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Molecular events during the induction of neurodegeneration and memory loss in estrogen-deficient rats

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ABSTRACT

This study aims to delineate the relationship among estrogen deficiency, neurodegeneration, and cognitive impairment of ovariectomized rats. Female Sprague–Dawley rats were ovariectomized and euthanized after 1–4 month periods (M_0–M_4 groups). Blood samples were collected for the determination of serum levels of 17β-estradiol (E_2), luteinizing hormone (LH), and follicle stimulating hormone (FSH). Five consecutive days before the euthanization, cognitive performance of the rats was examined by Morris water maze test. After euthanization, the hippocampus was collected, and expression of the genes associated with amyloid plaques (*App*, *Adam10* and *Bace1*) and neurofibrillary tangles (*Tau4* and *Tau3*) were examined by real-time PCR. Serum E_2 levels were declined following 2 weeks of ovariectomy. Conversely, serum FSH and LH levels were profoundly increased by 2 weeks of ovariectomy for approximately 4 and 22 times, respectively. Cognitive impairments, indicated by the longer latency and distance, were observed only in the M_3 and M_4 groups. The *Tau4* mRNA levels were significantly increased as early as 1 month after ovariectomy (in the M_1 group; $P < 0.05$), and tended to be increased further with the advancing time. Similarly, the *Tau3* mRNA levels were increased by ovariectomy, but with the highest level in the M_1 group, and decreased thereafter. The mRNA levels of *App*, *Adam10* and *Bace1* were increased by ovariectomy, but significant differences were observed only in the M_4 group. These results indicate that estrogen deficiency can induce a sequence of events that results in the production of neurofibrillary tangles, amyloid deposition, and spatial memory deficit in rats.

1. Introduction

Neurodegenerative disease is an age-related disease with increasing incidents over the past decade. The progressive loss of the structure and function of neurons in the brain, including neuronal death, is one of the causes of neurodegenerative diseases. The neuropathological hallmarks of neurodegenerative diseases are extracellular senile plaques and intracellular neurofibrillary tangles [20]. The extracellular senile plaques are composed largely of a 40- or 42-amino acid β-amyloid (Aβ) peptide. The intracellular neurofibrillary tangles are composed of aberrantly phosphorylated microtubule-associated proteins called 'TAU' [3]. These lesions are invariably found throughout the neocortex and hippocampus of patients with neurodegenerative disease that are linked to memory deficit and progressive loss of cognitive function [40].

TAU is a major microtubule-associated protein in brain cells, which is localized primarily in the axons. TAU protein helps neurons to maintain the cell shape and to facilitate axoplasmic transport [3,6,43]. Post-translational modification, such as phosphorylation, reduces the ability of TAU to promote microtubule assembly [48]. Abnormally hyperphosphorylated and filamentous TAU is the main component of the neurofibrillary tangles found in the brains of neurodegenerative diseases, especially in Alzheimer's disease [20,21]. The TAU proteins are encoded by a single gene consisting of 16 exons in humans [31]. In the adult human brain, six different *TAU* mRNA isoforms are expressed by an alternative splicing mechanism of exons 2, 3, and 10 of its pre-mRNA [1]. The exons 2 and 3 of *TAU* encode a 29 amino acid region located in the amino-terminal of the full-length TAU protein. Thus, the *TAU* mRNA isoform containing the exons 2 and 3, only the exon 2, or neither of them produces the long (L), short (S), or none (0) TAU protein isoform, respectively. Generally, the proportion of L, S, and 0 isoforms in the adult human brain is about 54%, 37%, and 9%, respectively [42], and this ratio does not differ between healthy people and patients with serious cognitive impairment,

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such as Alzheimer's patients [16]. On the other hand, the exon 10 encodes the second tubulin-binding repeat in the carboxy-terminal of the TAU protein [18], and thus alternative splicing of the exon 10 generates TAU isoforms with four or three tubulin-binding repeats (TAU4 or TAU3, respectively). The tubulin-binding repeat plays an important role in the interaction between TAU and microtubules. The proportion of TAU4 and TAU3 isoforms in the human brain differs between healthy adults [42,43] and patients with neurodegenerative disorders [19,46]. Thus, examination of the TAU4/TAU3 ratio should be considered to gauge the progress of neurodegenerative diseases.

Aβ, a major component of senile plaque, is generated from amyloid precursor protein (APP) by sequential proteolytic cleavages. The APP processing has two pathways, non-amyloidogenesis and amyloidogenesis, and is mediated by secretases (α, β, and γ). The non-amyloidogenic APP processing is mediated by α-secretase and results in the secretion of a non-toxic, large extracellular APP α fragment [51]. In this processing, A disintegrin and metalloproteinase (ADAM) family, including ADAM9, ADAM10 and ADAM17, has been suggested to function as α-secretase [40]. An increase in the expression of ADAM10 lowers the burden of amyloid load and precludes or abrogates Alzheimer's disease [10,11]. In contrast, the amyloidogenic pathway mediated by β-secretase and γ-secretase in neurons generates a variety of potentially amyloidogenic Aβ species from APP, which are insoluble and neurotoxic [13,40,51]. In this pathway, beta-site APP cleaving enzyme (BACE) functions as β-secretase. In the endocytic pathway, BACE1 and BACE2 cleave APP and generates APPβ and a longer C-terminal fragment, which is further processed by γ-secretase and generates Aβ. BACE1 is apparently the only enzyme with APP β-secretase activity in the brain, whilst BACE2 has limited expression in the normal human brain but is largely found in patients with Down syndrome [8,51]. In addition, BACE1 is highly expressed in the hippocampal and cortical regions of the brain of Alzheimer's disease [28,47]. Thus, α-secretase ADAM10 and β-secretase BACE1 were selected in this study as representative enzymes for the non-amyloidogenic and amyloidogenic pathways of the APP processing.

Recently, many reports indicated non-reproductive functions of estrogen, and the brain is one of the target organs. In the brain, estrogen plays a role in the control of neuronal functions, including neuronal proliferation, neuronal survival, and neuronal plasticity [4]. Estrogen also has great impacts over cognitive function, since it shows neurotrophic and neuroprotective actions over certain brain areas such as the hippocampus, cortex, and the striatum [4,30]. During the aging process or postmenopausal period in women, the reduction of estrogen level induces progressive neurodegeneration in the brain and results in cognitive impairment [4]. However, there is no report to indicate how estrogen deficiency induces neurodegenerative diseases. To delineate the relationships between estrogen deficiency, neurodegeneration, and cognitive impairment, we examined the effects of ovariectomy on the serum levels of 17β-estradiol (E_2), luteinizing hormone (LH) and follicle stimulating hormone (FSH), the expressions of the genes associated with amyloid plaques and neurofibrillary tangles in the hippocampal brain area, and the cognitive ability in adult female rats for 4 months.

2. Materials and methods

2.1. Animals

Adult female Sprague–Dawley rats, 2 months old, were obtained from the National Laboratory Animal Center, Mahidol University, Thailand. They were reared in stainless steel cages with sawdust bedding at five animals/cage in a room with controlled lighting (lights on 0600–1800 h) and temperature (25 ± 1 °C) at the Laboratory Animal Room, Faculty of Science, Chulalongkorn University, Thailand. The animals were fed with rat chow diet (Pokaphan Animal Feed Co. Ltd., Thailand) and water *ad libitum*. The rats were reared until they became 4 months old and used in this study following the study of Feng et al. [12]. The experimental protocol was approved by the Animal Ethical Committee in accordance with the guide for the care and use of laboratory animals prepared by Chulalongkorn University, Protocol Review No. 1123009.

2.2. Experimental design

At the age of 4 months, animals were divided into 5 groups; M_0, M_1, M_2, M_3 and M_4 with 9, 8, 7, 6 and 8 rats, respectively. In the M_0 (control) group, the rats of the diestrous phase were selected on the first day of the experiment. In the M_1, M_2, M_3 and M_4 groups, rats were bilaterally ovariectomized on the first day of the experiment under sodium pentobarbital anesthesia (40 mg/kg BW, i.p.), and kept for 1, 2, 3 and 4 months, respectively.

One-milliliter of blood sample was collected once a month in each group of rats at 0800–0900 h. In the last month of the experiment, the blood samples were additionally collected in two-week interval. Immediately after the blood clotted, blood serum was separated by centrifugation at 1000*g* for 30 min and then kept at −20 °C until being assayed for E_2, FSH and LH. Spatial memory test was performed in each group of rats for five consecutive days before the end of the experiment.

At the end of the experiment, the animals were euthanized and the brains were removed. The hippocampus region was then dissected, homogenized in 300 μl of TRIzol reagent (Invitrogen, USA), and kept at −80 °C until RNA extraction.

2.3. Hormone assays

Serum E_2 levels were determined by a double-antibody RIA system using ^{125}I-labeled radioligands as described previously [29]. The antisera against estradiol (GDN 244) were kindly provided by Dr. G.D. Niswender (Colorado State University, Fort Collins, CO, USA). The intra- and inter-assay coefficients of variation were 4.1% and 10.7%, respectively.

Serum FSH and LH levels were determined using the National Institute of Diabetes and Digestive and Kidney disease (NIDDK) kit for rat FSH and LH (Baltimore, MD, USA) as described previously [26]. Iodinated preparations used in this study were rat FSH-I-7 and rat LH-I-7, and the antisera were anti-rat FSH-S11 and anti-rat LH-S10, respectively. The results obtained are expressed using the rat FSH-RP-2 and rat LH-RP-2 reference standards. The intra- and inter-assay coefficients of variations were 6.5% and 7.6% for FSH and 6.4% and 8.6% for LH, respectively.

2.4. Spatial memory test

Morris water maze test was used to examine spatial memory of the rats [49]. The test was performed in a circular pool with 180 cm diameter and 70 cm deep, which was made of stainless steel, modified from Feng et al. [12]. The pool was filled with water to a depth of 50 cm and divided into four quadrants. A platform was submerged 1 cm below the surface of water in the center of north-east quadrant.

Each rat was given four trials per day for five consecutive days to find the hidden platform. The first trial was started by placing the rat into the water surface facing the pool wall in one of the four quadrants, and rotated the placing position clockwise to cover all four quadrants in the subsequent trials. From the next day onwards, the test was started from a quadrant different from the previous day. For each trial, the rat was allowed to swim in a maximum of 90 s to find the platform and given a 30 s rest period