Full-length article

Estrogen stimulates release of secreted amyloid precursor protein from primary rat cortical neurons via protein kinase C pathway¹

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Key words

Alzheimer disease; estrogen; amyloid precursor protein; estrogen receptors; protein kinase C

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Abstract

Aim: To investigate the mechanism of the action of estrogen, which stimulates the release of secreted amyloid precursor protein α (sAPP α) and decreases the generation of amyloid- β protein (A β), a dominant component in senile plaques in the brains of Alzheimer's disease patients. **Methods:** Experiments were carried out in primary rat cortical neurons, and Western blot was used to detect sAPP α in a culture medium and the total amount of cellular amyloid precursor protein (APP) in neurons. **Results:** 17 β -Estradiol (but not 17 α -estradiol) and β -estradiol 6-(*O*-carboxymethyl) oxime: BSA increased the secretion of sAPP α and this effect was blocked by protein kinase C (PKC) inhibitor calphostin C, but not by the classical estrogen receptor antagonist ICI 182,780. Meanwhile, 17 β -estradiol did not alter the synthesis of cellular APP. **Conclusion:** The effect of 17 β -estradiol on sAPP α secretion is likely mediated through the membrane binding sites, and needs molecular configuration specificity of the ligand. Furthermore, the action of the PKC-dependent pathway might be involved in estrogen-induced sAPP α secretion.

Introduction

Alzheimer's disease (AD) is characterized by two major pathological lesions in the brain: intracellular neurofibrillary tangles and extracellular deposition of senile plaques composed mainly of amyloid- β protein (A β)^[1]. Efforts have been made to explore the relative contribution of plaques and tangles to the pathogenesis of AD. In recent years, the amyloid hypothesis has been accepted by many researchers, who regard accumulation of A β in the brain as the primary factor driving AD pathogenesis^[2,3]. A β is derived from a larger ubiquitous transmembrane protein, amyloid precursor protein (APP). APP is cleaved through at least two different pathways: α -secretase pathway and β -secretase pathway. Through the β -secretase pathway, which involves β -secretase and γ -secretase, APP is cleaved into two fragments: sAPP β and A β . Alternatively, through the α -secretase pathway, which involves α -secretase and γ -secretase, APP is cleaved within the domain of amyloidogenic A β , thus precluding the generation of A β and producing non-

amyloidogenic secreted APP (sAPP α) and p3 (A β 17-40/42). Several studies demonstrated that the increased activity of the α -secretase pathway led to a decrease in the activity of the β -secretase pathway^[4].

Estrogen is considered as a neurotropic and neuro-protective agent^[5]. Estrogen replacement therapy (ERT) in postmenopausal women is related with a reduced risk and delayed onset of AD^[6]. It is therefore worthwhile investigating whether estrogen produces its neuroprotective effect through the regulation of APP processing^[7].

There are several lines of evidence supporting this hypothesis. In cell culture, the addition of estrogen resulted in increase in the production of sAPP α and a decrease in A β ^[8,9]. In animal models, estrogen treatment prevented the accumulation of A β in the brain of guinea pigs and transgenic mutant APP/PS1-expressing mice^[10,11]. It was suggested that the increased release of sAPP α by estrogen was mediated through the phosphorylation of extracellular-regulated kinase 1 and 2 (ERK1/2)^[12]. However, the precise mechanism of the neuroprotective effects of estrogen remains to be fur-

ther investigated. The purposes of the present study are to explore: (1) whether the effect of estrogen on sAPP α release is mediated through the membrane sites or the nuclear receptors; (2) whether the effect of estrogen depends on its molecular configuration specificity; (3) whether protein kinase C (PKC) is involved in the regulation of APP processing; and (4) whether classical estrogen receptor antagonist ICI 182 780 blocks the sAPP α secretion induced by estrogen.

Materials and methods

Cells and cell culture procedures Cortical neurons from 1-d postnatal Sprague-Dawley female or male rats were prepared as described previously^[13]. Cells were plated in DMEM (Life Technologies, Gaithersburg, Germany) containing 10% fetal bovine serum (GIBCO) in poly-*L*-lysine-coated (0.1 g/L; Sigma, St Louis, USA) 100-mm dishes (approximately 1.8×10^7 cells per dish) under standard conditions (37 °C, 5% CO₂). On the following day, the medium was replaced with phenol red-free Neurobasal Medium (GIBCO), supplemented with B27 (GIBCO), which produced cultures with >99% neuronal composition^[14]. The medium was replaced every 3 d. On the ninth day, the medium was replaced with phenol red-free Neurobasal Medium, supplemented with N₂ (10% volume as described in guideline, GIBCO, Rockville, USA). Estrogen and/or other chemicals were added to the dishes. The chemicals used were 17 β -estradiol (Sigma) dissolved in dimethyl sulfoxide (Me₂SO), 17 β -estradiol (Sigma) dissolved in Me₂SO; β -estradiol 6-(*O*-carboxymethyl) oxime: BSA (Sigma) dissolved in PBS, β -estradiol 17-hemisuccinate: BSA (Sigma, St Louis, USA) dissolved in PBS, ICI 182, 780 (TOCRIS) dissolved in Me₂SO, and PKC inhibitor calphostin C (Calbiochem, La Jolla, USA) dissolved in Me₂SO. After a 12-h incubation, the medium was collected for measurement of sAPP α . The medium was then centrifuged at 4000 $\times g$ for 10 min to remove the cellular debris. The cleared supernatant was concentrated with 30 kDa pore size Amicon Ultra (Millipore Co, Billerica, USA) for analysis. Cell monolayers were washed three times with ice-cold PBS and lysed in 1 $\times$ SDS loading buffer with protease inhibitors for 10 min on ice. The cell lysates were boiled for 5 min and then centrifuged for 10 min at 14 000 $\times g$. The proteins were stored at -20 °C.

Western blot analysis The protein concentrations were determined by BCA protein quantitative analysis kit (Shenergy Biocolor BioScience & Technology Company). An equal amount of protein from the medium was subjected to 7%–10% gradient SDS-poly-acrylamide gel electrophoresis (PAGE) (Amresco). After electrophoretic separation, proteins were transferred onto a PVDF membrane and the polyclonal antibody Rat A Beta (1:1000, Signet), which is

specific for Ab 3-16, was used to detect sAPP α . The efficiency of transfer was confirmed by staining the membrane with Ponceau S. The Ponceau S stain was then removed from the membrane by washing in PBST. Cellular APP was detected using monoclonal antibody 22C11 (1:200, Roche), which is specific for the amino terminus of APP. The amount of β -actin on the same membrane, which was determined by polyclonal antibody β -actin (1:1000, Santa Cruz), was taken as control. Antibody binding was detected by counter-staining with horseradish peroxidase-conjugated antibodies (1:1000, Calbiochem) and visualized using an ECL-detection kit (Pierce). The relative intensity of immunoreactive bands on the exposed film was quantified by a computer-assisted densitometry program (Smart view, Life Science Research Products and System Engineering).

Statistics Data from sAPP α measurement were analyzed by one-way ANOVA followed by Tukey *post hoc* test (SPSS software). Student's *t*-test was used to analyze the data of cellular APP content. $P < 0.05$ was considered to be statistically significant. Each experiment was repeated three to four times to verify the reproducibility of the results.

Results

Effect of 17 β -estradiol on sAPP α secretion and cellular APP content Primary cortical neurons were treated with 10, 100, and 1000 nmol/L 17 β -estradiol. Figure 1 shows that a 12-h treatment with increasing concentrations of 17 β -estradiol

Figure 1. Effect of 17 β -estradiol on sAPP α secretion from the rat cortical neurons. (A) Effect of 17 β -estradiol of various concentrations. $n=3$. Mean $\pm$ SD. ^b $P < 0.05$, ^c $P < 0.01$ vs control. (B) Electrophoresis results from a representative experiment. (1) Control; (2) 10 nmol/L 17 β -estradiol; (3) 100 nmol/L 17 β -estradiol; (4) 1000 nmol/L 17 β -estradiol.

resulted in a significant dose-dependent increase in sAPP α release into the medium as compared with the sAPP α level in the control. 17 β -Estradiol (100 nmol/L) did not alter the total cellular APP content in cultured cortical neurons (Figure 2).

Figure 2. Effect of 17 β -estradiol on total cellular APP content in the cultured rat cortical neurons. (A) Cellular APP content with or without 17 β -estradiol administration. $n=3$. Mean $\pm$ SD. (B) Results from a representative experiment for total cellular APP. (C) β -actin on the same film as (B).

Effect of β -estradiol 6-(*O*-carboxymethyl) oxime: BSA on the release of sAPP α To investigate whether the membrane-impermeable estradiol-BSA conjugates, β -estradiol 6-(*O*-carboxymethyl) oxime: BSA and β -estradiol 17-hemisuccinate: BSA, exerted similar actions on sAPP α secretion as 17 β -estradiol, neurons were incubated with these two agents. After a 12-h incubation, the supernatant was collected for measurement of sAPP α . β -Estradiol 6-(*O*-carboxymethyl) oxime:BSA (1000 nmol/L) increased sAPP α secretion, although the magnitude of the effect was smaller than that of 1000 nmol/L 17 β -estradiol. Estradiol 17-hemisuccinate: BSA (1000 nmol/L) also stimulated sAPP α secretion, but the difference was not statistically significant (Figure 3).

Effect of 17 α -estradiol on the release of sAPP α In order to investigate whether estradiol stimulates sAPP α secretion depending on its configuration specificity, we treated the neurons with 100 nmol/L, 1000 nmol/L 17 α -estradiol and 100 nmol/L 17 β -estradiol. Twelve hours later, the supernatant was collected and concentrated for Western blot analysis.

Figure 3. (A) Effects of 17 β -estradiol(E2), β -estradiol 6-(*O*-carboxymethyl) oxime:BSA(E2-6:BSA), and β -estradiol 17-hemisuccinate:BSA (E2-17:BSA), respectively, on the release of sAPP α . $n=4$. Mean $\pm$ SD. ^b $P<0.05$, ^c $P<0.01$ vs control. (B) Electrophoresis results from a representative experiment. (1) Control; (2) 17 β -estradiol; (3) β -estradiol 6-(*O*-carboxymethyl) oxime:BSA; (4) β -estradiol 17-hemisuccinate:BSA.

Figure 4. Effect of 17 α -estradiol on sAPP α release from rat cortical neurons. (A) Effects of 100, 1000 nmol/L 17 α -estradiol and 100 nmol/L 17 β -estradiol on the release of sAPP α . $n=3$. Mean $\pm$ SD. ^b $P<0.05$, ^c $P<0.01$ vs control.

The results showed that, in contrast to 17 β -estradiol, 17 α -estradiol did not alter sAPP α secretion (Figure 4).

Effect of PKC inhibitor on 17 β -estradiol-induced sAPP α secretion Calphostin C, a PKC inhibitor, was used to investigate the role of PKC on 17 β -estradiol-induced secretion of

sAPP α . Incubation with calphostin C (250 nmol/L) alone for 12 h did not change the sAPP α secretion. However, when the neurons were co-treated with calphostin C (250 nmol/L) and 17 β -estradiol (100 nmol/L) for 12 h, the stimulatory

Figure 5. Involvement of PKC in 17 β -estradiol effect on sAPP α secretion. (A) Effects of 17 β -estradiol, calphostin C, and 17 β -estradiol+calphostin C on the release of sAPP α , respectively. $n=3$. Mean $\pm$ SD. $^{\circ}P<0.01$ vs control. $^{\circ}P<0.05$ vs E2. (B) Results from a representative experiment. (1) control; (2) 17 β -estradiol; (3) calphostin C; (4) 17 β -estradiol+calphostin C.

effect of 17 β -estradiol on sAPP α secretion disappeared (Figure 5).

Effect of estrogen receptor antagonist on the 17 β -estradiol-induced sAPP α secretion To explore whether activation of the classical estrogen receptors is involved in 17 β -estradiol stimulating sAPP α secretion, rat cortical neurons were treated with 17 β -estradiol (100 nmol/L), estrogen receptor antagonist ICI 182 780 (1 μ mol/L) alone, and 17 β -estradiol (100 nmol/L) together with ICI 182 780 (1 μ mol/L). The results showed that ICI 182 780 alone produced no alteration in sAPP α secretion, while simultaneous administration of ICI 182 780 and 17 β -estradiol resulted in a significant increase in sAPP α secretion, similar to the effect of 17 β -estradiol (Figure 6).

Discussion

It appears that ERT prevents the onset of AD, but is not an effective treatment for AD^[6]. However, recent clinical studies revealed that ERT brought beneficial as well as adverse effects^[15]. Therefore, it is important to elucidate the mechanisms of the neuronal action of estrogen and to develop new drugs that retain the beneficial effects, but do not have the side effects of estrogen^[16]. It is generally accepted

Figure 6. No antagonism by ICI 182 780 against the 17 β -estradiol-induced sAPP α secretion. (A) Effects of 100 nmol/L 17 β -estradiol, 1 μ mol/L ICI 182 780 alone, and 100 nmol/L 17 β -estradiol+1 μ mol/L ICI 182 780 on the release of sAPP α . $n=3$. Mean $\pm$ SD. $^{\circ}P<0.01$ compared with the control. (B) Results from a representative experiment. (1) control; (2) 17 β -estradiol; (3) ICI 182 780; (4) 17 β -estradiol+ICI 182 780.

that the effect of estrogen in preventing AD is mediated through regulating APP processing^[2,7]. Our study provides evidence that estrogen increases sAPP α release without changing the total cellular APP content in cultured rat cortical neurons. Because the up-regulation of the α -secretase pathway mediating sAPP α production occurs within the same time course as down-regulation of the β -secretase pathway^[4], it is reasonable to suggest that estrogen could reduce the generation of A β secondary to increases in sAPP α secretion and, in turn, exert its neuroprotective functions.

In addition to classical intracellular estrogen receptor, estrogen binding sites are also present on the cell membrane^[5]. The actions of estrogen on its target tissues include a long-term “genomic” action mediated by intracellular estrogen receptors, and a rapid action through membrane binding sites that modulate a diversity of intracellular signal transduction pathways^[17]. Therefore, it is interesting to know whether the estradiol-induced release of sAPP α is brought about through the mediation of nuclear or membrane binding sites. Our data clearly demonstrated that β -estradiol 6-(*O*-carboxymethyl) oxime: BSA, a membrane-impermeable estradiol conjugate^[18,19], produced a similar effect like estrogen to increase the release of sAPP α , suggesting the involvement of the membrane binding sites. Furthermore, the estrogen receptor antagonist ICI 182 780 did not affect the effect of estrogen, suggesting that the classical estrogen receptors ER α or ER β

were not involved in the above-mentioned estradiol effect. The role of classical estrogen receptors in the neuronal effect of estrogen is controversial. Some studies showed that the estrogen-induced rapid intracellular signal transduction cascades were unaffected by the classical estrogen receptor antagonists^[18,20,21], such as tamoxifen and ICI 182 780, in cell lines that did or did not express estrogen receptors ER α and ER β ; while other studies showed that the rapid intracellular signal transduction was blocked by the classical estrogen receptor antagonists^[13,22–24]. The reason for the difference in these studies is unknown, but it is probably a result of the different cells the researchers used and different parameters chosen for study, which appear through different membrane binding sites. The results of the present study suggest that the structure of the membrane binding sites responsible for the estradiol-induced sAPP α secretion is probably different from that of the classical estrogen receptors.

In present study, 17 β -estradiol and its two BSA conjugates produced effects with different potencies. One of the possible explanations is that the combination of BSA alters the dimensional configuration of estradiol and thus changes its affinity for the membrane binding sites. In β -estradiol 6-(*O*-carboxymethyl) oxime: BSA, BSA attaches to estradiol at position 6, that is, on the “bottom” of the structure of estradiol, making the “top” (the C and D rings) of estradiol accessible. In contrast, in β -estradiol 17-hemisuccinate: BSA, BSA attaches to position 17 on the “top” of estradiol, making the “bottom” (the A and B rings) of the estradiol accessible. The difference in the configuration between the two conjugates can change the binding ability to the membrane sites and therefore change the effect in regulating sAPP α secretion. It is possible that a small amount of estradiol might dissociate from the conjugates, however, some studies reported that a very small amount of estradiol that dissociated from the conjugates did not exert effects on estrogen receptors^[25].

Behl *et al* indicated that both 17 β -estradiol and its isomer 17 α -estradiol exhibited neuroprotective actions against oxidative stress^[26]. 17 α -estradiol does not bind to the nuclear ER, but 17 α -estradiol was reported to stimulate phosphorylation of ERK1/2 in immature rat neurons^[27]. Our present study showed that 17 α -estradiol was unable to regulate the APP processing. Therefore, the mechanism for anti-oxidation effects of estrogens might be different from those of regulating APP metabolism.

Estrogen stimulates a rapid Ca²⁺ release from the intracellular calcium stores^[28]. Yeon *et al* indicated that PKC ϵ played an important role in modulating APP and that overexpression of the PKC ϵ V1 region, which specifically binds to the recep-

tor for activated C-kinase (RACK), blocked the phorbol ester-induced enhancement of sAPP α secretion^[29]. Recently, Beyer *et al* reported that estrogen activated G-protein-coupled phospholipase C, leading to the release of Ca²⁺ from intracellular stores and the activation of PKC^[30]. In the present study, we demonstrated that calphostin C prevented the increase of sAPP α secretion induced by 17 β -estradiol, indicating the involvement of PKC.

In summary, our data indicate that, in cultured rat cortical neurons, estrogen stimulates sAPP α secretion through mechanisms involving membrane binding sites and the activation of PKC. The effects of estradiol are not mediated by classical ER. The details of the estrogen membrane binding sites and the related signaling pathways remain to be further studied.

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