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Ovariectomy Enhances Acetylcholinesterase Activity But Does Not Alter Ganglioside Content in Cerebral Cortex of Female Adult Rats

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In the present work we investigated the effect of ovariectomy on acetylcholinesterase (AChE) activity and ganglioside content in cerebral cortex of female rats. We also studied the activity of butyrylcholinesterase (BuChE) in serum of these animals. Adult Wistar rats were divided into three groups: (1) naive females (control), (2) sham-operated females and (3) castrated females (ovariectomy). Thirty days after ovariectomy, rats were sacrificed by decapitation without anaesthesia. Blood was collected and the serum used for BuChE determination. Cerebral cortex was homogenized to determine AChE activity and extracted with chlorophorm:methanol for ganglioside evaluation. Results showed that rats subjected to ovariectomy presented a significant increase of AChE activity, but did not change the content and the profile of gangliosides in cerebral cortex when compared to sham or naive rats. BuChE activity was decreased in serum of rats ovariectomized. Our findings suggest that the alteration in the activity of brain AChE, as well as serum BuChE activity caused by ovariectomy may contribute to the impaired cognition and/or other neurological dysfunction found in post-menopausal women.

Key words: Acetylcholinesterase; butyrylcholinesterase; gangliosides; cerebral cortex; ovariectomy; female rats.

INTRODUCTION

In adult woman with a normal reproductive cycle the estrogenic compounds are secreted in great quantity mainly by ovaries, being the 17β estradiol considered the major estrogen (Rodrigues *et al*., 1999). Estrogen exerts also diverse nonreproductive actions on multiple organs, including the brain (Wise, 2002). It has been shown that estrogen deprivation is implicated in the pathogenesis of some neurodegenerative conditions, such as Alzheimer's disease and cerebral ischemia (Tang *et al*., 1996; Van Duijn, 1999; Zhang *et al*., 1998). In this context, there is a large body of literature suggesting that post-menopausal women are more vulnerable than young women to these diseases and cognitive deficit (Green and Simpkins, 2000; Wise *et al*., 2001a,b).

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Acetylcholinesterase (AChE) (E.C. 3.1.1.7), the enzyme involved in the hydrolysis of the neurotransmitter acetylcholine, contributes to the integrity and permeability of the synaptic membrane that occurs during neurotransmission and conduction (Grafius *et al*., 1971). This enzyme has been implicated in cholinergic and noncholinergic actions which may play a role in neurodegenerative diseases (Cummings, 2000; Henderson *et al*., 1996; Law *et al*., 2001). It has been also shown that AChE *per se* activates neuronal cell death (Calderón *et al*., 1998). On the other hand, it is known that estrogen withdrawal and replacement affect the cholinergic system in a variety of brain regions (Gibbs and Aggarwal, 1998; Simpkins *et al*., 1997).

Gangliosides are a family of sialic acid-containing glycosphingolipid present in high concentration in neural membranes. They play important roles in cell-cell interaction, cellular growth and differentiation, signal transduction, adaptation of plasma membrane to environmental variations and may be involved in neuronal development (Ando, 1983; Maccioni *et al*., 1984; Sanhoff and Van Echten, 1994). It has been proposed that gangliosides may play significant roles in memory and behavior (Rahmann, 1995). In addition, alterations in the content and composition of gangliosides have been reported in brain injuries such as hypoxia (Ramirez *et al*., 2003; Trindade *et al*., 2001, 2002), ischemia (Inokuchi *et al*., 1998), Alzheimer's disease and in other neurodegenarative disorders (Farooqui *et al*., 1988; Ohtani *et al*., 1996; Schneider *et al*., 1998; Yu and Ledeen, 1974). It was been shown that ethinylestradiol administration induces distinct responses on ganglioside concentrations, increasing or diminishing it in some regions of the forebrain of female adult rabbits, or not affecting it in others (Islam *et al*., 1986). On the other hand, there is a lack of studies analyzing the influence of female hormones reduction on neural connections which can be reflected by ganglioside content (DeKosky and Bass, 1982; Zeller and Marchase, 1992).

Considering that hormonal deprivation in post-menopausal women is implicated in the pathogenesis of cerebrovascular and Alzheimerś disease and that cholinesterases are altered in these conditions, in the present study we investigated the effect of ovariectomy on AChE activity and gangliosides content in cerebral cortex of female adult rats. We also determined BuChE activity in serum, a blood AChE marker.

MATERIALS AND METHODS

Subjects and Reagents

Female adult Wistar rats (3 months, 180–210 g BW) were obtained from the Central Animal House of the Department of Biochemistry, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on a 12/12 h light/dark cycle in an air-conditioned constant temperature (22 ± 1°C) colony room. Rats had free access to a 20% (w/w) protein commercial chow and water. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and was approved by the Ethics Committee of the Federal Rio Grande do Sul, Brazil. The chemicals were purchased from Sigma, St. Louis, MO.

Experimental Treatment

Animals were randomly assigned to one of the following groups ($n = 4$): naive (control), sham (only submitted to surgery without removing of ovaries) and ovariectomized. Rats were ovariectomized by surgery removing both the ovaries under anesthesia induced by i.p. injection of ketamine (90 mg/kg) and xylazine (10 mg/kg) to eliminate endogeneous ovarian steroids (Waynforth and Flecknell, 1992). One month after ovariectomy, rats were sacrificed by decapitation without anesthesia and the brain was immediately isolated, washed with saline solution and the cerebral cortex was dissected. Estradiol levels were evaluated in plasma after surgery by radioimmunoassay using a Biomedical kit (Biomedicals Technologies, Inc., Stoughton, MA). Estrogen levels in the ovariectomized rats were undetectable (data not shown) confirming the efficacy of the surgical procedures of ovariectomy.

Tissue Preparation

Rats were killed by decapitation without anaesthesia, the blood was rapidly collected, centrifuged at 3000 rpm for 10 min and the serum was separated and used for the BuChE assays. The brain was quickly removed and the cerebral cortex was dissected. For determination of AChE activity, cerebral cortex was homogenized in 10 volumes 0.1 mM potassium phosphate buffer, pH 7.5. For ganglioside extraction the cerebral cortex was weighed and extracted first with a 2:1 mixture of chloroform:methanol (C/M, 2:1, v/v) to a 20-fold dilution of tissue mass and centrifuged at 800 *g* for 10 min. The pellet was extracted with C/M 1:2 to a 10-fold dilution of original sample mass. The C/M extracts were combined and this pool was used for ganglioside evaluation (Roukema and Heijlman, 1970).

Acetylcholinesterase (AChE) Activity Assay

Acetylcholinesterase activity was determined according to Ellman *et al*. (1961), with some modifications (Villescas *et al*., 1981). Hydrolysis rates *v* were measured at acetylthiocholine (S) concentrations of 0.8 mM in 1 mL assay solutions with 30 mM phosphate buffer, pH 7.5, and 1.0 mM DTNB at 25°C. Fifty microliters of rat cerebral cortex supernatant was added to the reaction mixture and preincubated for 3 min. The hydrolysis was monitored by formation of the thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30s). All samples were run in duplicate.

Butyrylcholinesterase (BuChE) Assay

BuChE activity was determined by the method of Ellman *et al*. (1961) with some modifications. Hydrolysis rate v was measured at acetylthiocholine (S) concentration of 0.8 mM in 1 mL assay solutions with 100 mM phosphate buffer, pH 7.5, and 1.0 mM DTNB. Fifty microliters of rat serum was added to the reaction mixture and preincubated

for 3 min. The hydrolysis was monitored by formation of the thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30s) at 25°C. All samples were run in duplicate.

Ganglioside Evaluation

Aliquots from the total lipid extracts were used for ganglioside determination by the N-acetyl-neuramic acid (NeuAc) quantification with the resorcinol method described by Svennerholm (1957) and modified by Miettinen and Takki-Luukkainen (1959). Ganglioside species were analyzed by thin layer chromatography (TLC) and this technique was performed on 10 × 10 cm Merck plates of silica gel 60 using a developing tank described by Nores *et al*. (1994). Aliquots of the total lipid extracts containing 6 nmol of NeuAc suspended in C:M (1:1) were spotted on 8-mm lanes. TLC was developed, sequentially, with two mixtures of solvents, firstly C:M (4:1, v/v) and secondly C:M: 0.25% $CaCl_2$ (60:36:8, v/v). Ganglioside profile was visualized with resorcinol reagent (Lake and Goodwin, 1976; Svennerholm, 1957). The chromatographic bands were quantified by scanning densitometry at 580 nm with a CS 9301 PC SHIMADZU densitometer. Individual ganglioside values expressed as nmol ganglioside-NeuAc/mg tissue, were calculated by relating their respective percentage to the absolute total quantity of ganglioside-NeuAc. The terminology used herein for gangliosides is that recommended by Svennerholm (1963).

Protein Determination

Protein was measured by the method of Bradford (1976) using bovine serum albumin as standard.

Statistical Analysis

All assays were performed in duplicate and the mean was used for statistical analysis. Data were analyzed by one way ANOVA followed by the Duncan multiple test when F-test was significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer.

RESULTS

Figure 1 shows the effect of ovariectomy on AChE activity from cerebral cortex of rats. Ovariectomized rats present an increase (113%) of AChE activity when compared to control (naive) or rats submitted to surgery sham [$F(2, 9) = 20.72$; $p < 0.01$].

Since in our study the ovariectomized rats present an increase of brain AChE activity and that a recent report from the literature suggested that BuChE activity could hydrolyze acetylcholine glial (Mesulam *et al*., 2002) and that this enzyme could be used as a peripheral marker of brain AChE, in the present study we also verified the activity of BuChe in serum of female adult ovariectomized rats (Fig. 2). Results showed that rats submitted to ovariectomy presented an inhibition (45%) of BuChE activity when compared to naive and sham rats [$F(2, 9) = 11.43$; $p < 0.05$].

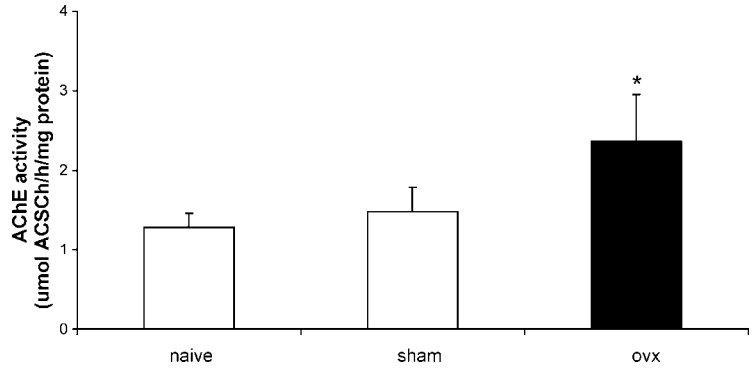

Figure 1. Effect of ovariectomy on acetylcholinesterase activity in cerebral cortex of rats. Data are expressed as mean ± S.D. for four independent experiments performed in duplicate. $^{*}p < 0.01$ compared to naive and sham groups (Duncan's multiple range test). AChE—acetylcholinesterase; ovx—ovariectomized.

Table 1 shows that ovariectomy did not cause changes in cerebral cortex weight [$F(2, 9) = 0.44$; $p > 0.05$] and total ganglioside content [$F(2, 9) = 0.54$; $p > 0.05$] in this brain structure of naive, sham and ovariectomized rats.

Thin layer chromatography (Fig. 3) shows the presence of four main cerebral gangliosides: GM1, GD1a, GD1b, and GT1b. The chromatogram reveals no difference on the ganglioside profiles between the studied groups.

DISCUSSION

Estrogen has been described to play an important role in cognitive functions and neuroprotection (Brinton, 2001; Gandy, 2003; Kampen and Sherwin, 1994). In this context, it has been shown that estrogen deprivation is implicated in the pathogenesis of neurodegenerative disorders, including stroke (Liao *et al.*, 2001) and Alzheimer's disease (Fillit,

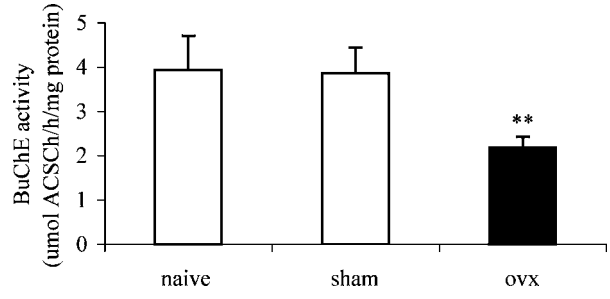

Figure 2. Effect of ovariectomy on butyrylcholinesterase activity in serum of rats. Data are mean ± S.D. for four independent experiments performed in duplicate. $^{**}p < 0.05$ compared to control (Duncan multiple range test). BuChE—butyrylcholinesterase; ovx—ovariectomized.

Table 1. Cerebral Cortex Weight and Ganglioside-NeuAc Content of Female Adult Wistar Rats

	Groups		
	Naive	Sham	Ovx
Cerebral cortex weight (mg)	668.5 ± 7.4	689.7 ± 30.9	661.0 ± 22.6
Ganglioside content (nmol NeuAc/mg tissue)	1.61 ± 0.05	1.61 ± 0.07	1.71 ± 0.10

Note. Control (naive), submitted to surgery (sham) and ovariectomized (ovx). Values are expressed as mean ± standard error; $n = 4$.

1994; Van Duijn, 1999). Evidences also show that post-menopausal estrogen replacement therapy reduces the risk and delay in the onset of these diseases (Tang *et al*., 1996; Van Duijn, 1999; Yaffe *et al*., 1998). In contrast, recent data from the literature showed that estrogen plus progestin therapy to post-menopausal women increased the risk for dementia in women aged 65 years or older and did not improve cognitive impairment in these women (Shumaker *et al*., 2003).

Reduction in cholinergic function and alteration in the content and composition of gangliosides have been reported as one of the causes of Alzheimer's disease and stroke (Bonnefont *et al*., 1998; Farooqui *et al*., 1988; Fredman, 1998; Inokuchi *et al*., 1998; Mesulam *et al*., 2002; Schneider, 1994). In addition, the interaction among estrogens, cholinergic system and especially ganglioside content has not studied.

In the present study, we investigated the effect of ovariectomy on AChE activity and on ganglioside content and profile in cerebral cortex of female adult rats. We used this animal model of steroid hormone deprivation because the ovariectomy is considered the most common animal model of post-menopausal changes in adult female rats (Savonenko and Markowska, 2003). We used cerebral cortex because the discovery of estrogen receptor, namely ER-β in this structure has provided novel sites for estrogen action in cerebral cortex (Shughrue and Merchenthaler, 2000). In addition, estrogen also appears to play a fundamental role in cortical neuroprotection, since estrogen treatment significantly reduces

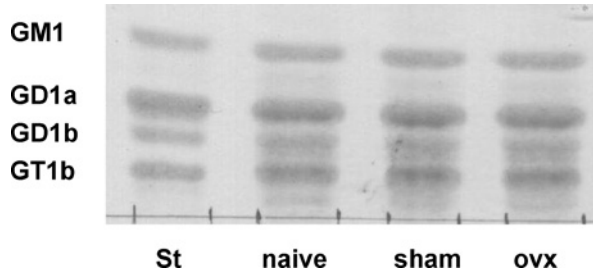

Figure 3. Thin-layer chromatography of ganglioside profile in cerebral cortex of female adult Wistar rats control (naive), submitted to surgery (sham) and ovariectomized (ovx). Ganglioside-NANA was estimated by the resorcinol-HCl reagent of Svennerholm (1957) as modified by Miettinen and Takki-Luukkainen (1959). A small volume of concentrated ganglioside extract containing 6 nmol was spotted for separation of the ganglioside fractions. The position of chromatographed ganglioside standards are indicated.

infarct size after ischemia in ovariectomized rats (Dubal *et al*., 1999). Our results showed that rats subject to ovariectomy presented a significant increase in AChE activity. However, we cannot establish at the present whether the increase of this enzyme activity following by ovariectomy would be a result of estrogen deprivation alone, since ovaries produce other substance such as progestin and inibin.

Changes in AChE activity in Alzheimer's disease patients have been previously reported (Arendt *et al*., 1992; Fishman *et al*., 1986; Gómez-Ramos and Morán, 1997). In this context, a reduction of this enzyme activity was demonstrated in cerebral cortex and hippocampus of patients affected by Alzheimer's (Fishman *et al*., 1986) and studies also show that alterations of AChE activity are associated with the cognitive alterations characteristic of these patients (Cummings, 2000; Law *et al*., 2001). On the other hand, degeneration of cholinergic nerve endings in specific regions of brain results not only in reduction of the tetrametric globular form (G4) of AChE, but also in a concomitant increase (300- to 400-fold) in the collagen-tailed form of this enzyme (Younkin *et al*., 1986). In this context, it was found that AChE (G1 globular form) is co-localized with senile plaques in the central nervous system (CNS), suggesting that this enzyme plays a role in the progressive β-amyloid aggregation and in senile plaque maturation characteristic of Alzheimer's disease (Arendt *et al*., 1992; Gómez-Ramos and Morán, 1997). In addition, recent studies suggest that amyloid-AChE complexes are formed when AChE accelarates the assembly of Aβ peptides into fibrils by interacting with the growing amyloid fibrils (Alvarez *et al*., 1997). Based on these findings, reversible inhibitors of cholinesterases have been used as cognitive stimulators in the treatment of Alzheimer's disease (Enz *et al*., 1993; Greig *et al*., 2001). Some studies also showed that ischemia transiently increases AChE activity in organotypic rat hippocampal slice cultures (Saez-Valero *et al*., 2003).

Considering that there is evidence showing that BuChe activity, which is considered a peripheral marker of neuronal AChE (Fossi *et al*., 1992), may have a role in the aggregation of AB that occurs in the early stages of senile plaque formation in Alzheimer's disease (Guillozet *et al*., 1997; Mesulam and Geula, 1994), we also examined the effect of ovariectomy on BuChE activity in serum of rats. Results showed that this enzyme activity was decreased (46%) in ovariectomized rats. The unexpected decrease of this BuChE activity in serum of ovariectomized rats may be possibly interpreted as a compensatory mechanism to decrease acetylcholine hydrolysis, since AChE activity is increased in brain. In fact, a similar pattern of these enzymes activity has been described in another study (Giacobini, 1997). In addition, other studies have reported that AChE activity is decreased in brain of patients with Alzheimeŕs disease, whereas BuChe activity is unchanged or increased (Davies and Maloney, 1976; Giacobini *et al*., 1989). So far we do not know the exact underlying mechanisms through which BuChE activity is decreased in our study.

We also showed in the present study that the content and profile of gangliosides in cerebral cortex was not changed in female rats ovariectomized. However, we cannot at this time, affirm whether the results here observed in cerebral cortex occur in other cerebral structures, because reports from literature show that estrogen administration decreases the content of total lipids in hypothalamus and increases the concentrations of gangliosides in hyppocampus, amygdaloid nucleus and olfactory bulbs, suggesting that the lipid contents and plasticity are affected differentially in the various areas of the brain by estrogen or phytoestrogen (Islam *et al*., 1986; Lephart *et al*., 2003).

Finally, it has been suggested that estrogen deprivation is likely to initiate or enhance neurodegenerative changes and to reduce the brain ability to maintain synaptic connectivity and cholinergic integrity, leading to the cognitive decline seen in post-menopausal individuals (Gandy, 2003). In this context, it has been shown that depletion of estrogen causes accumulation of Aβ peptide in the CNS of transgenic mice, which can be reversed by estradiol treatment (Zheng *et al*., 2002).

Summarizing, the present study demonstrates that female adults ovariectomized significantly increases AChE activity in cerebral cortex. This effect could decrease acetylcholine levels, leading to reduction of cholinergic neurotransmission. Assuming the possibility that these phenomena may occur in humans, our findings might be relevant to explain, at least in part, the cognitive impairment and the higher risk of neurodegenerative disease observed in post-menopausal woman.

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