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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 in men and women, and cognitive decline is greater in women than in men at the same stage of disease and at least in part on estradiol levels.

Objective: In our work we found that biological sex influences the effect of amyloid- β_{42} ($A\beta_{42}$) monomer on tau conformational change.

Methods: In this study we used transgenic mice expressing the wild-type human tau (hTau) which received intraventricular (ICV) injections of $A\beta$ peptides in nanomolar concentration.

Results: We found that $A\beta_{42}$ produces pathological conformational changes and hyperphosphorylation 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 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 interaction of these factors should be considered in women, in order to identify the best appropriate therapeutic approach in preventing cognitive impairment.

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

INTRODUCTION

Hallmarks of Alzheimer's disease (AD) are the accumulation of amyloid- β ($A\beta$) peptides in amy-

loid plaques, and the aggregation of tau protein to form neurofibrillary tangles.

$A\beta$ derives from the amyloid precursor protein (APP) through β site APP cleavage (BACE) 1 and γ -secretase processing. APP has multiple C-termini, most ending at residues 31 and 42. $A\beta_{42}$ aggregates more readily than $A\beta_{40}$ through sequential

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which were subjected to intravitreal (ICV) injections of A β peptides in nanomolar concentration. We discovered that A β ₄₂ 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 GSK3 β , 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, A β , and tau [8].

In this paper, we pursue the hypothesis that biological sex influences the effect of A β ₄₂ monomers on pathological tau conformational change. Our data revealed that A β ₄₂ 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.

5') mouse tau gene (forward CCCACCTGTAAC-3', reverse GTATGTCCACCC-3'), and di (forward 5'-CAGGCTTTGAA reverse 5'-TGAACCTGTGGG 3'). Mice were maintained on 129/SvJae/C57BL/6 background and were kept on a 12 h light/dark cycle and water available *ad libitum*. All procedures on live animals were performed under the supervision of a licensed veterinarian. The protocol was approved by the European Communities (November 24, 1986; 86/609/EEC) and the Italian Ministry of Health and University. All experiments followed the institutional guidelines on animal welfare and care on Care and Protection of living animals. All experimental or other scientific publications must be registered with the Italian Ministry of Health (No. 17/2010-B, June 30, 2010) and the *hoc* Ethical Committee of the University of Turin (http://www.unito.it/unitoWAR/pubblica/Ricerca1/Ricerca_comitato1).

Two groups of 2-month-old male and female mice were treated for 3 h ($n = 60$) with 100% O₂/N₂O anesthesia, hTau mice were intracerebrally injected with A β peptides or saline. The sites used for injection were: anteroposteriorly, 0.5 mm lateral, 1.2 mm relative to Bregma; dorsoventrally, 1.7 mm from the dural surface. The procedure was validated by injecting one mouse with a fluorescent dye (1 μ l).

Ovariectomy

Two groups of 2-month-old female mice underwent bilateral ovariectomy (OVX) or sham-operated groups. The bilateral ovariectomy was performed on anesthetized female mice were exposed by a midline incision in the dorsal skin and m

centration) with sterile double distilled water, centrifuged at 10000 g for 10 min to remove possibly aggregates and then intraventricularly injected. The quality of A β 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 μ M with PBS, and 50 μ l of solution was spotted onto a freshly cleaved 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 17 β -estradiol (E₂) for three weeks (1 μ g/Kg) [11]. Animals were allowed to recover for at least three weeks before experiments were performed and subsequently were subjected to intracerebroventricular injection of A β ₄₂ 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.

150 mM NaCl, 1 mM EGTA, 1 mM PMSF, phosphatase and protease inhibitors) then centrifuged at 10,000 g for 10 min to isolate soluble proteins. Supernatants (supernatant solution) were collected and incubated with detergent sarkosyl (5% final concentration) at 4°C. The sarkosyl mixtures were then centrifuged in Beckman SW 55 Ti rotor for 1 h at 4°C. Pellets were resuspended in sample buffer to obtain sarkosyl-free lysates (20 μ g) were run on 3–15% gradient PAGE gel (Invitrogen) and transferred to nitrocellulose membrane. Blots were probed (5% no fat milk) and incubated with primary antibodies. Peroxidase-conjugated secondary antibodies were incubated at room temperature (RT) and developed using ECL-Plus Forte Western substrate (Wako Pure Chemical Industry, Japan). Densitometric values were normalized to β -actin.

Total antioxidant capacity

To evaluate the antioxidant capacity of brain tissues, we performed the total antioxidant capacity (TAC) dosage kit (ab65329, Abcam) according to the manufacturing protocol.

Quantitative determination of 17 β -estradiol

To quantify E2 levels, blood samples were collected to obtain plasma. To perform the measurement, a commercially available ELISA kit (E2-ELISA kit, catalog # ADI-901-174) according to the manufacturer's protocol.

Fig. 1. Intracerebroventricular treatment with $A\beta_{42}$ causes a change in tau conformation only in male or ovariectomized female mice. Representative western blot of brain samples from control (saline) and treated $A\beta_{42}$ peptides for ICV male and female mice using conformational tau antibody (MC1) and a total tau antibody (Tau 5) for detection. Some female mice were subjected to ovariectomy (OVX) and some were not (CTR). $A\beta_{42}$ was injected or not (MONO). Densitometric quantification shows an increase of the total protein level of both MC1 and Tau 5 in OVX and MONO groups. The data are mean $\pm$ standard error of the mean (SEM); * $p < 0.05$ versus CTR; § $p < 0.05$ 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 Mi-croRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and a RT-primer pool con-

taining microRNA-specific stem loop primers for miR218 (Mature miRNA Sequence: GAUCUAACCAUGU) and for snRNA (Mature snRNA Sequence: GTGCTCGCTTCGACTAAAATTGGAACGATACAGCATGGCCCCCTGCGCAAGGATTCGTGAAGCGTTCCATATTTT).

Each qPCR contained 1.3 μ L of cDNA, 1 μ L 20X TaqMan MicroRNA Assay, and 1 μ L 2X TaqMan Universal PCR Master Mix.

Fig. 2. The conformational change mediated by A β ₄₂ is induced by protein hyperphosphorylation. A) Representative extracts from control (saline) and treated A β ₄₂ 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 as loading control. Densitometric analysis shows a significant increase of total protein levels of AT8, pS422, and pS396 in ovariectomized female mice. B–D) Representative western blot of brain extracts from control (saline) and treated male and female hTau mice using p JNK (B), pERK1/2 (C), and pGSK3 β (D). Some female mice were subjected to ovariectomy as loading control. Densitometric analysis shows a significant increase of total protein levels of all the three kinases in ovariectomized female mice. The data are mean $\pm$ standard error of the mean (SEM); * p < 0.05; ** p < 0.01 versus OVX by one-way ANOVA followed by Bonferroni post test n = 5.

All values were presented as mean $\pm$ standard error of the mean (SEM). Means were compared by one or two-way analysis of variance (ANOVA) with Bonferroni as a *post-hoc* test. Values of $*p < 0.05$ were considered significant, $**p < 0.01$ very significant, and $***p < 0.001$ extremely significant.

RESULTS

Intracerebroventricular treatment with A β ₄₂ 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 A β ₄₂ 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 A β ₄₂ 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 A β ₁₄₂.

The conformational change mediated by A β ₄₂ 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.

(+3-fold increase). A similar result was obtained when measuring the nuclear levels of p-tau, which increase in the same group of mice 3 times compared to the controls (F

Estradiol hormone therapy protects female mice against A β ₄₂-mediated tau conformational change

To confirm that the presence of estradiol is involved in the different effect exerted by treatment with A β ₄₂ on the pathological change of tau, groups of female mice were treated or not were subcutaneously treated with estradiol (E₂) (1 μ g/kg) and fed with a soy-free diet for 4 weeks. We first tested the validity of the method for measuring the levels of estradiol in the groups. As can be seen, oophorectomy significantly decreases circulating estradiol levels. Estradiol treatment determines an increase in estradiol levels, which becomes significantly higher with respect to control females (+100%) (Fig. 3). Figure 4 shows that treatment with estradiol completely blocks the pathological conformational change of total tau mediated by A β ₄₂ in ovariectomized females. Then, to further confirm the effect of estradiol, we studied the insolubilization of tau by the Sarkosyl detergent technique, and a tau band at approximately 75 kDa was observed, revealed with Tau 46 antibody. Figure 5 shows that A β ₄₂ in ovariectomized female mice, with estradiol blocks the aggregation of tau (Fig. 4B).

Estradiol therapy protects female mice against A β ₄₂-mediated tau hyperphosphorylation

Figure 5 shows that enrichment of tau in the insoluble fraction is also followed by complete phosphorylation of tau.

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).

and the simultaneous intracerebral injection of Aβ₄₂ induces a further decrease of this parameter (−70%). Treatment with estradiol restores the drop-in antioxidant capacity back to control values (Fig. 6A).

Finally, we measured levels of miR29a. Recent discoveries demonstrate that miRNAs are able to modulate the expression of genes involved in tau phosphorylation [14]. It has been found that increase of miR29a levels has been found that increase of miR29a level of target protein tyrosine phosphorylation consequent enhancement of tau phosphorylation.

We observed that levels of miR29a are higher in ovariectomized female mice treated with Aβ₄₂, whereas the E₂ treatment provides a total protection of the miRNA levels. It is interesting to note that the levels of miR29a in male hTau mice treated with Aβ₄₂ are similar to those obtained in females after oophorectomy.

DISCUSSION

In our work we pursued the hypothesis that estradiol influences the effect of Aβ₄₂ monomer on the conformational tau conformational change. Our results show that Aβ₄₂ produced pathological conformational change and hyperphosphorylation of tau in ovariectomized female mice and in intact female. The risk of developing Alzheimer's disease progression and severity are known to be different in men and women [14]. The drop-in estrogen and the pathological tau are supported by data indicating that early menopause increases the risk of developing dementia. The risk of developing dementia and cognitive decline is greater in women than in men at the same stage of disease and is inversely correlated with estrogen levels [14]. Our results suggest the therapeutic potential of estrogen therapy in Alzheimer's disease.

Fig. 4. Estradiol hormone therapy protects against A β ₄₂-mediated tau conformational change. A) Representative samples from control (saline) and treated A β ₄₂ peptides for ICV female hTau mice using a conformational tau anti-tau antibody (Tau 5) for detection. Some female mice were subjected to ovariectomy and/or to E₂ (1 μ g/kg) and 4 weeks. β -actin served as loading control. Densitometric quantification shows an increase of the total protein level in ovariectomized female mice injected or not with A β ₄₂; the treatment with estradiol completely protects the increase of tau protein. B) Representative western blot of insoluble tau fraction by sarkosyl detergent technique (saline) and treated A β ₄₂ peptides for ICV female hTau using Tau 46 antibody for detection. β -actin served as loading control. To show the increase of A β ₄₂ in ovariectomized females, we showed a band at approximately 75 kDa molecular weight revealed with Tau 46 antibody. The data are mean $\pm$ standard error of the mean (SEM); * p < 0.05; ** 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 protected against the induction of neuroinflammation and neurodegeneration [16–18]. The encouraging results from *in vitro* clashed with clinical trials of

Fig. 5. Estradiol hormone therapy protects against $A\beta_{42}$ -mediated tau hyperphosphorylation. A) Representative western blots of brain extracts from control (saline) and treated $A\beta_{42}$ peptides for ICV female hTau mice using antibodies specific for the detected phosphorylation sites such as AT8, pS422, and pS396. Some female mice were subjected to ovariectomy and/or to E₂ diet for three weeks. β -actin served as loading control. Densitometric analysis shows an increase of total protein levels of tau protein phosphorylated at pS396 in ovariectomized female mice injected or not with $A\beta_{42}$; the treatment with estradiol completely protects the levels of tau protein. B–D) Representative western blot of brain extracts from control (saline) and treated $A\beta_{42}$ peptides for ICV female hTau mice using antibodies specific for the detected phosphorylation sites such as pJNK (B), pERK1/2 (C), and pGSK3 β (D). Some female mice were subjected to ovariectomy and/or to E₂ (1 μ M) diet for three weeks. β -actin served as loading control. Densitometric analysis shows an increase of total protein levels of pJNK, pERK1/2, and pGSK3 β in ovariectomized female mice injected or not with $A\beta_{42}$; the treatment with estradiol completely protects the activation of these kinases. The data are mean $\pm$ standard error of the mean (SEM); * p < 0.05; ** p < 0.01 versus control; § p < 0.05 versus OVX; followed by Bonferroni post test n = 5.

Fig. 6. Estradiol hormone therapy protects male mice against A β ₄₂-mediated tau conformational change as well as Representative western blot of brain samples from control (saline) and treated A β ₄₂ peptides for ICV male hTau mice. tau (MC1) and a specific antibody for tau pathological phosphorylation (AT8) for detection. Some male mice were su and soy-free diet for three weeks. β -actin served as loading control. Densitometric quantification shows an increase of both MC1 and AT8 in mal mice injected or not with A β ₄₂; the treatment with estradiol completely protects the con the increase of tau phosphorylation. The data are mean $\pm$ standard error of the mean (SEM); ** p < 0.01 versus control 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, emerging evidence suggests that protein tau could be a potential target for estrogens. It has been demonstrated that 17 β estradiol 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 GSK3 β 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 results suggest that estrogens exert their effects through their alpha-type receptor, which interacts 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 this signal pathways leads to an inhibition of GSK3 β and therefore to a reduction in tau phosphorylation [25]. Our results confirm the effects of estrogens on the pathological hyperphosphorylation of tau mediated by intracerebroventricular injection with A β in transgenic mice. From our results, it cannot be concluded if estradiol acts as an antioxidant or as a neuroprotective agent, therefore, in a mode independent of its action on the estrogen receptor or modulates at the receptor level downstream pathways. We showed that it is not only estradiol that, under A β treatment, hyperphosphorylates tau, these results suggest a cellular mechanism of estradiol.

Fig. 7. Estradiol hormone therapy protects against oxidative stress and downregulate miRNA 218. A) Evaluation of antioxidant capacity in female mice subjected to ovariectomy and or to E₂ (1 µg/kg) and soy-free diet for three weeks. We found that ovariectomy significantly decreases the antioxidant capacity respect to control females, whereas the treatment with estradiol protects against this decrease. B) miRNA 21 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 found that Aβ₄₂ decreases the antioxidant capacity respect to control males. D) miRNA 21 levels capacity in male mice treated or not with Aβ₄₂. We found that Aβ₄₂ significantly decreases miRNA level respect to control males. **p* < 0.05; ***p* < 0.01 versus control; §*p* < 0.05 versus control. 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. In models of biological reducing agents [27]. In experimental models, we found that the decrease in antioxidant levels was followed by a decrease in 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 A β 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 estradiol 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 ER α and ER β , and both are widely distributed in the brain [31]. In the brain of patients with AD, both ER α and ER β are defective. Mitochondrial ER β is reduced in the frontal cortex of female patients with AD [32], and the alternative splicing of ER α mRNA is decreased in the AD brain especially in female patients [33]. Moreover, in the hippocampus of AD patients the ER α -expressing neurons are reduced [34], whereas ER β 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 A β 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. ER α overexpression increased miRNA 218 expression and the hyperphosphorylation of tau, whereas ER β decreased miRNA 218 expression and tau phosphorylation [13]. Interestingly, a number of miRNA

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