrogen. *, $P < 0.05$ compared to estrogen-treated neurons in the absence of astrocytes (unpaired two-tailed t test). All values represent means ± SEM of 3 to 5 independent experiments.

ular link between astrocytosis and AD. Whether astrocytes are able to mediate the same effect as well as the estrogen-dependent decrease in Aβ in cells expressing wild-type APP_{695} remains to be elucidated. Nevertheless, more detailed work needs to be done in order to determine the biological basis for the regulation of APP processing by estrogen and the molecular events that underlie the effect of astrocytes.

In conclusion, our results provide strong evidences that astrocytes, independently of the apoE genotype and probably via secreted factor(s), play a role in the regulation of neuronal Swedish-mutated APP metabolism via the estrogenic pathway. Although we must be cautious in extrapolating from *in vitro* findings to multifactorial human disease like AD, our data further support an important role for astrocytes in the regulation of neuronal APP metabolism and may lead to insight into the molecular basis of estrogen contributions in the context of AD pathogenesis.

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