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Estradiol receptors mediate estradiol-induced inhibition of mitochondrial Ca^{2+} efflux in rat caudate nucleus and brain stem

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Abstract: Our earlier studies found that in vitro estradiol modulates mitochondrial Ca^{2+} transport in discrete brain regions. The present study examined the role of estradiol receptors (ERs) in estradiol-induced inhibition of Ca^{2+} efflux from synaptosomal mitochondria isolated from rat caudate nuclei and brain stems. Radioactively labeled $CaCl_2$ (0.6–0.75 μCi $^{45}CaCl_2$) was used for Ca^{2+} transport monitoring. The results revealed that in the presence of ER antagonist 7α,17β-[9[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol (ICI 182,780) (1 μmol/L), the inhibitory effect of estradiol on mitochondrial Ca^{2+} efflux was more than 60% decreased, suggesting the involvement of ER in this mode of estradiol neuromodulatory action. When particular contributions of ERα and ERβ were tested, it was found that ERβ agonist 2,3-*bis*(4-hydroxy phenyl)-propionitrile (10 nmol/L) inhibited Ca^{2+} efflux more than 20%, while the inhibition with ERα agonist 4,4',4"-(4-propyl-[1*H*]-pyrazole-1,3,5-triyl)*tris*phenol (10 nmol/L) was about 10%, both compared to the control. Both agonists demonstrated attenuation of Ca^{2+} efflux decrease in the presence of mitochondrial Na^+/Ca^{2+} exchanger antagonist 7-chloro-5-(2-chlorophenyl)-1,5-dihyhdro-4,1-benzothiazepin-2(3*H*)-one (10 μmol/L), showing interference with the inhibitory action of that agent. Our results strongly indicate ERs as the mediators of estradiol-induced mitochondrial Ca^{2+} efflux inhibition in rat caudate nucleus and brain stem synaptosomes.

Key words: Ca^{2+} efflux, estradiol receptors, synaptosomal mitochondria, caudate nucleus, brain stem

1. Introduction

Estradiol exerts its physiological function by 2 main pathways: genomic, via the transcription of certain target genes, and nongenomic, which is independent of direct gene activation (Stormshak and Bishop, 2008; Yu and Chaudry, 2009). While genomic effects are quite well described (Chen et al., 2004; Vasudevan and Pfaff, 2008), nongenomic estradiol effects are insufficiently known and studied. In different brain regions estradiol affects, in a nongenomic manner, the growth of dendritic branches, activity of GABA receptors, and the concentration of sodium and potassium ions (Kelly et al., 2002; Kow et al., 2006; Vasudevan and Pfaff, 2008). Modulation of synaptosomal and mitochondrial Ca^{2+} transport, previously reported from our laboratory, also relates to rapid, nongenomic methods of estradiol action (Nikezic et al., 1996, 1997; Horvat et al., 2000, 2001; Petrovic et al., 2005, 2011, 2012). The estradiol receptors (ERs) ERα and ERβ appear to be the key mediators of estradiol effects in the brain. They are primarily localized in the cytoplasm and nucleus, while small amounts of ER are associated with the cell membrane and organelles (Kisler et al., 2013). Both receptors are present in the mitochondrial matrix, being involved in different aspects of hormone influence. Acting through ERα, estradiol increases mitochondrial cytochrome c protein, mRNA, and mitochondrial aconitase activity in brain endothelial cells (Razmara et al., 2008). On the other hand, ERβ knockdown results in a lower resting mitochondrial membrane potential, an increase in resistance to oxidative stressors, and maintained ATP concentrations in primary hippocampal neurons (Yang et al., 2009). Results of our in vitro studies on mitochondria isolated from hippocampal synaptosomes strongly suggest the involvement of ER in estradiol-induced modulation of mitochondrial Ca^{2+} flux (Petrovic et al., 2011). However, obtained results do not exclude the possibility of direct hormone action through estrogen-specific binding sites (other than ERs) associated with mitochondrial membranes. We already reported that specific estradiol binding to synaptosomal mitochondria is involved in

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extragenomic hormone action on mitochondrial and synaptosomal content of Ca^{2+} (Horvat et al., 2001). In addition, estradiol showed beneficial effects on fatty acid composition of membrane phospholipids (Petrovic et al., 2014).

Calcium (Ca^{2+}) transport across the plasma membrane and membranes of subcellular organelles plays a fundamental role in neuronal activity and functions, such as synaptogenesis, neurite outgrowth, neurotransmitter release, synaptic plasticity, and cell differentiation and apoptosis. Among others, dopamine metabolism in the caudate nucleus (NC) and the development of the auditory brain stem also depend on Ca^{2+} flux regulation (Etou et al., 1996; Hirtz et al., 2011). In these regions, as well as in whole brain tissue, compromised Ca^{2+} homeostasis leads to neuronal dysfunction and cell death, being connected with multiple CNS disorders (Carafoli, 2002). Due to specific Ca^{2+} transport mechanisms, ruthenium red (RR)-sensitive uniporter for Ca^{2+} influx and mitochondrial Na^+/Ca^{2+} exchanger (mtNCX) for Ca^{2+} efflux mitochondria are organelles critical for Ca^{2+} buffering in neural cells. The large population of neuronal mitochondria can store and release relatively large amounts of calcium, leading to transient changes in the intracellular Ca^{2+} concentrations. Mitochondrial damage and disturbance of mitochondrial Ca^{2+} flux adversely affect the overall Ca^{2+} homeostasis in the brain (Szabadkai and Duchen, 2008).

The predominant Ca^{2+} efflux mechanism in synaptosomal mitochondria is the mtNCX. Estradiol was already found to inhibit mtNCX activity and consequently to decrease Ca^{2+} efflux from whole rat brain, hippocampal, NC, and brain stem (BS) synaptosomal mitochondria (Petrovic et al., 2012). Even more, the involvement of ER in Ca^{2+} efflux inhibition from hippocampal mitochondria has been confirmed (Petrovic et al., 2011). With this in mind, it was hypothesized that ER can be involved in transducing estradiol's inhibitory effects on mitochondrial Ca^{2+} efflux in 2 other brain regions, the NC and BS. Thus, the aim of this study was to examine if, and to what extent, ERs participate in estradiol-induced inhibition of mitochondrial Ca^{2+} efflux in rat NC and BS.

2. Materials and methods

2.1. Animals

Mature, chronically (3 weeks prior to use) ovariectomized female Wistar rats were used. The animals were obtained from the special animal breeding unit of our laboratory and maintained under constant conditions (lights on: 0500–1700 hours, temperature: 24 °C, free access to food and water). All procedures were approved by the Ethics Committee of the Serbian Association for the Use of Animals in Research and Education and were in accordance with the guidelines published in the 1996 NIH Guide for the Care and Use of Laboratory Animals and the principles presented in the 'Guidelines for the Use of Animals in Neuroscience Research by the Society for Neuroscience.

2.2. Isolation of synaptosomal mitochondria

After decapitation, whole brains were removed on ice. Synaptosomes were prepared from pools of NC and BS (10 NCs/pool and 10 BSs/pool). Purified synaptosomal mitochondria were isolated using a Ficoll gradient: 4.5% and 6% resolved in medium containing 0.24 mol/L mannitol, 60 mmol/L sucrose, 50 μmol/L EDTA, and 10 mmol/L Tris-HCl (pH 7.4), as described previously (Petrovic et al., 2011). Proteins were determined by the method of Lowry et al. (1951) as modified by Markwell et al. (1978).

2.3. Pharmacological agents

$^{45}CaCl_2$ (specific activity: 16.8 mCi/mg) was purchased from PerkinElmer (USA). Cellulose nitrate filters (pore size: 0.45 μm) were from Whatman International (USA).

7α,17β-[9[(4,4,5,5,5-Pentafluoropentyl)sulfinyl] nonyl] estra-1,3,5(10)-triene-3,17-diol (ICI 182,780), 4,4',4"-(4-propyl-[1*H*]-pyrazole-1,3,5-triyl)*tris*phenol (PPT), 2,3-*bis*(4-hydroxy phenyl)-propionitrile (DPN), and 7-chloro-5-(2-chlorophenyl)-1,5-dihyhdro-4,1-benzothiazepin-2(3*H*)-one (CGP) were obtained from Tocris Cookson Ltd. (USA). RR, 17β-estradiol (E2), 17β-estradiol conjugated to bovine serum albumin (E-BSA), and other chemicals were purchased from Sigma Chemical Co. (USA).

2.4. Mitochondrial Ca^{2+} transport

Ca^{2+} transport in mitochondria isolated from NC and BS synaptosomes was performed in the same conditions as already published (Petrovic et al., 2012). After preincubation at 23 °C for 10 min in a medium containing 300 mmol/L mannitol, 10 mmol/L KCl, 1 mmol/L malate, 5 mmol/L glutamate, and 10 mmol/l Tris-HCl (pH 7.4), mitochondria (0.2 mg protein/mL in a final volume of 300 μL) were loaded with Ca^{2+} in the presence of 0.2 mol/L $CaCl_2$ (0.6–0.75 μCi $^{45}CaCl_2$) for 5 min. Ca^{2+} influx was stopped by RR (17.5 μg/mg protein), a specific inhibitor of the mitochondrial Ca^{2+}-uniporter. Aliquots of 100 μL in size were vacuum-filtered on 0.45-μm pore-size cellulose-nitrate filters. After being washed twice with 3 mL of 0.25 M sucrose, the Ca^{2+} retained in mitochondria was calculated from radioactivity counting (corrected for blank without mitochondria and for nonspecific binding).

For Ca^{2+} efflux monitoring, mitochondria (0.2 mg protein/mL in the remaining volume of 200 μL) were incubated in the absence or presence of 0.5 nmol/L E2 or E-BSA (E2 concentration: 0.5 nmol/L) for an additional 10 min. Ca^{2+} efflux was initiated by adding 100 mmol/L NaCl and 0.2 mmol/L EDTA and lasted for 5 min.

The process was stopped with 2 mL of 0.25 M sucrose. Aliquots of 1 mL in size were vacuum-filtered and washed as described above. Na^+-dependent Ca^{2+} efflux was calculated by subtracting the Ca^{2+} concentration retained in mitochondria after Na^+/EDTA addition from the Ca^{2+} concentration in mitochondria after addition of RR.

The effects of the estrogen receptor antagonist (ICI 182,780 (1 μmol/L)), ERα agonist (PPT (10 nmol/L)), ERβ agonist (DPN (10 nmol/L)), and mitochondrial Na^+/Ca^{2+} exchanger specific antagonist (CGP (10 μmol/L)) on Na^+-dependent Ca^{2+} efflux in the presence and absence of E2 were measured by incubating Ca^{2+}-preloaded mitochondria with ICI 182,780 for 20 min, PPT and DPN for 10 min, or CGP for 3 min before Na^+/EDTA efflux initiation.

2.5. Statistical analysis

Changes in Ca^{2+} efflux observed after administering ICI 182,780, PPT, DPN, or CGP were assessed by one-way ANOVA followed by Tukey's post hoc test for intergroup comparison. The values represent the mean ± SEM of at least 3 experiments (with triplicate determinations). The differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Effect of ER antagonist ICI 182,780 on mitochondrial Ca^{2+} efflux inhibition

The effects of ER antagonist ICI 182,780 on the inhibitory effect of E2 and E-BSA on Ca^{2+} efflux from NC and BS synaptosomal mitochondria are shown in Figure 1. In control conditions, Ca^{2+} efflux from NC mitochondria was 4.25 ± 0.28 nmol/mg protein. In the presence of E2, Ca^{2+} efflux was reduced to 2.51 ± 0.18 nmol/mg protein ($P < 0.001$, when comparing E2 to the control) and in the presence of E-BSA to 3.36 ± 0.26 nmol/mg protein ($P < 0.001$ when comparing E-BSA to the control). Thus, E2 inhibited Ca^{2+} efflux from NC mitochondria by 41% and E-BSA by 21%. When mitochondria were pretreated with ICI 182,780 before E2 or E-BSA addition, an increase of Ca^{2+} efflux could be seen when compared to E2 or E-BSA alone. Ca^{2+} efflux measured in the presence of ICI 182,780 and E2 was 3.62 ± 0.26 nmol/mg protein ($P < 0.001$ when compared ICI + E2 to E2 alone) and in the presence of ICI 182,780 and E-BSA was 4.08 ± 0.20 nmol/mg protein ($P < 0.001$ when comparing ICI + E-BSA to E-BSA alone). Thus, pretreatment with ICI 182,780 decreased E2- and E-BSA-induced inhibition of Ca^{2+} efflux from NC mitochondria by 64% and 81%, respectively. In the BS mitochondria, control Ca^{2+} efflux was 2.63 ± 0.22 nmol/mg protein, while E2 reduced this efflux to 1.66 ± 0.20 nmol/mg protein ($P < 0.001$ when comparing E2 to the control) and E-BSA to 2.18 ± 0.13 nmol/mg protein ($P < 0.001$ when comparing E-BSA to the control). Thus, E2 inhibited Ca^{2+} efflux from

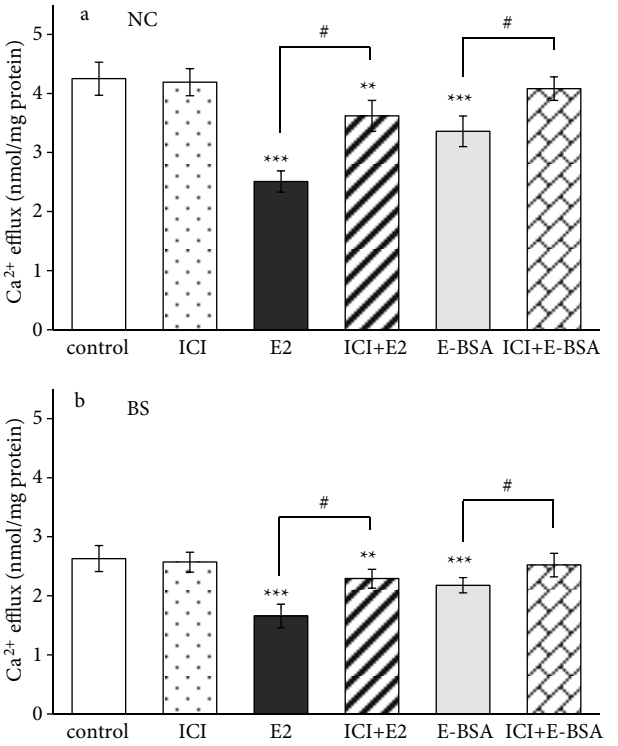

Figure 1. Prevention of E2 and E-BSA inhibitory action on Ca^{2+} efflux by ER antagonist ICI 182,780: a) in NC mitochondria, b) in BS mitochondria. Ca^{2+}-preloaded mitochondria were incubated for 20 min in the absence or presence of 1 μmol/L ER antagonist ICI 182,780 at 23 °C. Incubation continued for an additional 10 min in the absence or presence of 0.5 nmol/L E2 or E-BSA (E2 concentration 0.5 nmol/L). Efflux was measured after the addition of 100 mmol/L NaCl and 0.2 mmol/l EDTA. The amount of released Ca^{2+} was estimated as indicated in Section 2. One-way ANOVA followed by Tukey's post hoc test showed significant decrease of Ca^{2+} efflux from mitochondria treated with E2 or E-BS when compared to the control (***$P < 0.001$) and significant increase of Ca^{2+} efflux when mitochondria were treated with ICI 182,780 before E2 or E-BSA addition ($^{\#}P < 0.001$, when comparing ICI + E2 to E2, and ICI + E-BSA to E-BSA). The results represent the mean ± SEM of 3 experiments (with triplicate determinations).

BS mitochondria by 37% and E-BSA by 18%. As in NC mitochondria, pretreatment of BS mitochondria with ICI 182,780 before E2 or E-BSA addition elevated Ca^{2+} efflux when compared to E2 or E-BSA alone. In the presence of ICI 182,780 and E2, Ca^{2+} efflux was 2.29 ± 0.16 nmol/mg protein ($P < 0.001$ when comparing ICI + E2 to E2 alone), and in the presence of ICI 182,780 and E-BSA, Ca^{2+} efflux was 2.52 ± 0.20 nmol/mg protein ($P < 0.001$ when comparing ICI + E-BSA to E-BSA alone). Thus, ER antagonist ICI 182,780 diminished the inhibitory effect of E2 on Ca^{2+} efflux from BS mitochondria by 65% and E-BSA by 76%. Such an effect of ER antagonist strongly suggests

the involvement of ER in E2 and E-BSA inhibitory action on mitochondrial Ca^{2+} efflux.

3.2. Effects of ERα agonist PPT and ERβ agonist DPN on mitochondrial Ca^{2+} efflux inhibition

To test the assumption about the involvement of ER in Ca^{2+} efflux inhibition and to examine the respective roles of ERα and ERβ, mitochondrial Ca^{2+} efflux was measured in the presence of a PPT-specific agonist of ERα and a DPN-specific agonist of ERβ (Figure 2). PPT decreased Ca^{2+} efflux from NC mitochondria by 10% (control Ca^{2+} efflux: 4.71 ± 0.26 nmol/mg protein; in the presence of PPT: 4.23 ± 0.17 nmol/mg protein; $P < 0.05$) and from BS mitochondria by 15% (control Ca^{2+} efflux: 2.36 ± 0.15 nmol/mg protein; in the presence of PPT 2.01 ± 0.13 nmol/mg protein; $P < 0.01$). The inhibitory effect of DPN was even stronger, reducing Ca^{2+} efflux from NC mitochondria by 25% (Ca^{2+} efflux in the presence of DPN 3.53 ± 0.1 Ca^{2+}/mg protein, $P < 0.001$ when compared to control) and from BS mitochondria by 21% (Ca^{2+} efflux in the presence of DPN 1.86 ± 0.17 Ca^{2+}/mg protein, $P < 0.001$ when compared to control). In mitochondria from both brain regions, NC and BS, the separate inhibitory potential of PPT or DPN was significantly lower when compared to E2, at $P < 0.001$. A significant difference between Ca^{2+} efflux in the presence of PPT and DPN was observed in NC mitochondria ($P < 0.001$).

3.3. Effect of specific inhibitor of mitochondrial Na^+/Ca^{2+} exchanger CGP

In addition, both ER agonists expressed their effects in experiments with CGP, the specific inhibitor of mtNCX (Figure 3). CGP inhibited Ca^{2+} efflux from NC mitochondria by 94% (control Ca^{2+} efflux: 4.46 ± 0.22 nmol/mg protein; in the presence of CGP: 0.27 ± 0.045 nmol/mg protein; $P < 0.001$) and from BS mitochondria by 92% (control Ca^{2+} efflux: 2.51 ± 0.24 nmol/mg protein; in the presence of CGP 0.20 ± 0.075 nmol/mg protein; $P < 0.001$). On the other hand, the incubation of mitochondria with ERβ agonist DPN before the addition of CGP increased Ca^{2+} efflux from NC mitochondria by 2.3-fold when compared with CGP alone. In the presence of DPN and CGP, Ca^{2+} efflux was 0.62 ± 0.057 nmol/mg protein ($P < 0.001$ when comparing DPN + CGP to CGP alone). Incubation of BS mitochondria with DPN before the addition of CGP increased Ca^{2+} efflux by 3.2-fold (0.64 ± 0.081 nmol/mg protein) when compared with CGP alone ($P < 0.001$ when comparing DPN + CGP to CGP). ERα agonist PPT was less effective, with a 1.5-fold Ca^{2+} efflux increase from NC mitochondria, in the presence of CGP (0.4 ± 0.091 nmol/mg protein, $P < 0.001$ when comparing PPT + CGP to CGP) and almost no increase of efflux from BS mitochondria when compared with CGP alone. In mitochondria from both regions, a significant difference between Ca^{2+} efflux in the presence of PPT + CGP and DPN + CGP was observed at $P < 0.001$.

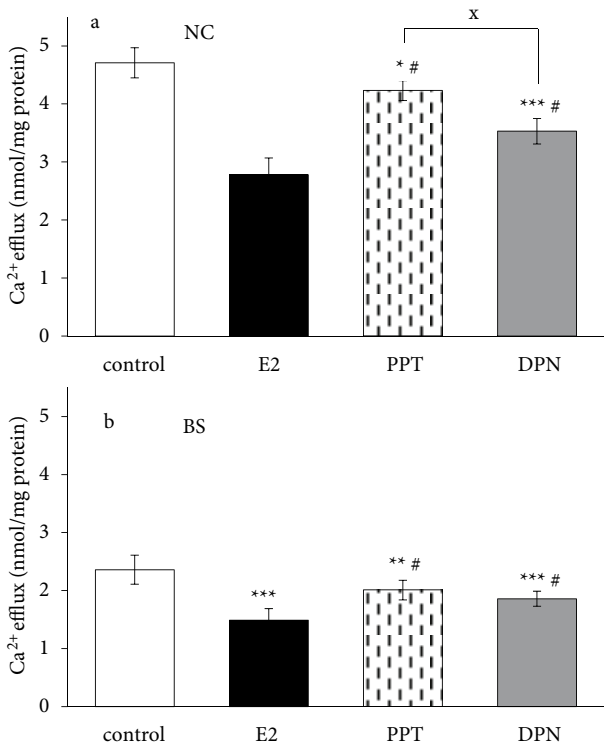

Figure 2. Involvement of ERα and/or ERβ in Ca^{2+} efflux modulation: a) in NC mitochondria, b) in BS mitochondria. Ca^{2+}-preloaded mitochondria were incubated for 10 min in the absence or presence of 0.5 nmol/L E2, 10 nmol/L selective ERα agonist PPT, or 10 nmol/L selective ERβ agonist DPN at 23 °C. Efflux was measured after the addition of 100 mmol/L NaCl and 0.2 mmol/L EDTA. The amount of released Ca^{2+} was estimated as indicated in Section 2. One-way ANOVA followed by Tukey's post hoc test showed significant decrease of Ca^{2+} efflux from mitochondria treated with E2, PPT, or DPN when compared to the control (for NC mitochondria the significance was ***$P < 0.001$, **$P < 0.05$, *$P < 0.001$, respectively, and for BS mitochondria ***$P < 0.001$, **$P < 0.01$, *$P < 0.001$, respectively); significantly different Ca^{2+} efflux from PPT- or DPN-treated mitochondria when compared to E2-treated mitochondria ($^{\#}P < 0.001$); and significant decrease of Ca^{2+} efflux from DPN-treated NC mitochondria when compared to PPT-treated NC mitochondria ($^{X}P < 0.001$). The results represent the mean ± SEM of 3 experiments (with triplicate determinations).

4. Discussion

In the present study, the role of mitochondrial ER in E2-induced inhibition of Ca^{2+} efflux from synaptosomal mitochondria isolated from rat brain NCs and BSs was evaluated by using specific ER antagonist ICI 182,780 and agonists PPT and DPN. We reported for the first time that both ERα and ERβ are involved in the inhibitory effect of E2 on Ca^{2+} efflux from NC and BS synaptosomal mitochondria.

Estradiol is well known to affect different aspects of mitochondrial function. By promoting mitochondrial

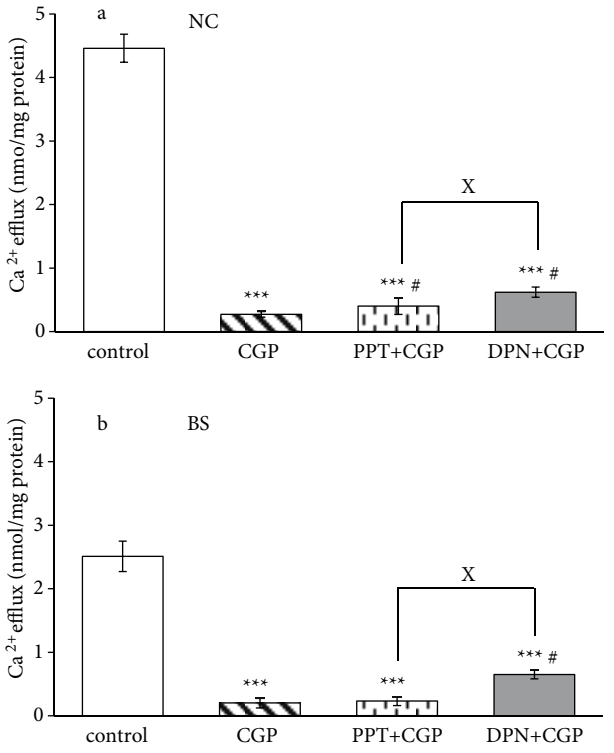

Figure 3. Involvement of ERα and/or ERβ in Ca^{2+} efflux modulation by mitochondrial Na^+/Ca^{2+} exchanger inhibitor CGP: a) in NC mitochondria, b) in BS mitochondria. Ca^{2+}-preloaded mitochondria were incubated for 10 min in the absence or presence of 10 nmol/L selective ERα agonist PPT or 10 nmol/L selective ERβ agonist DPN at 23 °C. Incubation continued for an additional 3 min in the absence or presence of 10 μmol/L CGP. Efflux was measured after the addition of 100 mmol/L NaCl and 0.2 mmol/L EDTA. The amount of released Ca^{2+} was determined as indicated in Section 2. One-way ANOVA followed by Tukey's post hoc test showed significant decrease of Ca^{2+} efflux from mitochondria treated with CGP (***$P < 0.001$, when compared to the control), significant increase of Ca^{2+} efflux from mitochondria treated with PPT (only in NC mitochondria) or DPN before CGP addition when compared to CGP alone ($^{\#}P < 0.001$), and significant increase of Ca^{2+} efflux from DPN + CGP-treated mitochondria when compared to PPT + CGP-treated mitochondria ($^{X}P < 0.001$). The results represent the mean ± SEM of 4 experiments (with triplicate determinations).

energetic efficiency, ATP supply, free radical defense system, and maintenance of Ca^{2+} homeostasis, E2 contributes to the preservation of mitochondrial ultrastructure and membrane potential (Simpkins et al., 2009; Richardson et al., 2012; Sastre-Serra et al., 2013). Even more, E2 is known to reverse the damage of neural cell mitochondria structure and ATP production caused by gonadal hormone deficiency (Xu et al., 2008). Since many of E2's neuroprotective mechanisms converge upon mitochondria (Nilsen et al., 2003), any insight into the pathways of E2 effects on mitochondrial function, including their ability to accumulate and release Ca^{2+}, could be of particular interest. As we previously reported, 17β-estradiol acting in a nongenomic manner inhibits Ca^{2+} efflux from whole rat brain synaptosomal mitochondria, as well as from synaptosomal mitochondria isolated from specific brain regions: the hippocampus, NC, and BS (Horvat et al., 2001; Petrovic et al., 2011, 2012). There are 2 possible mechanisms of observed estradiol action: one going through ERα/β localized in the mitochondrial matrix (Chen et al., 2004; Psarra and Sekeris, 2009) and the other, non-ER mediated, going through estrogen-specific binding sites associated with mitochondrial membranes (Moss et al., 1997). Our recent results on hippocampal mitochondria speaking in favor of ER's role in E2-induced inhibition of mitochondrial Ca^{2+} efflux in this brain region suggest that ER could be involved in transduction of E2 effects in 2 other brain regions, the NC and BS.

As expected, when isolated NC and BS synaptosomal mitochondria were pretreated with ER antagonist ICI 182,780 to prevent E2 binding to ER, the inhibitory effects of E2 and E-BSA on Ca^{2+} efflux were significantly decreased (65%–80%), highlighting an important role for ER in E2-induced modulation of mitochondrial Ca^{2+} flux. As seen in the literature, ICI 182,780 is a potent inhibitor of E2 action in the nervous system. Among others, treatment with ICI 182,780 blocks E2's effects on $P2X_3$ receptor activity in sensory neurons (Ma et al., 2005), Akt1 activity in hippocampal CA1 neurons (Yang et al., 2010), mitochondrial superoxide production in brain endothelial cells (Razmara et al., 2008), and mitochondrial dysfunction in astrocytes (Guo et al., 2012). Still, in our study, E2 and E-BSA kept between 20%–35% of their inhibitory potential even in the presence of ICI 182,780, suggesting that E2-inhibition of mitochondrial Ca^{2+} efflux in the NC and BS could at least partially be due to hormone action through membrane binding-sites other than ERs. As previously reported, E2 binds to an oligomycin-sensitive subunit of the mitochondrial enzyme F_0F_1-ATPase (Ramirez et al., 2001). Thus, by binding to such membrane-binding sites near mtNCX, E2 may indirectly disrupt the function of that transport protein.

However, our experiments with selective ERα agonist PPT and ERβ agonist DPN confirmed the critical role of ER in E2 action observed in this study. Both agonists mimicked the inhibitory effect of E2 on Ca^{2+} efflux from NC and BS mitochondria, although they were less effective. On the other hand, DPN exerted stronger inhibitory potential than PPT, suggesting the dominant role of ERβ. In line with our results, ERβ mediated E2 reduction due to ischemic damage in the NC of ovariectomized mice (Carswell et al., 2004), while its agonist DPN decreased the nitric oxide-mediated hypotensive effects of E2 in

the rostral ventrolateral medulla of the BS (Shih, 2009). Moreover, DPN enhances and PPT reduces ATP-evoked response in nociceptive trigeminal BS neurons (Tashiro et al., 2012). Further results in favor of ERβ were obtained in experiments with selective mtNCX inhibitor CGP. In our model system, Ca^{2+} efflux inhibition by CGP was almost complete (>90%) when compared to the control. In contrast to Ca^{2+} efflux inhibition by E2 in physiological conditions, pretreatment with E2 significantly increased mitochondrial Ca^{2+} efflux in the presence of CGP (Petrovic et al., 2012). Moreover, in the current study, in mitochondria isolated from both brain regions, DPN protected mtNCX activity against CGP's inhibitory action in the same manner as E2, while ERα agonist PPT had a weaker effect on NC and no effect on BS mitochondria.

The obtained results indicate for the first time that ERα and ERβ participate in E2-induced modulation of Ca^{2+} efflux from NC and BS synaptosomal mitochondria. A dominant role of ERβ has been shown in mitochondria from both brain regions. Since the experiments were performed in vitro on isolated mitochondria, these findings represent new evidence about genome-independent E2 effects in specific brain regions.

In summary, our study has shed light on the pathways through which estradiol regulates mitochondrial Ca^{2+} sequestration and consequently global cell Ca^{2+} homeostasis in the NC and BS. The role of ER has been confirmed. Considering the vital role of Ca^{2+} in regulation of total neural cell activity, from propagation of nerve impulse to cell metabolism and apoptosis, the effects on mitochondrial Ca^{2+} flux via ER could be the one of the important routes of estradiol neuromodulatory action in the brain.

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References

Carafoli E (2002). Calcium signaling: a tale for all seasons. P Natl Acad Sci USA 99: 1115–1122.

Carswell HVO, Macrae IM, Gallagher L, Harrop E, Horsburgh KJ (2004). Neuroprotection by a selective estrogen receptor beta agonist in a mouse model of global ischemia. Am J Physiol Heart C 287: H1501–1504.

Chen JQ, Delannoy M, Cooke C, Yager JD (2004). Mitochondrial localization of ERα and ERβ in human MCF7 cells. Am J Physiol Endoc M 286: E1011–1022.

Etou K, Kuroki T, Tatebayashi Y, Tashiro N, Hirano M (1996). Effects of calcium antagonists nifedipine and flunarizine on phencyclidine-induced changes in the regional dopaminergic metabolism of the rat brain. Biochem Pharmacol 51: 83–86.

Guo J, Duckles SP, Weiss JH, Li X, Krause DN (2012). 17β-Estradiol prevents cell death and mitochondrial dysfunction by an estrogen receptor-dependent mechanism in astrocytes after oxygen-glucose deprivation/reperfusion. Free Radical Biol Med 52: 2151–2160.

Hirtz JJ, Boesen M, Braun N, Deitmer JW, Kramer F, Lohr C, Muller B, Nothwang HG, Striessnig J, Lohrke S et al. (2011). Cav1.3 calcium channels are required for normal development of the auditory brainstem. J Neurosci 31: 8280–8294.

Horvat A, Nikezic G, Petrovic S, Kanazir DT (2001). Binding of estradiol to synaptosomal mitochondria: physiological significance. Cell Mol Life Sci 58: 636–644.

Horvat A, Petrovic S, Nedeljkovic N, Martinovic JV, Nikezic G (2000). Estradiol affects Na-dependent Ca^{2+} efflux from synaptosomal mitochondria. Gen Physiol Biophys 19: 59–71.

Kelly MJ, Ronnekleiv OK, Ibrahim N, Lagrange AH, Wagner EJ (2002). Estrogen modulation of K^+ channel activity in hypothalamic neurons involved in the control of the reproductive axis. Steroids 67: 447–456.

Kisler K, Chow RH, Dominguez R (2013). Fluorescently-labeled estradiol internalization and membrane trafficking in live N-38 neuronal cells visualized with total internal reflection fluorescence microscopy. J Steroids Hormon Sci S12: 002.

Kow LM, Devidze N, Pataky S, Shibuya I, Pfaff DW (2006). Acute estradiol application increases inward and decreases outward whole-cell currents of neurons in rat hypothalamic ventromedial nucleus. Brain Res 1116: 1–11.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275.

Ma B, Rong W, Dunn PM, Burnstock G (2005). 17β-Estradiol attenuates α, β-meATP-induced currents in rat dorsal root ganglion neurons. Life Sci 76: 2547–2558.

Markwell MA, Haas SM, Bieber LL, Tolbert NE (1978). A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal Biochem 87: 206–210.

Moss RL, Gu Q, Wong M (1997). Estrogen: nontranscriptional signaling pathway. Recent Prog Horm Res 52: 33–69.

Nikezic G, Horvat A, Nedeljkovic N, Martinovic JV (1996). 17β-Estradiol in vitro affects Na-dependent and depolarization-induced Ca^{2+} transport in rat brain synaptosomes. Experientia 52: 217–220.

Nikezic G, Nedeljkovic N, Horvat A, Kanazir D, Martinovic JV (1997). Estradiol in vitro modulates Na^+-dependent Ca^{2+} uptake by synaptic plasma membrane vesicles of rat brain regions. J Endocrinol Invest 20: 664–668.

Nilsen J, Brinton RD (2003). Mechanism of estrogen-mediated neuroprotection: regulation of mitochondrial calcium and Bcl-2 expression. P Natl Acad Sci USA 100: 2842–2847.

Petrovic S, Demajo M, Horvat A (2005). Estradiol affects calcium transport across mitochondrial membrane in different brain regions. Ann NY Acad Sci 1048: 341–343.

Petrovic S, Milosevic M, Drakulic D, Grkovic I, Stanojlovic M, Mitrovic N, Horvat A (2012). 17β-Estradiol modulates mitochondrial Ca^{2+} flux in rat caudate nucleus and brain stem. Neuroscience 220: 32–40.

Petrovic S, Takic M, Arsic A, Vucic V, Drakulic D, Milosevic M, Horvat A (2014). Effect of sex hormones on plasma phospholipid fatty acid composition in intact rats and rats with bilaterally occluded carotid arteries. Physiol Res 63: 331–339.

Petrovic S, Velickovic N, Stanojevic I, Milosevic M, Drakulic D, Stanojlovic, M, Horvat A (2011). Inhibition of mitochondrial Na^{+}-dependent Ca^{2+} efflux by 17 beta-estradiol in the rat hippocampus. Neuroscience 192: 195–204.

Psarra AM, Sekeris CE (2009). Glucocorticoid receptors and other nuclear transcription factors in mitochondria and possible functions. Biochim Biophys Acta 1787: 431–436.

Ramirez VD, Kipp JL, Joe I (2001). Estradiol, in the CNC, targets several physiologically relevant membrane- associated proteins. Brain Res Rev 37: 141–152.

Razmara A, Sunday L, Stirone C, Wang XB, Krause DN, Duckles SP, Procaccio V (2008). Mitochondrial effects of estrogen are mediated by estrogen receptor alpha in brain endothelial cells. J Pharmacol Exp Ther 325: 782–790.

Richardson TE, Yu AE, Wen Y, Yang S, Simpkins JW (2012). Estrogen prevents oxidative damage to the mitochondria in Friedreich's ataxia skin fibroblasts. PLoS One 7: e34600.

Sastre-Serra J, Nadal-Serrano M, Pons DG, Roca P, Oliver J (2013). The over-expression of ERβ modifies estradiol effects on mitochondrial dynamics in breast cancer cell line. Int J Biochem Cell B 45: 1509–1515.

Shih C (2009). Activation of estrogen receptor β-dependent nitric oxide signaling mediates the hypotensive effects of estrogen in the rostral ventrolateral medulla of anesthetized rats. J Biomed Sci 16: 60.

Simpkins JW, Yi KD, Yang SH (2009). Role of protein phosphatases and mitochondria in the neuroprotective effects of estrogens. Front Neuroendocrin 30: 93–105.

Stormshak F, Bishop CV (2008). Board-invited review: Estrogen and progesterone signaling: genomic and nongenomic actions in domestic ruminants. J Anim Sci 86: 299–315.

Szabadkai G, Simoni AM, Bianchi K, De Stefani D, Leo S, Wieckowski MR, Rizzuto R (2006). Mitochondrial dynamics and Ca2+ signaling. Biochim Biophys Acta 1763: 442–449.

Vasudevan N, Pfaff DW (2008). Non-genomic actions of estrogens and their interaction with genomic actions in the brain. Front Neuroendocrin 29: 238–257.

Xu XW, Shi C, He ZQ, Ma CM, Chen MM, Shen YP, Guo Q, Shen CJ, Xu J (2008). Effects of phytoestrogen on mitochondrial structure and function of hippocampal CA1 region of ovariectomised rats. Cell Mol Neurobiol 28: 875–886.

Yang LC, Zhang QG, Zhou CF, Yang F, Zhang YD, Wang RM, Brann DW (2010). Extranuclear estrogen receptors mediate the neuroprotective effects of estrogen in the rat hippocampus. PLoS One 5: e9851.

Yang SH, Sarkar SN, Liu R, Perez EJ, Wang X, Wen Y, Yan LJ, Simpkins JW (2009). Estrogen receptor beta as a mitochondrial vulnerability factor. J Biol Chem 284: 9540–9548.

Yu H, Chaudry IH (2009). The role of estrogen and receptor agonists in maintaining organ function after trauma-hemorrhage. Shock 31: 227–237.