Estrogens Inhibit Amyloid-β-Mediated Paired Helical Filament-Like Conformation of Tau Through Antioxidant Activity and miRNA 218 Regulation in hTau Mice

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Abstract

Background: The risk of developing Alzheimer’s disease as well as its progression and severity are known to be different in men and women, and cognitive decline is greater in women than in men at the same stage of disease and could be correlated at least in part on estradiol levels.

Objective: In our work we found that biological sex influences the effect of amyloid-β42 (Aβ42) monomers on pathological tau conformational change.

Methods: In this study we used transgenic mice expressing the wild-type human tau (hTau) which were subjected to intraventricular (ICV) injections of Aβ peptides in nanomolar concentration.

Results: We found that Aβ42 produces pathological conformational changes and hyperphosphorylation of tau protein in male or ovariectomized female mice but not in control females. The treatment of ovariectomized females with estradiol replacement protects against the pathological conformation of tau and seems to be mediated by antioxidant activity as well as the ability to modulate the expression of miRNA 218 linked to tau phosphorylation.

Conclusion: Our study indicates that factors as age, reproductive stage, hormone levels, and the interplay with other risk factors should be considered in women, in order to identify the best appropriate therapeutic approach in prevention of cognitive impairment.

Keywords: Alzheimer’s disease, antioxidants, estradiol, miRNA, tau protein

INTRODUCTION

Hallmarks of Alzheimer’s disease (AD) are the accumulation of amyloid-β (Aβ) peptides in amyloid plaques, and the aggregation form neurofibrillary tangles. Aβ derives from the amyloid-precursor (AβPP) through β site APP cleaving enzyme 1 (BACE) 1 and γ-secretase processing that generates multiple C-termini, most end at residue 40 and 42. Aβ42 aggregates more stably than Aβ40 through sequential
which were subjected to intraventricular (ICV) injections of Aβ peptides in nanomolar concentration. We discovered that Aβ42 monomers, but not oligomers: 1) produce paired helical filament-like conformation of tau protein, and 2) induce two phosphorylated epitopes which are not present in normal tau (Ser396 and Ser422) through the activation of GSK3β, JNK, and ERK 1/2 kinases [4].

Recent epidemiological studies showed that two-thirds of AD patients are women [5], and this fact cannot be attributed only to their higher life expectancy. In this connection, the loss of estradiol might be one of the factors leading to declining cognitive function in women [6].

Of note, we found that oxidative stress, together with important oxidative stress-related risk factors related to AD, such as hypoxia, hyperglycemia, and hypercholesterolemia, are potential causes of the increased BACE1 activity [7]. In AD, estrogen neuroprotective activity is exerted at multiple levels. Preclinical data showed that, in addition to their action against neuroinflammation and oxidative stress, estrogens are able to influence both the main players of neurodegeneration, Aβ, and tau [8].

In this paper, we pursue the hypothesis that biological sex influences the effect of Aβ42 monomers on pathological tau conformational change. Our data revealed that Aβ42 monomers produce the pathological conformational changes and hyperphosphorylation of tau protein in male or ovariectomized female mice but not in control female. The treatment of ovariectomized females with estradiol replacement protects against the pathological conformation of tau. The hypothesized protective mechanism is mediated both by their antioxidant activity and by their ability to modulate the expression of miRNA 218 linked to tau phosphorylation.
The quality of Aβ preparations was controlled using atomic force microscopy (AFM). AFM was carried out on a Multimode AFM with a Nanoscope V system operating in Tapping Mode using standard antimony(n)-doped Si probes (T: 3.5–4.5 mm, L: 115–135 mm, W: 30–40 mm, f0:313–370 kHz, k: 20–80 N/m) (Bruker). The scan rate was tuned proportionally to the area scanned and was kept in the 0.5–1.2 Hz range. The sample was then diluted to 5 μM with PBS, and 50 μl of solution was spotted onto a freshly cleaned muscovite mica disk and incubated for 5 min. The disk was then washed with ddH2O and dried under a gentle nitrogen stream. Samples were analyzed with the Scanning Probe Image Processor (SPIP Version 5.1.6 released April 13, 2011) data analysis package (Nanoscience Instruments, Phoenix, AZ, USA). SPIP software was used to analyze the distribution of the molecular assemblies of the different populations in terms of height and diameter, as previously described [10]. Our controls were hTau mice ICV injected with saline. The experiments were done four weeks after ovariectomy. After two weeks from ovariectomy surgery, one group of female mice was subjected to a daily subcutaneous injection of 17β-estradiol (E2) for three weeks (1 μg/Kg) [11]. Animals were allowed to recover for at least three weeks before experiments were performed and subsequently were subjected to intracerebroventricular injection of Aβ42 monomers (200 nM) or saline and sacrificed after 3 h.

Antibodies and immunoblot analysis

Immunoblot analysis was performed using the following antibodies: MC1 (kind gift from Dr. P. Davies, Albert Einstein College of Medicine, New York, 1:500); Tau5 (Millipore, #577801, 1:1000); ERK1/2 (Santa Cruz Biotechnology, Sc-93, 1:500); pERK1/2 (Cell Signaling Technology, #43765, 1:1000); GSK3β (Abcam, #22261, 1:1000); GSK3α (Abcam, #22261, 1:1000); JNK1/2 (Cell Signaling Technology, #9252, 1:500); pJNK1/2 (Cell Signaling Technology, #9251, 1:500); AT8 (Innogenetics, #90206, 1:500); Tau 46 (Millipore, #577801, 1:1000). The samples were run on 3–8% Tris-HCl gradient gels and transferred to nitrocellulose membrane. Blots were blocked (5% no fat milk) and incubated with primary antibodies. Peroxidase secondary antibodies were incubated at room temperature (RT) and developed with Forte Western substrate (WBLUF0100, Milli-pore). Densitometric values were normalized to β-actin.

Total antioxidant capacity

To evaluate the antioxidant capacity in mice tissues, we performed the total antioxidant capacity (TAC) dosage kit (ab65329, Abcam). All analyses were performed on total extracts according to the manufacturer protocol.

Quantitative determination of 17β-estradiol

To quantify E2 levels, blood mice was collected to obtain plasma. To perform the measurement, we used a commercially available ELISA kit (catalog # ADI-901-174) according to the manufacturer protocol.
Fig. 1. Intracerebroventricular treatment with Aβ42 causes a change in tau conformation only in male or ovariectomized female mice. Representative western blot of brain samples from control (saline) and treated Aβ42 peptides for ICV male and female mice using a conformational tau antibody (MC1) and a total tau antibody (Tau 5) for detection. Some female mice were subjected to ovariectomy. Densitometric quantification shows an increase of the total protein level of both MC1 and Tau 5 in treated female mice injected or not with Aβ42. The data are mean ± standard error of the mean (SEM); *p < 0.05; **p < 0.01 versus control; §p < 0.05 versus OVX by one-way ANOVA followed by Bonferroni post test n = 5.

MicroRNA isolation and quantitative real time PCR

MicroRNA was isolated from brains of female mice using the MagMAXmirVana kit and according to manufacturer’s protocol (Applied Biosystems, Foster City, CA, USA). Subsequently, cDNA synthesis was performed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and a RT-primer pool containing microRNA-specific stem-loop primers for miR218 (Mature miRNA Sequence: UUGUGCUUGAUCUAACCAUGU) and for small nuclear RNA U6 (Control Sequence: GTGCTCGCTTCGGCAGCATACTAAATTTGGAACGATACAGAGAAGATTAGCATGGCCCCTGCGCAAGGATGACACGCAAATTCGTGAAGCGTTCCATATTTT).

Each qPCR contained 1.3 μL cDNA, 1 μL 20X TaqMan MicroRNA Assay and 10 μL 2X TaqMan Universal PCR Master Mix.
Fig. 2. The conformational change mediated by Aβ42 is induced by protein hyperphosphorylation. A) Representative western blot of brain extracts from control (saline) and treated Aβ42 peptides for ICV male and female hTau mice using antibodies specific for pathological tau phosphorylation sites such as AT8, pS422, and pS396. Some female mice were subjected to ovariectomy (OVX) as loading control. Densitometric analysis shows a significant increase of total protein levels of AT8, pS422, and pS396 only in male mice and ovariectomized female mice. B–D) Representative western blot of brain extracts from control (saline) and treated Aβ42 peptides for ICV male and female hTau mice using p JNK (B), pERK1/2 (C), and pGSK3β (D). Some female mice were subjected to OVX as loading control. Densitometric analysis shows a significant increase of total protein levels of all the three kinases only in male and ovariectomized female mice. The data are mean ± standard error of the mean (SEM); *p < 0.05; **p < 0.01 versus control; §p < 0.05 versus OVX by one-way ANOVA followed by Bonferroni post test n = 5.
RESULTS

Intracerebroventricular treatment with Aβ42 causes a change in tau conformation only in male or ovariectomized female mice

Figure 1 shows that, as previously demonstrated, the intracerebroventricular injection of 200 nM Aβ42 in male hTau mice is able to determine a pathological conformational change of the tau protein. The same treatment is able to determine this effect only in female mice after ovariectomy. The same result was obtained by evaluating the total tau protein levels. As can be seen, treatment with Aβ42 significantly increases total tau levels in male (1.5-fold increase) and ovariectomized female mice (1.5-fold increase) with respect to control female mice injected or not with Aβ142.

The conformational change mediated by Aβ42 is induced by protein hyperphosphorylation

To understand if the conformational change of tau was due to hyperphosphorylation, we measured the phosphorylation of some specific sites related to the pathology by western blotting. Phosphorylation levels were studied through the use of the AT8 antibody (which recognizes the Ser 202/Thr 205 epitopes), the antibody S396 and S422. These are all phosphorylation sites closely associated with disease progression. (which increase in the same groups by about three times compared to the controls (Fig. 3). Figure 4A shows that the treatment with Aβ42 significantly increases pJNK levels in nuclear extracts of male (E2)(+3-fold increase). A similar result was obtained measuring the nuclear levels of pJNK in ovariectomized females (E2)(+3-fold increase). A similar result was obtained measuring the nuclear levels of pJNK in ovariectomized females (E2)(+3-fold increase).

Estradiol hormone therapy protects against Aβ42-mediated tau conformational change

To confirm that the presence of estradiol is involved in the different effect exerted by the treatment with Aβ42 on the pathological conformational change of tau, groups of female mice were or not were subcutaneously treated with estradiol (E2) (1 μg/kg) and fed with a soy diet for 12 weeks. We first tested the validity of the treatment measuring the levels of estradiol in different groups. As can be seen, oophorectomy significantly decreases circulating estradiol levels (–60%), while E2 treatment determines an increase (+100%) in ovariectomized females (+100%) (Fig. 3). Figure 2B shows that Aβ42 treatment with estradiol completely prevents hyperphosphorylation of the sites taken into consideration in male and ovariectomized female mice, while in control females, phosphorylation of the sites is extremely significant for all the phosphorylation sites studied: (+4–5-fold increase AT8; 3–4-fold increase H9252 Cytoskeletal Phosphorylation).


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Fig. 3. Estradiol hormone therapy significantly increases E2 levels. Groups of female mice, ovariectomized or not, were subcutaneously treated with E2 (1 μg/kg) and fed with a soy-free diet for three weeks. To test the validity of the treatment, we measured E2 levels in serum of our experimental groups. We observed that the ovariectomy induces a significant decrease in hormone levels, whereas the treatment protects the decrease of E2 levels, that significantly increased with respect to controls. The data are mean ± standard error of the mean (SEM); *p < 0.05; **p < 0.01 versus control by one-way ANOVA followed by Bonferroni post test n = 6.

mediated phosphorylation, after oophorectomy, of pathology-related sites (Fig. 5A). As expected, the kinases involved in the phosphorylation of the above sites do not appear induced; thus, as observed in Fig. 5B, the levels of nuclear pJNK, pERK, and pGSK3β are absolutely comparable to the levels of the control females.

Estrogen hormone therapy protects male hTau mice against Aβ42-mediated tau conformational change and hyperphosphorylation

To further confirm the protective role of estradiol on the pathological conformational change of tau and its hyperphosphorylation, we also treated hTau male mice with estradiol, following the same protocol as the females. As can be seen in Fig. 6, the treatment with estradiol is able to completely protect both the conformational change of tau as revealed by the significant increase of the band revealed with MC1 antibody as well as its hyperphosphorylation revealed by using AT8 antibody that recognizes Ser202/Thr205 phospho-epitopes (Fig. 6).

DISCUSSION

In our work we pursued the hypothesis that gender differences in the risk of developing AD are related to the therapeutic potential of estrogen. We observed that levels of miR218 are significantly higher in ovariectomized female mice with Aβ42, whereas the E2 treatment is followed by a total protection of the miRNA levels. Finally, we measured levels of miR218, since recent discoveries demonstrate that estrogen receptors are able to modulate the expression of microRNA. It is well known that E2 treatment, at least during the early stage of AD pathology, significantly influences the effect of Aβ42 monomers on pathological conformational change. Our data show that Aβ42 produced pathological conformational change and hyperphosphorylation of tau in female mice and in ovariectomized female mice but not in control females. The risk of developing dementia and the severity of cognitive decline is greater in women than in men and the simultaneous intracerebroventricular injection of Aβ42 induces a further drop in antioxidant capacity (–70%). Treatment with estradiol reverses the drop-in antioxidant capacity bringing it back to control values (Fig. 6A).

Finally, we measured levels of nuclear pJNK, pERK, and pGSK3β, involved in tau phosphorylation [13]. In particular it has been found that increase of miR218 reduces levels, whereas the treatment protects the decrease of E2 levels, that significantly increased with respect to controls. The data are mean ± standard error of the mean (SEM); *p < 0.05; **p < 0.01 versus control by one-way ANOVA followed by Bonferroni post test n = 6.
Fig. 4. Estradiol hormone therapy protects against Aβ42-mediated tau conformational change. A) Representative western blot samples from control (saline) and treated Aβ42 peptides for ICV female hTau mice using a conformational tau antibody (MC1) for detection. Some female mice were subjected to ovariectomy and or to E2 (1 μg/kg) and soy-free diet for three weeks. β-actin served as loading control. Densitometric quantification shows an increase of the total protein level of both MC1 and Tau 5 in ovariectomized female mice injected or not with Aβ42; the treatment with estradiol completely protects the conformational change and the increase of tau protein. B) Representative western blot of insoluble tau fraction by sarkosyl detergent technique extracts from control (saline) and treated Aβ42 peptides for ICV female hTau using Tau 46 antibody for detection. β-actin served as loading control. After injection of Aβ42 in ovariectomized females, we showed a band at approximately 75 kDa molecular weight revealed with Tau 46 antibody, whereas estradiol blocks the aggregation of tau protein. The data are mean ± standard error of the mean (SEM); *p < 0.05; **p < 0.01 versus control; §p < 0.05 versus OVX by one-way ANOVA followed by Bonferroni post test n = 5.

active in the last years and recently it has taken new life, thus finding new therapeutic approaches for AD is one of the most important challenges of modern medicine. Numerous experimental evidences have shown that estrogens have protective effects against induction of neuroinflammation and neurodegeneration [16–18]. The encouraging results obtained in vitro clashed with clinical trials outcomes. In 2003,
Fig. 5. Estradiol hormone therapy protects against Aβ42-mediated tau hyperphosphorylation. A) Representative western blot of brain extracts from control (saline) and treated Aβ42 peptides for ICV female hTau mice using antibodies specific for the detection of phosphorylation sites such as AT8, pS422, and pS396. Some female mice were subjected to ovariectomy and or to E2 (1 g/kg) and soy-free diet for three weeks. β-actin served as loading control. Densitometric analysis shows an increase of total protein levels of pS396 in ovariectomized female mice injected or not with Aβ42; the treatment with estradiol completely protects the hyperphosphorylation of tau protein. B–D) Representative western blot of brain extracts from control (saline) and treated Aβ42 peptides for ICV female hTau mice using p JNK (B), pERK1/2 (C), and pGSK3β (D). Some female mice were subjected to ovariectomy and or to E2 (1 g/kg) and soy-free diet for three weeks. β-actin served as loading control. Densitometric analysis shows an increase of total protein levels of all the three kinases in ovariectomized female mice injected or not with Aβ42; the treatment with estradiol completely protects the activation of the kinases. The data are mean ± standard error of the mean (SEM); *p < 0.05; **p < 0.01 versus control; §p < 0.05 versus OVX by one-way ANOVA followed by Bonferroni post test n = 5.
Fig. 6. Estradiol hormone therapy protects male mice against Aβ42-mediated tau conformational change as well as hyperphosphorylation. Representative western blot of brain samples from control (saline) and treated Aβ42 peptide ICV male hTau mice using a conformational tau (MC1) and a specific antibody for tau pathological phosphorylation (AT8) for detection. Some male mice were subjected to E2 (1 g/kg) and soy-free diet for three weeks. β-actin served as loading control. Densitometric quantification shows an increase of both MC1 and AT8 in male mice injected or not with Aβ42; the treatment with estradiol completely protects the conformational change and the increase of tau phosphorylation. The data are mean ± standard error of the mean (SEM); **p < 0.01 versus control by one-way ANOVA followed by Bonferroni post test n = 5.

Clinical trials had shown that the use of replacement therapy significantly increased the risk of dementia and cognitive decline [19, 20]. These studies suggested that patients aged 65 and over, already in menopause for a long time, did not represent an adequate experimental group, because replacement therapy is indicated for women who have just gone through menopause, and suggested the presence of a therapeutic window useful for this type of therapeutic approach [21]. More recently, evidence suggests that protein tau could be a potential target for estrogens. It has been demonstrated that 17β estriadiol promotes tau dephosphorylation in vitro in rat cortical neurons and SH-SY5Y neuronal cells [22]. Other authors confirmed these results showing that E2 prevent the phosphorylation of tau in an estrogen receptor-mediated and dose-dependent manner [23]. In vivo studies have shown that estrogenic treatment increases GSK3β phosphorylation in Ser 9/21, a site that inactivates the kinase activity, protecting the phosphorylation of pathological sites related to disease progression [24]. Moreover, these studies suggest that estrogens exert their effect through their alpha-type receptor with insulin-like growth factor 1 receptor by incorporating itself into a macromolecular complex that includes phosphoinositide 3-kinase and protein kinase A (AKT). The activation of these signal pathways leads to an inhibition of GSK3β and therefore to a reduction in tau phosphorylation [25]. Our results confirm the protective effect of estrogens on the pathological conformation and hyperphosphorylation of tau mediated by intracerebroventricular injection with Aβ42 in hTau mice. From our results, it cannot be determined if estradiol acts as an antioxidant compound, therefore, in a mode independent of its receptor or modulates at the receptor level protective signal pathways. We showed that it inhibits the kinases that, under Aβ treatment, hyperphosphorylate tau; these results suggest a cellular signaling effect of estradiol.
Fig. 7. Estradiol hormone therapy protects against oxidative stress and downregulate miRNA 218. A) Evaluation of the total antioxidant capacity in female mice subjected to ovariectomy and or to E₂ (1 μg/kg) and soy-free diet for three weeks. We show that ovariectomy significantly decreases the antioxidant capacity respect to control females, whereas the treatment with estradiol protects the antioxidant capacity. B) miRNA 218 levels capacity in female mice subjected to ovariectomy and or to E₂ (1 μg/kg) and soy-free diet for three weeks. We show that ovariectomy significantly increases the levels of miRNA 218 with respect to controls female, the treatment with estradiol completely protects the increase. C) Evaluation of the total antioxidant capacity in male mice treated or not with Aβ₄₂. We show that Aβ₄₂ decreases the antioxidant capacity respect to control males. D) miRNA 218 levels capacity in male mice treated or not with Aβ₄₂. We show that Aβ₄₂ significantly decreases miRNA level respect to control males. *p<0.05; **p<0.01 versus control; §p<0.05 versus OVX by one-way ANOVA followed by Bonferroni post test n=5.

A lot of protective mechanisms relating to estrogens have been described in the literature [26]. Most recent studies have revealed that estrogens exert an antioxidant action not only by direct chemical neutralization of reactants, but also by modulating the expression of antioxidant enzymes and levels of biological reducing agents [27]. In our experimental models, we found that the decrease of estrogenic levels was followed by a decrease of antioxidant activity and that this event...
with important oxidative stress-related risk factors related to AD such as hypoxia, hyperglycemia, and hypercholesterolemia, are potential causes of the increased BACE1, the crucial enzyme for Aβ production, activity [7].

Recently emerging evidence suggests that estrogens are involved in regulation of microRNAs in many pathological conditions [28]. Rao et al. showed that estriol regulates particular target miRNAs in a specific tissue and age manner in ovariectomized rats [29]. Furthermore, the deprivation of estrogens caused the progressive loss of regulation of the miRNA, leading to a lack of regulation even after the reintroduction of the estrogens [30]. In our work we focused our attention on the miRNA 218, because it is implicated in the phosphorylation of tau upon estrogen receptor (ER) α and β activation. There are two known ERs, usually referred to as ERα and ERβ, and both are widely distributed in the brain [31]. In the brain of patients with AD, both ERα and ERβ are defective. Mitochondrial ERβ is reduced in the frontal cortex of female patients with AD [32], and the alternative splicing of ERα mRNA is decreased in the AD brain especially in female patients [33]. Moreover, in the hippocampus of AD patients the ERα-expressing neurons are reduced [34], whereas ERβ immunoreactivity is increased [35]. These findings indicate a potential role of these two receptors in the pathogenesis of AD. Then it has been reported that the neuroprotection against Aβ toxicity by estrogens requires the expression of both receptors and the activation of mitogen-activated protein kinase pathway [36]. Specifically, Xiong et al. demonstrated opposite effects of these two receptors on tau phosphorylation. ERα overexpression increased miRNA 218 expression and the hyperphosphorylation of tau, whereas ERβ decreased miRNA 218 expression and tau phosphorylation [13]. Interestingly, a number of miRNA

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