J Bioenerg Biomembr (2008) 40:625–630
DOI 10.1007/s10863-008-9195-1

# Increased vulnerability of brain to estrogen withdrawal-induced mitochondrial dysfunction with aging

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Received: 19 September 2008 / Accepted: 18 December 2008 / Published online: 13 January 2009


**Abstract** In the present study, to determine whether aging could increase the vulnerability of the brain to estrogen withdrawal-induced mitochondrial dysfunction, we measured the cytochrome c oxidase (COX) activity and mitochondrial adenosine triphosphate (ATP) content in hippocampi of 2 groups of ovariectomized (OVX) Wistar rats aged 2 months (young) and 9 months (middle-aged), respectively. In addition, effects of genistein and estradiol benzoate (EB) were tested also. We observed only a transient alteration of COX activity and mitochondrial ATP content in hippocampi of young OVX rats but a prolonged lowering of COX activity and mitochondrial ATP content in hippocampi of middle-aged OVX rats. This suggested that with aging compensatory mechanisms of mitochondrial function were attenuated, thus exacerbated estrogen withdrawal-induced mitochondrial dysfunction in hippocampi. Significantly, EB/genistein treatment reversed this estrogen withdrawal-induced mitochondrial dysfunction in both young and middle-aged rats suggesting that genistein may be used as a substitute for estradiol to prevent age-related disease such as Alzheimer's disease in post-menopausal females.

**Keywords** Mitochondrial dysfunction · COX · ATP · Ovariectomy · Aging · Neurodegeneration

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## Introduction

Mitochondria are an important source of free radicals, and mitochondrial components are also major targets for the free-radical damage associated with aging (Sastre et al. 1996; Garcia de La Asunción et al. 1996; Miquel et al. 1980; Viña et al. 2005). But oxidant production by mitochondria from females is significantly lower than that from males (Viña et al. 2005). As tested in hepatic and brain mitochondria from mice or rats, mitochondria from females produce approximately half the amount of hydrogen peroxide as those from males and have higher levels of mitochondrial reduced glutathione, manganese superoxide dismutase, and glutathione peroxidase than males (Viña et al. 2005). Oxidative damage to mitochondrial DNA is also four-fold higher in males than in females (Borras et al. 2007). Moreover, the 16S rRNA expression, which decreases significantly with aging, is four times higher in mitochondria from females than in those from males of the same chronological age (Borras et al. 2007). These differences may be explained by estrogens. Therefore, estrogen withdrawal may induce mitochondrial dysfunction in females.

To test the above possibility, we examined in the hippocampal CA1 region of Sparague-Dawley rats the mitochondrial ultrastructure, the ATP content and ATP synthesis at different phases after ovariectomy. Significantly, we observed a temporal alteration of mitochondrial ultrastructure and a transient lowering of ATP content and ATP synthesis in mitochondria within 12 days after ovariectomy (Xu et al. 2008; Shi et al. 2008). In addition, with genistein or EB as substitutes for endogenous estradiol, these alterations could be effectively prevented (Xu et al. 2008; Shi et al. 2008). However, these results only showed a temporal alteration in mitochondria after

ovariectomy, possibly due to the strong compensatory mechanisms of young rats (2–3 months old) that made a prompt response to estrogen withdrawal-induced mitochondrial damage. Therefore, in the present study, to determine whether aging can promote a prolonged mitochondrial dysfunction in hippocampi of OVX rats, we measured the COX activity and mitochondrial ATP content in hippocampi of OVX Wistar rats of 2 and 9-months of age. In addition, EB and genistein as substitutes of endogenous estradiol were given to the OVX rats. The results may contribute to better understanding of the mitochondrial mechanisms of neurodegeneration in postmenopausal women and the protective effects of genistein.

## Methods

### Experimental animals and drug administration

Two groups of 132 female Wistar rats aged 2 months (young) and 9 months (middle-aged), respectively, were provided by the animal facility of Sun-Yat Sen University. Before experiments, they were kept under conditions of constant temperature and humidity and with a 12 h light–12 h dark cycle. Rats in each age group were subdivided into thirteen subgroups; i.e., sham-operated, OVX-3,6,9,12,15,18 day; estradiol benzoate treated-12, 15, 18 day (OVX-EB-12, 15, 18 day); and genistein-treated-12, 15, 18 day (OVX-genistein-12, 15, 18 day) subgroups (for sham-operated and OVX-15, 18 day groups, $n=16$; for OVX-EB-15, 18 day and OVX-genistein-15, 18 day subgroups, $n=6$; for the other six subgroups, $n=10$). Half of the rats in each subgroup were used for COX activity measurement and the other half were for mitochondrial ATP content determination. The bilateral ovariectomy and drug administration were performed as described previously (Xu et al. 2007).

### COX activity measurement

Isolation of mitochondria from hippocampi and protein concentration determination were performed as described previously (Shi et al. 2008). COX activity was then measured using a commercially available cytochrome c oxidase assay kit (Sigma, USA).

### Mitochondrial ATP content measurement

Mitochondrial ATP content measurement was performed as described previously (Shi et al. 2008). Briefly, 2 μl of ice-cold 1.6 M perchloric acid was added to 1 ml mitochondrial suspension. The mixture was then centrifuged at 15,000 rpm for 5 min. The resulting supernatant was neutralized by adding an equal volume of saturated potassium hydroxide solution and then centrifuged at 15,000 rpm for 5 min. The mitochondrial ATP content was then measured with a reverse-phase high performance liquid chromatograph (Waters, USA).

### Statistical analysis

Statistical analysis was performed by SPSS 15.0 software. All data were submitted to a Kolmogorov-Smirnov test for normal distribution and one-way ANOVA for the comparison of population variance of all the groups. If significant differences were observed, the least significant difference t-test (LSD-t) was applied to multiple comparisons of means between each group.

## Results

### Change of COX activity in 2-month and 9-month old OVX rats

As shown in Fig. 1, after ovariectomy, COX activity in hippocampi of both 2-month and 9-month old rats was time-dependent. For 2-month old rats, after ovariectomy, COX activity increased on the 9th day, decreased significantly on the 12th day, and returned to normal on the 15th day (Fig. 1). In addition, no significant difference of COX activity between OVX-15 day and OVX-18 day groups was found suggesting that, COX activity in OVX young rats could be maintained at normal level after a temporal decrease (Fig. 1). In contrast, for 9-month old rats, after ovariectomy, COX activity declined significantly after peaking on the 9th day and was maintained at a low level during the remaining process of observation, since no significant difference of COX activity among OVX-12 day,

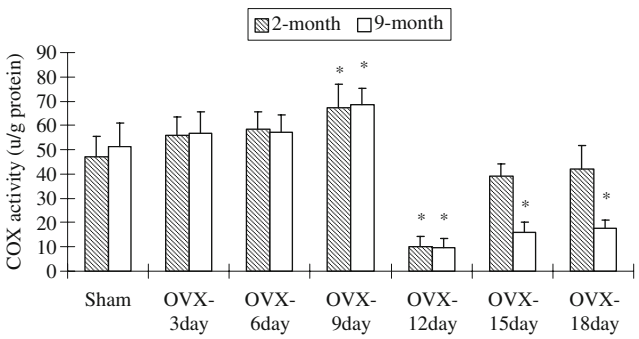


**Fig. 1** The hippicampi of 2 and 9 months old rats were isolated at different times after surgery as described under Methods. Then COX activity was assayed also as described under Methods. Differences were compared to a sham-operated group and found to be significant at $*p<0.01$

OVX-15 day and OVX-18 day groups could be found (Fig. 1).

In addition, for 2-month old OVX rats, a 12-day EB/genistein treatment could completely prevent the decrease of COX activity (Fig. 2), but no significant effects of 15-day or 18-day EB/genistein treatment on COX activity were observed (Figs. 3 and 4). By contrast, treatment of 9-month old OVX rats with EB/genistein for 12 days, 15 days or 18 days could effectively reverse the decrease of COX activity (Figs. 2, 3 and 4).

### Change of mitochondrial ATP content in 2-month and 9-month old rats after ovariectomy

After ovarietomy in both 2-month and 9-month old rats, the mitochondrial ATP content decreased in a time-dependent manner (Fig. 5). For the 2-month old rats, only a temporal decrease of mitochondrial ATP content on the 12th day was detected (Fig. 5). In addition, the mitochondrial ATP content of OVX-15 day and OVX-18 day groups was not statistically significantly different from that of the control group suggesting that, mitochondrial ATP content in young OVX rats could be maintained at a normal level after a temporal decrease. However, for 9-month old rats, mitochondrial ATP content declined following the 12th day after ovariectomy, and was maintained at a low level during the remaining process of observation. No significant difference of mitochondrial ATP content among OVX-12 day, OVX-15 day and OVX-18 day groups could be found (Fig. 5).

In addition, for 2-month old OVX rats, a 12-day EB/genistein treatment was able to reverse the decrease of mitochondrial ATP content completely (Fig. 6), but no significant effects of 15-day or 18-day EB/genistein treatment on mitochondrial ATP content were detected (Figs. 7 and 8). By contrast, treatment of 9-month old OVX rats with EB/genistein for 12 days, 15 days or 18 days could prevent a decrease of mitochondrial ATP content effectively (Figs. 6, 7 and 8).

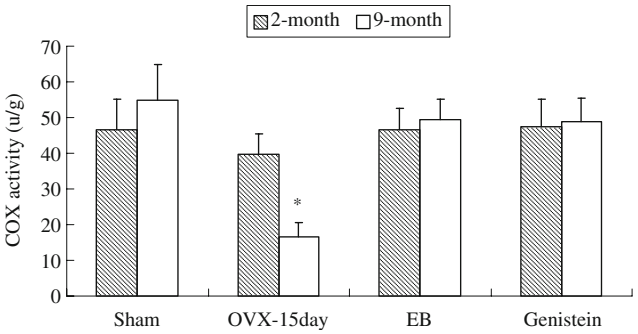


**Fig. 3** Rats (2 and 9 months old) were treated with or without EB/genistein from the second day after surgery, once a day for 15 days. Then the hippicampi was isolated and COX activity was measured as described under Methods. Differences were compared to a sham-operated group and found to be significant at $*p < 0.01$

## Discussion

Our previous and present studies suggested that after ovariectomy, there was a temporal mitochondrial dysfunction in hippocampi from young rats (2–3 month old). Decrease of COX activity and mitochondrial ATP content may be associated with lowering of antioxidant functions after estrogen withdrawal. After ovariectomy, the lowered antioxidant functions result in oxidative stress and lipid peroxidation (Borras et al. 2003; Ho et al. 2003; Xu et al. 2007). Oxidative stress may decrease mRNA levels for electron transport chain enzymes including COX and increase the mutation of these enzymes, consequently decreasing the ATP content (Aksenov et al. 1999; Borras et al. 2007).

However, after a temporal decrease, mitochondrial function in young rats could return to normal. Actually,

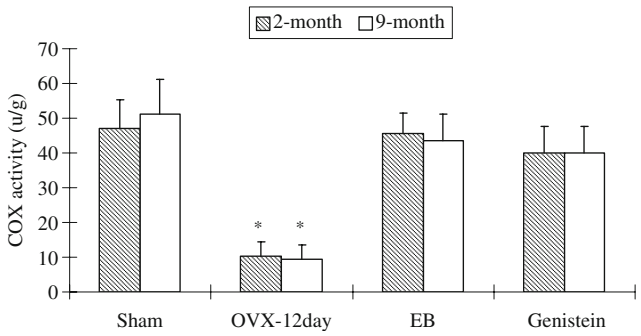


**Fig. 2** Rats (2 and 9 months old) were treated with or without EB/genistein from the second day after surgery once a day for 12 days. Then the hippicampi was isolated and COX activity was measured as described under Methods. Differences were compared to a sham-operated group and found to be significant at $*p < 0.01$

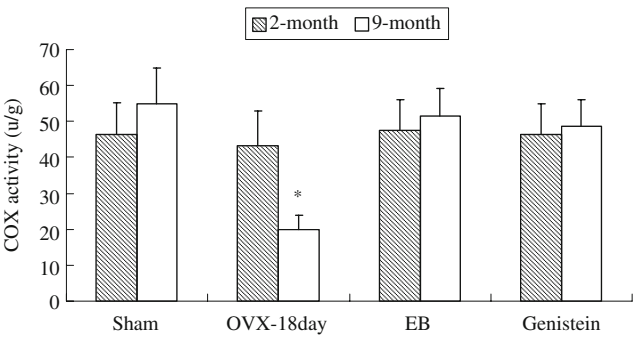


**Fig. 4** Rats (2 and 9 months old) were treated with or without EB/genistein from the second day after surgery, once a day for 18 days. Then the hippicampi was isolated and COX activity was measured as described under Methods. Differences were compared to a sham-operated group and found to be significant at $*p < 0.01$

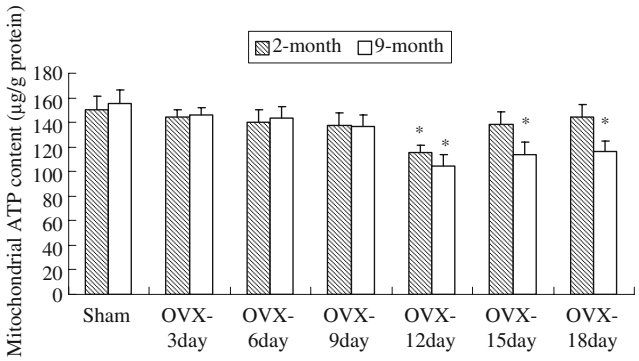


**Fig. 5** The hippicampi of 2 month and 9 month old rats were isolated at different times after surgery as described under Methods. Then, mitochondrial ATP content was assayed also as described under Methods. Differences were compared to a sham-operated group and found to be significant at $*p < 0.01$

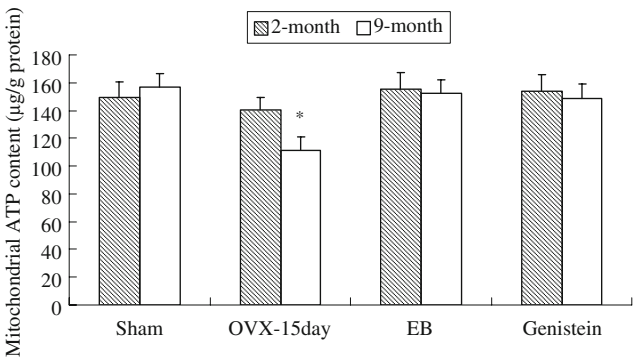


**Fig. 7** Rats (2 and 9 months old) were treated with or without EB/genistein from the second day after surgery, once a day for 15 days. Then the hippicampi was isolated and mitochondrial ATP content was measured as described under Methods. Differences were compared to a sham-operated group and found to be significant at $*p < 0.01$

there are several compensatory mechanisms that can compensate for the oxidative phosphorylation defects. A possible mechanism could be the existence of an excess of active respiratory chain complexes that could be used as a reserve to compensate for a deficit, i.e., an oxidative phosphorylation defect could be compensated by turning some inactive complexes into active ones (Rossignol et al. 2003). The second mechanism may be a direct chemical regulation of oxidative phosphorylation enzyme activity in response to a decrease in energy production (Rossignol et al. 2003). For instance, COX activity can be regulated by cAMP-dependent phosphorylation (Kadenbach et al. 2000) or ATP binding (Beauvoit and Rigoulet 2001). Theses two mechanisms were supported by this study showing that on the 9th day after ovariectomy, COX activity temporally increased to maintain the mitochondrial ATP content at normal level. The third mechanism may be the 'network attenuation' (Rossignol et al. 2003). This concept derives from metabolic control theory. According to this theory, at a given steady state rate of respiration, moderate inhibition of the activity of one respiratory chain complex leads to variations in the concentrations of intermediary metabolites (coenzyme Q, cytochrome c, electrochemical gradient of protons), which in turn modulates the activities of the other enzymes of the network (respiratory chain complexes) to maintain the respiratory flux unchanged (Kacser and Burns 1973; Heinrich and Rapoport 1974; Reder 1988). Additionally, compensation of defects in oxidative phosphorylation can also occur at the cellular level through stimulation of mitochondrial ATP production by the action of various hormones or neurotransmitters (Arendt et al. 1983; Assimacopoulos-Jeannet et al. 1986; Brand et al. 1990; Korzeniewski 2001; Rossignol et al. 2003). Still, at the tissue level, defects in oxidative phosphorylation can be further compensated for by an increase in oxygen delivery by the circulatory system via an acceleration of cardiac

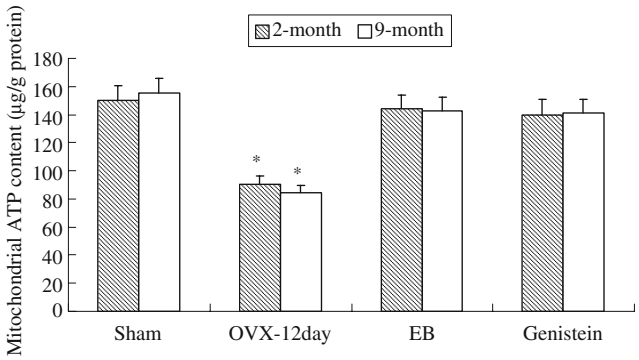


**Fig. 6** Rats (2 and 9 months old) were treated with or without EB/genistein from the second day after surgery once a day for 12 days. Then the hippicampi was isolated and mitochondrial ATP content was measured as described under Methods. Differences were compared to a sham-operated group and found to be significant at $*p < 0.01$

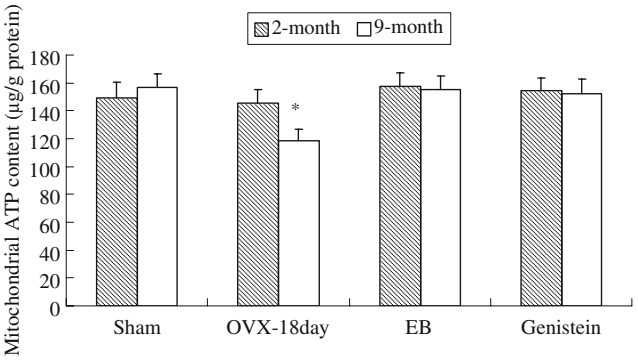


**Fig. 8** Rats (2 and 9 months old) were treated with or without EB/genistein from the second day after surgery, once a day for 18 days. Then the hippicampi was isolated and mitochondrial ATP content was measured as described under Methods. Differences were compared to a sham-operated group and found to be significant at $*p < 0.01$

output and breathing (Taivassalo et al. 2002; Rossignol et al. 2003).

Therefore, in this study, recovery of COX activity and mitochondrial ATP content in young OVX rats may mainly be associated with the allosteric modulation and control of COX synthesis (Abelenda and Puerta 1999). In addition, responses of endogenous hormones and neurotransmitters to estrogen withdrawal might be another major compensatory mechanism. It was found that, various endogenous hormones or neurotransmitters may regulate COX activity and mitochondrial ATP synthesis directly (e.g. vasopressin, glucagon, adrenaline) (Assimacopoulos-Jeannet et al. 1986; Brand et al. 1990) and indirectly via antioxidant actions (e.g. melatonin) (Arendt et al. 1983).

However, with aging, all these compensatory mechanisms may be attenuated, and thereby increase the vulnerability and susceptibility of cell/organ to various damages (Toescu 2005). For instance, in short-term cultures of hippocampal neurons, an N-methyl-D-aspartate (NMDA)-induced excitotoxic lesion resulted in a minimal degree of neuronal death in neurons derived from 3 day old animals, whereas more than 90% of the neurons obtained from 21 day old animals died (Marks et al. 2000), reproducing *in vitro* results obtained *in vivo* in rodents (Liu et al. 1996). In addition, Ventura et al. (2002) also found that, a similar decrease in complex I activity (between 30 and 50% inhibition) will lead to a dramatic decrease in the rate of mitochondrial respiration in old rats, while having no effect in young animals. In this study, young and middle-aged rats showed similar COX activity and mitochondrial ATP content in hippocampi. But after ovariectomy, COX activity and mitochondrial ATP content in hippocampi of middle-aged rats could only be maintained at a low level. Similarly, luminometric measurements of the ATP content and rate of ATP production in resting conditions using mitochondria acutely obtained from Fischer 344 rats showed no difference between adult (12 months) and old (24 months) animals (Drew and Leeuwenburgh 2003). Moreover, in measuring ATP from whole brain slices, little difference in ATP content between young and old cerebellar slices was observed, but following neuronal activity (45 min after a 5 min pulse of 75 mM KCl) the ATP content in the old slices (from 20 to 23 months old animals) was about 50% less than in the younger slices (12 months old) (Toescu 2005). These data suggested a decreased functional reserve of mitochondria during aging.

Notably, increased vulnerability, as that associated with aging, does not mean increased neuronal death (Toescu 2005). The process of aging is a continuous decrease of the homeostatic reserve, defined as the ability of cells to fight various metabolic stressors and maintain the cells/organs on the steady-state level of homeostasis (Toescu 2005). With age, acute surges of metabolic activities become more dangerous as they approach the limits of homeostatic reserve defenses, which underlies the age-dependent increase in vulnerability (Toescu 2005). The process of neurodegeneration, characterized by extensive neuronal death, becomes mostly manifest at the older ages, on the background of decreased homeostatic reserve, and could result either from an increased level of metabolic load, or from an accelerated reduction of the homeostatic reserve, or from a combination of both (Toescu 2005). As our previous results suggested that, mitochondrial dysfunction may be a mechanism underlying neurodegeneration in postmenopausal women (Xu et al. 2007; Xu et al. 2008; Shi et al. 2008). In the present study, we further showed that aging may catalyze this estrogen withdrawal-induced mitochondrial dysfunction.

On the other hand, our results also suggested that, EB/genistein treatment could prevent the estrogen withdrawal-induced mitochondrial dysfunction in hippocampi from both young and middle aged rats. EB/genistein may help to up-regulation of antioxidant enzymes by binding to the estrogen receptors and activating the mitogen activated protein (MAP) kinase and nuclear factor kappa B (NFkappaB) signalling pathways, thus offer protection to mitochondria against premature oxidative damage (Borras et al. 2007). In addition, EB/genistein may preserve ATP levels via increased oxidative phosphorylation and reduced ATPase activity followed by increased mitochondrial respiration via lowered oxidative load, increased transcript levels of the mitochondrial genome-encoded genes cytochrome oxidase subunits I, II and III, subsequently increasing the ATP content (Nilsen et al. 2004; Miquel et al. 2006). In addition, it has been found that phytoestrogen could reverse ATP depletion in the mitochondria with reduced glutamate-induced neurotoxicity (Lecanu et al. 2005). These findings suggest that, genistein, like estrogen, helps to recover mitochondrial energy metabolism.

However, our study also showed that, after 15 days or 18 days of EB/genistein treatment, no enhancement of mitochondrial function in young rats was observed. In addition, increase of COX activity induced by ovariectomy in young Wistar rats could be effectively reversed after 10 days of estradiol treatment (Abelenda and Puerta 1999). These results suggest to us that, modulation of the internal environment of cell/organism may contribute to the protective effect of EB/genistein on mitochondrial function. Estrogenic effects of EB/genistein may alleviate effects of estrogen withdrawal on mitochondrial function. But the detailed mechanisms underlying the protective action of EB/genistein have to be further explored.

In summary, the COX activity and mitochondrial ATP content in hippocampi from OVX Wistar rats of 2-months and 9-months of age were measured and the effects of genistein and EB tested. It was found compared with young

rats that middle-aged rats showed a prolonged lowering of COX activity and mitochondrial ATP content in their hippocampi after ovariectomy. This suggests that with aging the functional capacity of mitochondria decreases, likely because of less estrogen production. However, EB/genistein treatment prevented this estrogen withdrawal-induced mitochondrial dysfunction in hippocampi of young and middle aged rats. This suggests that a phyto-estrogen like genistein may be used as a substitute for estradiol to prevent central neurodegeneration in post-menopausal women.

**Acknowledgements** Many thanks for support from the GuangDong Provincial Natural Science Foundation (No. C04105332) and the Key Guidance Project of GuangDong Provincial Science and Technology Planning (No. 2005B33801004).

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