CrossMark

ORIGINAL RESEARCH

Repeated Estradiol Treatment Attenuates Chronic Cerebral Hypoperfusion-Induced Neurodegeneration in Rat Hippocampus

Miloš Stanojlović[1] · Ivana Guševac[1] · Ivana Grković[1] · Nataša Mitrović[1] ·
Jelena Zlatković[1] · Anica Horvat[1] · Dunja Drakulić[1]

Abstract Although a substantial number of pre-clinical and experimental studies have investigated effects of 17β-estradiol, its precise molecular mechanism of action in the early state of chronic cerebral hypoperfusion remains controversial. The present study attempted to verify whether post-ischemic estradiol treatment (33.3 µg/kg for seven consecutive days) affects previously reported number of hippocampal apoptotic cells and amount of DNA fragmentation characteristic for apoptosis as well as the expression of key elements within synaptosomal Akt and Erk signal transduction pathways (NF-κB, Bax, Bcl-2, cytochrome C, caspase 3, and PARP). Additionally, alterations of aforementioned molecules linked to protection in various neurodegenerative disorders were monitored in the cytosolic, mitochondrial, and nuclear fractions associating investigated kinases and NF-κB with gene expression of their downstream effectors—Bcl-2, Bax, and caspase 3. The results revealed that an initial increase in the number of apoptotic cells and amount of DNA fragmentation induced by chronic cerebral hypoperfusion was significantly reduced by 17β-estradiol. In synaptic regions, an altered profile with respect to the protein expression of Bcl-2 and phosphorylated Akt was detected, although the level of other examined proteins was not modified. In other investigated sub-cellular fractions, 17β-estradiol elicited phosphorylation and translocation of Akt and Erk along with modulation of the expression of their subsequent effectors. Our findings support the concept that repeated post-ischemic 17β-estradiol treatment attenuates neurodegeneration induced by chronic cerebral hypoperfusion in hippocampus through the activation of investigated kinases and regulation of their downstream molecules in sub-cellular manner indicating a time window and regime of its administration as a valid therapeutic intervention.

Keywords Estradiol · Neuroprotection · Chronic cerebral hypoperfusion · Hippocampus

Introduction

Aside from traditional performance as regulator and maintainer of female reproductive functions, and due to its lipophilic nature, estradiol easily diffuses across cellular membranes and elicits its effects in various tissues including cardiovascular, immune and central nervous systems (Petrone et al. 2014). Within the brain, one of the prime neuronal targets for its action is the hippocampus, which comprises three types of estrogen receptors (ER): ERα, ERβ, and GPR30 (G-protein coupled receptor) (Arevalo et al. 2015; Brann et al. 2012; Liu et al. 2009; Manthey and Behl 2006). Its gradual genomic (classical) or/and rapid non-genomic effects emerge due to modulation of ligand-dependent ER signaling pathways and thus the expression and activity of various downstream molecules. In the non-genomic pathway that occurs within seconds or minutes, a ligand-bounded ER complex can remain at the plasma membrane (GPR30) or in the cytoplasm (ERα and ERβ) and acts as a signaling molecule through inhibition of caspase 3 and caspase 8 activity and activation of caspase 12 (Ma et al. 2013; Jia et al. 2009; Jover et al. 2002), protein kinase B (PKB/Akt) and mitogen-activated kinases (MAPK/Erk) (Brann et al. 2012;

✉ Dunja Drakulić
drakulic@vinca.rs

[1] Department of Molecular Biology and Endocrinology, VINCA Institute of Nuclear Sciences, University of Belgrade, P.O.Box 522, Belgrade 11001, Republic of Serbia

Springer

Jover-Menguala et al. 2010; Yang et al. 2010), etc. In addition to the non-genomic pathway, following various types of insults, estradiol can transmit signal through genomic mechanism when both ERα and ERβ serve as ligand-activated transcription factors, form dimmers, and translocate into the nucleus. In such manner, it increases the expression of several proteins involved in cell survival, including phosphoinositide 3 kinase (PI3K) (Wang et al. 2006), Bcl-2 (Choi et al. 2004; Dubal et al. 1999; Singer et al. 1998), Bcl-x (Stoltzner et al. 2001), NF-κB (Bondeau et al. 2001), PARP (McCullough et al. 2005), and reduces expression of molecules involved in cell death-like Bax and subsequently decreases release of cytochrome C (Choi et al. 2004).

Protective properties of this female sexual steroid hormone in ovariectomized female (Dubal et al. 1998; Simpkins et al. 1997), young and middle-aged animals (Dubal and Wise 2001), in vivo models of focal cerebral ischemia (Manthey and Behl 2006), as well as various neurodegenerative disorders including traumatic brain injury, stroke, Alzheimer's disease have been previously documented (Garcia-Segura et al. 2001). Its neuroprotective actions are associated with the activation of NADPH oxidase, reduction of oxidative stress, regulation of superoxide and reactive oxygen species (ROS) generation (Brann et al. 2012), suppressive actions on inflammatory processes via TNFα and NF-κB (Koellhoffer and McCullough 2013) or enhancing the neurogenesis (Li et al. 2011), etc. Our recently published study implicates estradiol treatment with the absence of apoptotic cascade activation in prefrontal cortex of adult male rats submitted to chronic cerebral hypoperfusion as well as reduction of DNA fragmentation and number of degenerating neurons through the initiation of Akt and Erk signaling pathways and regulation of their downstream effectors (Stanojlovic et al. 2015). However, opposite to its widely acknowledged neuroprotective role in cerebral ischemia, some reports indicate negative outcome or no effect of estradiol treatment in this type of brain injury (Lobo 2013; Grodstein et al. 2008; Bingham et al. 2005; Gordon et al. 2005; Harukuni et al. 2001). The discrepancy between neuroprotective effects versus ineffective or even negative outcomes may lie in the type and severity of the insult, timing of hormone administration, duration of treatment, manner of application (Sherwin 2009), gender (Gibson 2013), etc.

Our previous report indicated that modulations in proapoptotic biochemical cascades triggered in synaptic terminals could provoke time-dependent neurodegeneration in hippocampus of adult male rats (Stanojlović et al. 2014b), while estradiol was sufficient to exert profound protective effects in prefrontal cortex in ischemic brain (Stanojlović et al. 2015). Hence, the aim of the current study was to evaluate the impact of chronic post-ischemic estradiol treatment in the regime of repeated administration on hippocampal complex apoptotic signaling pathways and cell survival. Conducted experiments provide observations of protein expression, distribution, and activation not only in synaptosomal but also in cytosolic, nuclear, and mitochondrial sub-cellular fractions, along with mRNA expression and cytochemical analysis highlighting the potential involvement of Akt and Erk signaling and thus give insight into the possible neuroprotective effects of estradiol after an onset of chronic cerebral hypoperfusion.

Materials and Methods

Animals

All animal procedures were approved by the Ethical Committee for the Use of Laboratory Animals of University of Belgrade, VINCA Institute of Nuclear Sciences, Belgrade, Republic of Serbia according to the guidelines of the EU registered Serbian Laboratory Animal Science Association (SLASA).

Three-month-old male Wistar rats (300–350 g, $n = 100$), obtained from the local colony and separate litters, were maintained under the standard conditions: group housed (4 per cage) with free access to food (commercial pellet) and tap water, regular 12-h light/12-h dark cycle, and constant temperature (21 ± 2 °C) and humidity.

Surgical Procedure and Treatment

To induce chronic cerebral hypoperfusion (CCH) in rats ($n = 74$), permanent bilateral occlusion of common carotid arteries was done as previously described (Stanojlović et al. 2015, 2014a, b). Briefly, CCH surgeries were performed on 5 % chloral hydrate (400 mg/kg) anesthetized animals. A neck ventral midline incision was made to expose both carotid arteries. The arteries were carefully separated from the carotid sheath, cervical sympathetic, and vagus nerve and double ligated with 5–0 silk suture. The same procedure was performed on sham group ($n = 26$) but without actual ligation of carotid arteries. During post-operative recovery, all rats were closely monitored on their physical health condition on a daily basis.

Following ligation of carotid arteries, animals were randomly assigned in two groups. Rats in the first group (CCH + E) were injected subcutaneously with 17β-estradiol (Sigma-Aldrich Co., St Louis, MO, USA) dissolved in commercial flax oil in dose 33.3 µg/kg/day, while the animals in the second group (CCH + V) received an equal volume of vehicle (commercial flax oil) (Stanojlović et al. 2015). Sham-operated animals were subjected to vehicle (commercial flax oil, Sham + V). Following the treatments

that were administrated from 09.00 to 10.00 AM for seven consecutive days starting immediately after both types of surgeries, animals were sacrificed.

Tissue and Sample Preparation

For Fluoro-Jade B staining, animals ($n = 5$ per each CCH group and $n = 2$ per sham group) were euthanized with an overdose of 5 % chloral hydrate, perfused transcardially with saline and then with 4 % paraformaldehyde (PFA).

The animals for other analyses were quickly decapitated with guillotine (Harvard Apparatus, Holliston, MA, USA). For immunoblotting of proteins in cytosolic, nuclear, and mitochondrial extracts ($n = 8$ per each CCH group and $n = 6$ per sham group), DNA fragmentation assay ($n = 8$ per each CCH group and $n = 6$ per sham group), and qRT-PCR analysis ($n = 8$ per each CCH group and $n = 6$ per sham group), the hippocampi obtained from all experimental groups were quickly isolated, immediately frozen in liquid nitrogen, and stored at -70 °C for subsequent processing. To prepare samples for Western blotting analysis of proteins in synaptosomal fraction, after decapitation, the hippocampi ($n = 8$ per each CCH group and $n = 6$ per sham group) were rapidly removed for immediate processing.

Preparation of Sub-cellular Fractions for Western Blot Analysis

For immunoblotting of proteins in cytosolic, nuclear, and mitochondrial extracts, individual frozen hippocampal tissue was weighed and homogenized with 20 strokes of Potter–Elvehjem Teflon-glass homogenizer in ice-cold 20 mM Tris–HCl (pH 7.2) buffer containing 10 % glycerol, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2 mM DTT, and several protease (20 mM Na_2MoO_4, 0.15 mM spermine, 0.15 mM spermidine, 0.1 mM PMSF, 5 µg/ml antipain, 5 µg/ml leupeptin, 5 µg/ml aprotinin, 10 µg/ml trypsin inhibitor and 3 mM benzamidine) and phosphatase inhibitors (20 mM glycerophosphate, 5 mM $Na_4P_2O_7 \times 10H_2O$, 2 mM Na_3VO_4, 25 mM NaF) (1:2 w/v). Samples were centrifuged at $2000\times g$, 10 min, 4 °C to gain supernatant and pellet. Supernatant was further centrifuged at $20000\times g$, 30 min, 4 °C to obtain crude mitochondrial pellet. The resulting supernatant was ultracentrifuged at $105000\times g$, 60 min, 4 °C to get final supernatants used as cytoplasmic fraction. The crude mitochondrial pellet was washed (three times) in 0.5 ml of homogenization buffer and centrifuged at $20000\times g$, 30 min, 4 °C. Mitochondrial pellets were then lysed in buffer containing 50 mM Tris–HCl (pH 7.4), 5 % glycerol, 1 mM EDTA, 5 mM DTT, protease inhibitors, and 0.05 % Triton X-100 and incubated on ice for 90 min with frequent vortexing. The resulting

fraction was used as a final mitochondrial extract. The final pellets containing nuclear and cell remains were weighed, resuspended (1:1 w/v) in the homogenizing buffer supplied with 0.5 M KCl, incubated 2 h on ice (with frequent vortexing), and centrifuged at $8000\times g$, 10 min, 4 °C. Supernatant was used as nuclear extract (Drakulić et al. 2013).

For immunoblotting of proteins in synaptosomal fraction, hippocampi were homogenized with 20 strokes in Potter–Elvehjem Teflon-glass homogenizer at 900 rpm in ice-cold 0.32 M sucrose and centrifuged at $1000\times g$, 10 min. The supernatant was decanted, while pellet was resuspended in 10 ml of the same medium and centrifuged for a second time under the same conditions. The supernatants were pooled and centrifuged at $12000\times g$, 30 min, 4 °C, and obtained pellet was resuspended in 5 mM Tris–HCl.

All samples were stored at -70 °C until further processing.

Protein concentration of samples was measured by the modified method of Markwell et al. (1978) using bovine serum albumin (BSA) (Sigma-Aldrich Co., St Louis, MO, USA) as a standard.

Western Blotting

Equal amounts of total proteins (20 µg of cytosolic, nuclear and mitochondrial extracts, 40 µg of synaptosomal fraction) were separated on 10 or 12 % SDS-PAGE gel, depending on the protein molecular mass, and transferred on PVDF membranes (Imobilion-P membrane, Millipore, USA). Membranes were blocked in TBS containing 5 % non-fat milk (NFM) or 5 % BSA and 0.1 % Tween 20 for 2 h and incubated overnight at 4 °C with the following primary antibodies: anti-Bax (sc-7480, Santa Cruz Biotechnology Inc., CA, USA, dilution 1:1000), anti-Bcl-2 (sc-492, Santa Cruz Biotechnology Inc., CA, USA, dilution 1:1000), anti-procaspase 3 (sc-7148, Santa Cruz Biotechnology Inc., CA, USA, dilution 1:1000), anti-NF-kB (Santa Cruz Biotechnology Inc., CA, USA, dilution 1:1000), anti-cytochrome C (sc-13561, Santa Cruz Biotechnology Inc., CA, USA, dilution 1:1000), anti-PARP (#9542, Cell Signaling Technology Inc., Beverly, MA, USA, dilution 1:1000), anti-Akt (#9272, Cell Signaling Inc., Beverly, MA, USA, dilution 1:1000), anti-phospho Akt (p-Akt) (#9271S, Cell Signaling Inc., dilution 1:1000), anti-Erk 44/42 kDa (#9102, Cell Signaling Inc., Beverly, MA, USA, dilution 1:1000), anti-phospho Erk 44/42 kDa (p-Erk 44/42 kDa) (#9101S, Cell Signaling Inc., Beverly, MA, USA, dilution 1:1000), and anti-β-actin (sc-1615, Santa Cruz Biotechnology Inc., CA, USA, dilution 1:5000). After washing, the membranes were incubated for 2 h with horseradish peroxidase-conjugated goat anti-rabbit antibody (sc-2030, Santa Cruz Biotechnology Inc., CA, USA,

dilution 1:5000) for Bcl-2, procaspase 3, PARP, Akt, p-Akt, Erk 44/42 kDa, p-Erk 44/42 kDa and NF-kB; horseradish-peroxidase-conjugated donkey anti-mouse antibody (sc-2318, Santa Cruz Biotechnology Inc., CA, USA, dilution 1:5000) for Bax and cytochrome C; horse-radish peroxidase-conjugated donkey anti-goat antibody (sc-2033, Santa Cruz Biotechnology Inc., CA, USA, dilution 1:5000) for β-actin. The antigen–antibody complex was detected using enhanced chemiluminescence (ECL) system (Amersham Bioscience, Piscataway, NJ, USA), and densitometric analysis was performed using *Image J* software package. The level of all proteins in sham operated group was taken as 100 %, whereas changes were calculated with respect to this value and normalized to β-actin.

Additional experiments confirming the purity of the cell fractions using specific antibody have been done originally with anti-c-jun (sc-1694, Santa Cruz Biotechnology, Inc., CA, USA, dilution 1:1000), anti-α-tubulin (T9026, Sigma-Aldrich, Co., St Louis, MO, USA, dilution 1:1000), and anti-mHsp60 (sc-13115, Santa Cruz Biotechnology, Inc., CA, USA, dilution 1:1000) antibodies for the nuclear, cytosolic, or mitochondrial cell compartments, respectively, to exclude the possibility of cross-contamination (data not shown).

DNA Fragmentation Assay

Diphenylamine (DPA) colorimetric assay was carried out as previously described (Drakulić et al. 2013). Briefly, frozen hippocampi were homogenized in lysis buffer containing 5 mM Tris–HCl (pH 8.0), 20 mM EDTA and 0.5 % Triton X-100. Homogenates were then centrifuged at $27000\times g$, 20 min, 4 °C to separate intact chromatin in the pellets from fragmented DNA in the supernatant fractions. Pellets were resuspended in 0.5 N perchloric acid (PCA), while 5.5 N PCA was added to supernatant fractions to final concentration of 0.5 N. Samples were heated at 90 °C, 15 min and centrifuged at $1500\times g$, 10 min, 4 °C to remove proteins. Supernatant fractions were reacted with DPA for 16–20 h at RT, and absorbance was measured at 600 nm.

Results are presented as a percentage of control fragmentation obtained by formula: % of fragments = [OD600 nm T/OD600 nm (T + B)] × 100, where T is fragmented DNA and B is intact DNA obtained from rats subjected to sham operation and vehicle treatment (commercial flax oil, Sham + V) as well as chronic cerebral hypoperfusion and vehicle (commercial flax oil, CCH + V) or estradiol treatment (CCH + E). In the sham-operated group, DNA fragmentation was expressed as a percentage of total DNA appearing in the supernatant fraction and taken as 100 %, whereas in other experimental groups, those were also calculated as a percentage of total DNA appearing in the supernatant fraction and reported as a percentage of DNA fragmentation detected in sham.

Fluoro-Jade Staining and Image Analysis

Following transcardiac perfusion, brains were carefully removed on ice, fixed in 4 % PFA for 24 h at 4 °C, and cryoprotected in a sucrose gradient (10, 20 and 30 % sucrose in 0.2 M phosphate-buffered saline) for 24 h at 4 °C. The brains were frozen in isopentane, cooled on dry ice, and stored at −70 °C. The hippocampal brain areas were cut, and every 3rd coronal section (16 or 25 μm thick) was mounted on a slide, allowed to dry overnight, and stored at −20 °C. The slides were first immersed in a basic alcohol solution containing 5 % NaOH in 70 % ethanol and dH_2O and incubated in 0.06 % $KMnO_4$ solution for 10 min and then transferred for 20 min into a 0.0001 % solution of Fluoro-Jade B (Chemicon International, Millipore, Billerica, CA, USA) dissolved in 0.1 % acetic acid and rinsed three times in dH_2O for 1 min. Slides were additionally immersed in 0.01 % Hoechst 33258 (Acros Organics, Fair Lawn, NJ, USA) staining solution for 10 min and cover slipped with glycerol. The sections were examined and transformed into digital images by the Axio Observer Microscope Z1 (Zeiss, Jena, Germany) using a filter system suitable for visualizing fluorescein isothiocyanate. Cells labeled with Fluoro-Jade B were observed as individual, shiny green spots that were clearly discernible from the background while Hoechst staining of nuclei showed chromatin condensation in the nuclei of Fluoro-Jade B-positive cells, which is indicative of neurodegeneration. The number of degenerating neurons labeled by Fluoro-Jade B and Hoechst staining was counted in 3 fields under the area of the screen (0.38 mm^2) in 5 sections per animal by three independent researchers. The Fluoro-Jade assay was run with sham-operated rats treated with vehicle as a control for neurodegeneration.

RNA Extraction and Reverse Transcription

Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA quality was determined using spectrophotometric analyses. For the synthesis of cDNAs, a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used according to the manufacturer's instructions. MultiScribe Reverse Transcriptase was used to transcribe 2 μg of total RNA in the presence of 2 μl Random Primers. The cDNAs were stored at −20 °C until further use.

qRT-PCR

The studied gene products were amplified by Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) in a 7500 Realtime PCR System. Specific primers were used to selectively amplify Bcl-2, Bax, caspase 3 and PARP mRNA. Housekeeping genes GAPDH and RPL-19 were used as the internal controls. Since it had shown better performance, RPL-19 was used to normalize absolute values obtained for all samples. The sequences of the used primers and length of their amplification products are described in Table 1.

cDNAs were amplified using the following conditions: hold 95 °C/10 min, denaturation 95 °C/15 s, annealing 60 °C/1 min, extension 60 °C/1 min, and final extension 72 °C/5 min. Melt curve analysis was performed at the end of every experiment to confirm formation of a single PCR product.

As additional controls, PCR samples lacking only template were run for each set of reactions. A single peak melting profile was obtained for all reactions. Each sample was run in triplicate and all experiments were repeated three times. Standard curves were included for each primer pair to estimate the efficiency of amplification and values were obtained from the slope of cycle threshold versus log concentration (Rutledge and Cote 2003). The relative changes in mRNA expression were analyzed using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2009).

Data Analysis

The results are presented as percentage of the mean of the values in the animals submitted to sham operation and treated with vehicle ± SEM. The differences among the groups were analyzed by one-way analysis of variance (ANOVA) test followed by the post hoc Tukey test using Origin 7.5 software package. A p value of 0.05 or less was considered to be significant for all statistical analyses.

Results

Estradiol Suppresses Pro-apoptotic Signaling in the Hippocampal Synaptosomal Fraction of CCH Rats
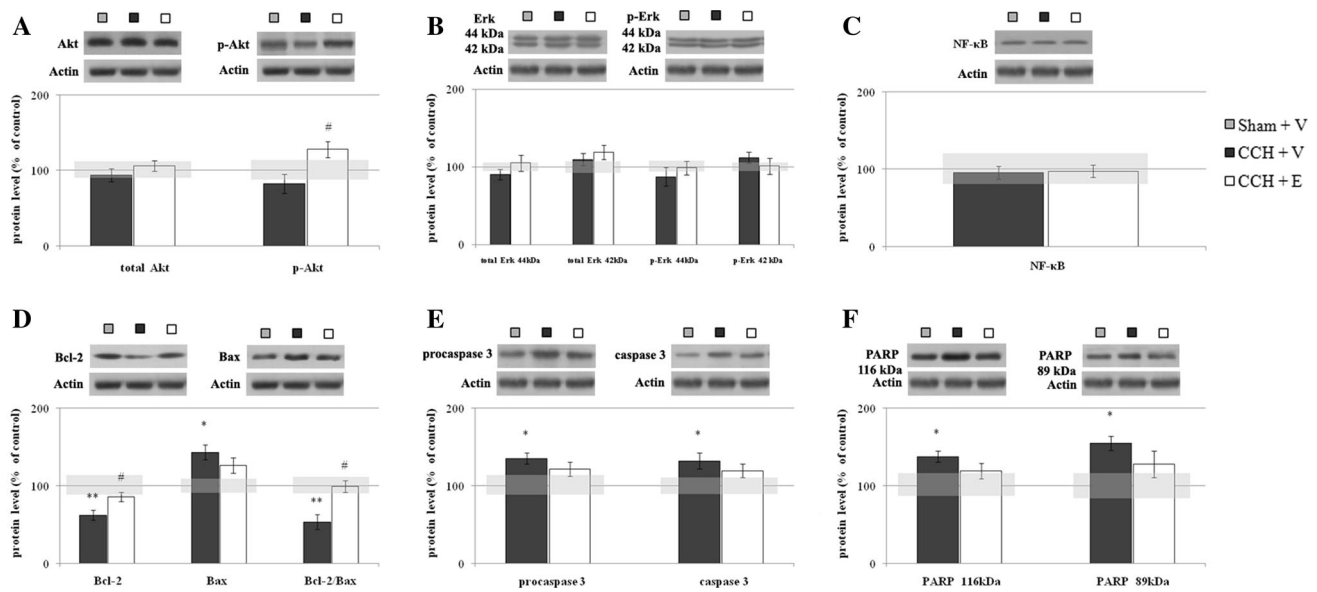
The hippocampal synaptosomal expression of total forms of both investigated kinases involved in cell survival, Akt, and Erk (Erk 44/42 kDa), remained unaltered in all experimental groups (Fig. 1a, b). Interestingly, estradiol treatment induced augmentation of phosphorylated Akt (p-Akt) about 46 % when compared to vehicle treated hypoperfused rats ($p < 0.05$, CCH + V vs. CCH + E) but failed to change the levels of both phosphorylated Erk forms (p-Erk 44/42 kDa, CCH + V vs. CCH + E) (Fig. 1a, b).

The protein expression of NF-κB retained similar in all experimental groups (Fig. 1c).

Figure 1d illustrates that in rats submitted to chronic cerebral hypoperfusion and vehicle when compared to sham group, Bcl-2 expression was decreased about 38 % ($p < 0.01$, Sham + V vs. CCH + V) and about 23 % in contrast to estradiol-treated group ($p < 0.05$, CCH + V vs. CCH + E). In parallel, level of Bax protein was significantly increased ($p < 0.05$, Sham + V vs. CCH + V) while estradiol managed to recover it to control level (Fig. 1d). Observed modulations in Bcl-2 and Bax expression induced significant alterations in their protein ratio between sham-operated group and one subjected to hypoperfusion ($p < 0.01$ Sham + V vs. CCH + V) while

Table 1 The sequences of the used primers and length of their amplification products

Gene name	Sequences of primers (5'–3')	Melting temperature (°C)	Product (bp)
GAPDH	F: GGCAAGTTCAATGGCACAGTCAAG	65	125
	R: ACATACTCAGCACCAGCATCACC	65	
RPL-19	F: TCGCCAATGCCAACTCTCGTC	63	92
	R: AGCCCGGGAATGGACAGT	65	
Bax	F: TGCTACAGGGTTTCATCCAG	58	135
	R: CCAGTTCATCGCCAATTC	57	
Bcl-2	F: TGGAAAGCGTAGACAAGGAGATGC	65	88
	R: CAAGGCTCTAGGTGGTCATTCAGG	67	
NF-κB p65	F: GTGCAGAAAGAAGACATTGAGGTG	64	131
	R: AGGCTAGGGTCAGCGTATGG	63	
PARP	F: CGCTCAAGGCTCAGAACGAG	63	130
	R: CAGGATTGCGGACTCTCCA	59	
caspase 3	F: GATGTCGATGCAGCTAACC	57	321
	R: TGTCTCAATACCGCAGTCC	57	

Fig. 1 Alterations in protein expression of apoptotic molecules in hippocampal synaptosomal fraction in rats subjected to chronic cerebral hypoperfusion treated with vehicle (commercial flax oil, CCH + V) or estradiol (CCH + E) compared to sham-operated animals treated with vehicle (Sham + V). Graphics represent relative protein levels with representative Western blots of total Akt and p-Akt (**a**), total Erk 44/42 kDa and p-Erk 44/42 kDa (**b**), NF-κB (**c**), Bcl-2, Bax and Bcl-2/Bax protein ratio (**d**), procaspase 3 and cleaved caspase 3 (**e**), PARP (full length (116 kDa) and cleaved (89 kDa)) (**f**). Data are presented as the mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test (*$p < 0.05$, **$p < 0.01$ Sham + V vs. CCH + E, and #$p < 0.05$ CCH + V vs. CCH + E)

estradiol provoked shifting for about 46 % towards an anti-apoptotic molecule, Bcl-2 ($p < 0.05$, CCH + V vs. CCH + E) returning it to control level (Sham + V vs. CCH + E) (Fig. 1d).

In comparison to sham group, hypoperfusion led to augmentation of procaspase 3 and its cleaved form (17 kDa) (Fig. 1e) as well as full length PARP (116 kDa) and its cleaved form (89 kDa) protein levels (Fig. 1f) ($p < 0.05$, Sham + V vs. CCH + V). Although estradiol attenuated the effects of chronic cerebral hypoperfusion by returning the expressions of investigated proteins to control levels, no statistical differences were obtained between both hypoperfused groups (CCH + V vs. CCH + E).

Modulations of Apoptotic Signaling in Hippocampus of Rats Subjected to Chronic Cerebral Hypoperfusion and Estradiol Treatment

Effects of estradiol treatment in the state of chronic cerebral hypoperfusion were also evaluated based on the level of redistribution and activation of Akt and Erk by analyzing their protein expression in hippocampal cytosolic and nuclear fractions in contrast to sham. Data showed that protein expressions of all total kinases were on the similar level in both cytosol and nucleus in all investigated groups (Fig. 2a, b). However, the level of p-Akt was augmented in both sub-cellular fractions of rats subjected to chronic

cerebral hypoperfusion and estradiol compared to sham ($p < 0.01$, Sham + V vs. CCH + E). Furthermore, in contrast to chronic cerebral hypoperfused animals treated with vehicle, estradiol increased p-Akt expression in cytosolic about 70 % ($p < 0.001$) and in nuclear fraction for 68 % ($p < 0.001$) (Fig. 2a) along with the enhancement of p-Erk 44 kDa in cytosol about 36 % ($p < 0.05$) and in nucleus around 54 % ($p < 0.01$) as well as p-Erk 42 kDa in cytosol for 52 % ($p < 0.01$) and in nucleus around 60 % ($p < 0.01$) (Fig. 2b).

As shown in Fig. 2c, the expression of NF-κB remained statistically unaltered in cytosolic fraction isolated from both hypoperfused groups compared to sham, however, its level in nucleus was altered about 44 % between animals exposed to hypoperfusion and treated with vehicle and estradiol ($p < 0.01$, CCH + V vs. CCH + E).

Although protein levels of both investigated members of Bcl-2 family, Bcl-2 and Bax, remained statistically unaltered in cytosolic fractions of all investigated groups (Sham + V vs. CCH + V and Sham + V vs. CCH + E), estradiol induced significant modulations in their mitochondrial expression as well as Bcl-2/Bax protein ratio when comparing rats subjected to chronic cerebral hypoperfusion and vehicle with ones submitted to estradiol (CCH + V vs. CCH + E) (Fig. 2d). Namely, under the influence of estradiol, the level of Bcl-2 protein was increased about 33 % ($p < 0.05$), while Bax was decreased
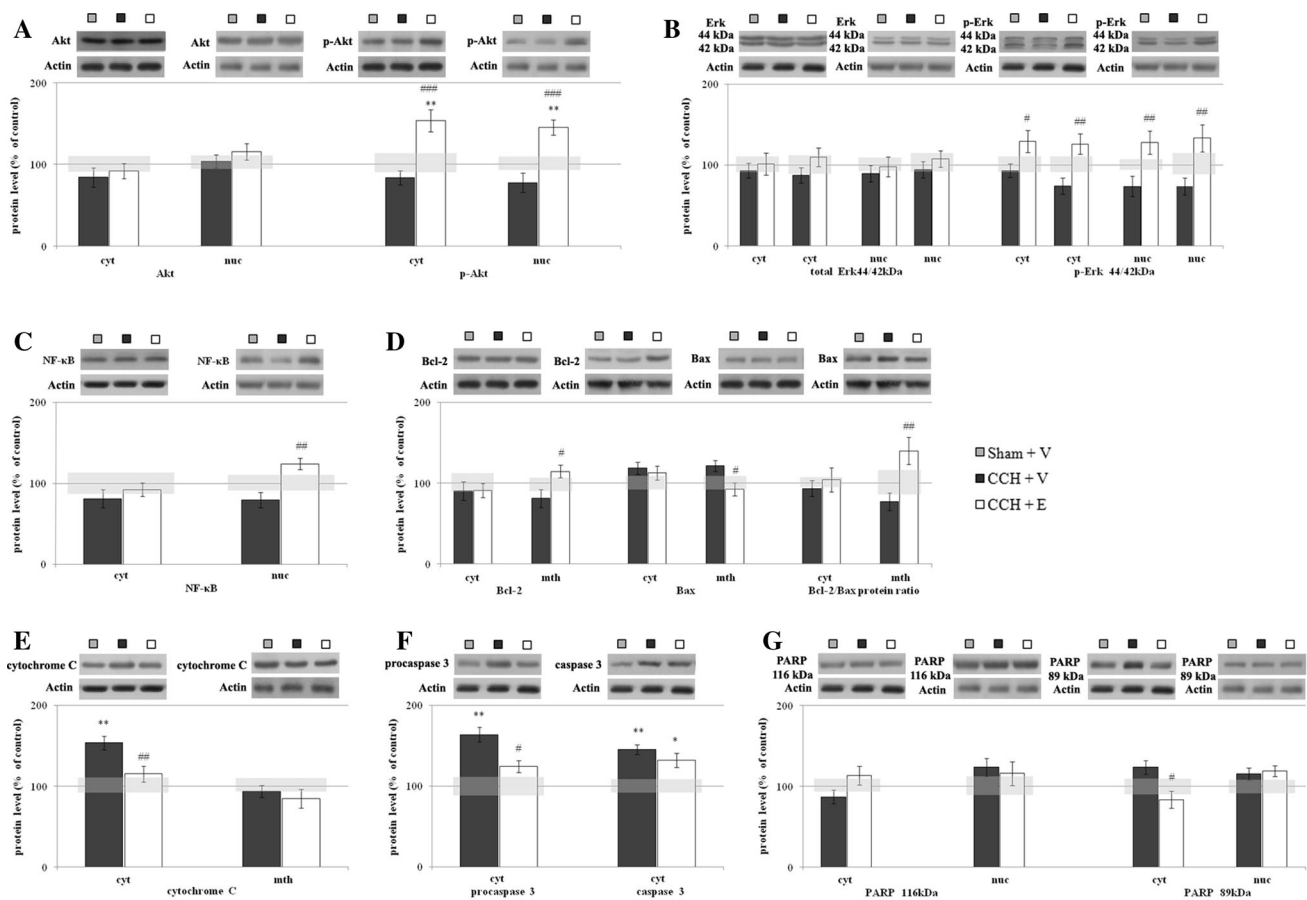
Fig. 2 Estradiol modulates protein expression of apoptotic molecules in hippocampal cytosolic (cyt), nuclear (nuc) or mitochondrial (mth) fractions. Graphics represent relative protein levels with representative Western blots of total Akt and p-Akt (**a**), total Erk 44/42 kDa and p-Erk 44/42 kDa (**b**), NF-κB (**c**), Bcl-2, Bax and Bcl-2/Bax protein ratio (**d**), cytochrome C (**e**), procaspase 3 and cleaved caspase 3 (**f**), PARP (full length (116 kDa) and cleaved (89 kDa)) (**g**) in rats subjected to chronic cerebral hypoperfusion treated with vehicle (commercial flax oil, CCH + V) or estradiol (CCH + E) compared to sham-operated animals treated with vehicle (Sham + V). Data are presented as the mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test (*$p < 0.05$, **$p < 0.01$ Sham + V vs. CCH + E, and #$p < 0.05$, ##$p < 0.01$ CCH + V vs. CCH + E)

about 29 % ($p < 0.05$), which induced predominance of anti-apoptotic molecule Bcl-2 against pro-apoptotic Bax in mitochondrial fraction about 62 % ($p < 0.01$) (Fig. 2d).

Protein expression of cytochrome C in cytosol of rats subjected to chronic cerebral hypoperfusion treated with vehicle was statistically enhanced when compared to sham ($p < 0.01$, Sham + V vs. CCH + V), while its expression was decreased almost to control level due to estradiol treatment (reduction about 38 %, $p < 0.01$, CCH + V vs. CCH + E) (Fig. 2e). Level of investigated protein was similar in mitochondrial fractions isolated from sham and both chronic cerebral hypoperfused groups (Fig. 2e).
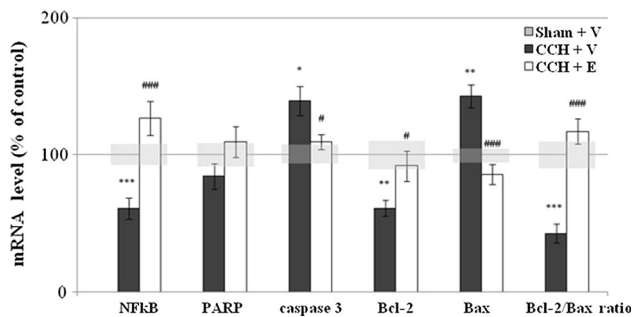
Chronic cerebral hypoperfusion increased expression of procaspase 3 in cytosol comparing to sham operation ($p < 0.01$, Sham + V vs. CCH + V), whereas estradiol treatment lowered its expression around 39 % ($p < 0.01$, CCH + V vs. CCH + E) (Fig. 2f). Interestingly, in contrast to sham, the level of its cleaved form was augmented

in hypoperfused group treated either with vehicle ($p < 0.01$, Sham + V vs. CCH + V) or estradiol ($p < 0.05$, Sham + V vs. CCH + E) (Fig. 2f).

The data demonstrated that both full length PARP (116 kDa) and its cleaved fragment (89 kDa) were unaltered in cytosol and nucleus of chronic cerebral hypoperfused rats treated with either vehicle or estradiol in contrast to sham-operated group; however, estradiol diminished the expression of PARP 89 kDa in cytosol of hypoperfused rats about 40 % ($p < 0.05$, CCH + V vs. CCH + E) (Fig. 2g).

Estradiol-Induced Modulation of mRNA Expression in Hippocampus of CCH Rats

As Fig. 3 demonstrates, compared to sham, chronic cerebral hypoperfusion reduced level of NF-κB mRNA about 39 % ($p < 0.001$, Sham + V vs. CCH + V) while its level

Fig. 3 Nf-κB, PARP, caspase 3, Bcl-2, Bax and Bcl-2 to Bax mRNAs levels in hippocampus of rats subjected to sham operation treated with vehicle (commercial flax oil, Sham + V), chronic cerebral hypoperfusion treated either with vehicle (CCH + V) or estradiol (CCH + E). Data are presented as the mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ Sham + V vs. CCH + E, and #$p < 0.05$, ###$p < 0.001$ CCH + V vs. CCH + E)

was upregulated for 65 % in response to estradiol ($p < 0.001$) associating this transcription factor and modulations in redistribution and activation of investigated kinases in regulation of Bcl-2 and Bax gene expressions. In contrast to vehicle-treated chronic cerebral hypoperfused rats, estradiol induced increase in Bcl-2 mRNA level for about 32 % ($p < 0.05$, CCH + V vs. CCH + E) and decrease in Bax expression about 58 % ($p < 0.001$, CCH + V vs. CCH + E). This further caused shifting of the ratio between apoptotic molecules within Bcl-2 family towards pro-apoptotic in hypoperfused group treated with vehicle in contrast to sham ($p < 0.001$, Sham + V vs. CCH + V) and anti-apoptotic for about 75 % when compared to estradiol group ($p < 0.001$, CCH + V vs. CCH + E). Caspase 3 mRNA level was also affected by cerebral hypoperfusion ($p < 0.05$, Sham + V vs. CCH + V) and decreased about 30 % when treated with estradiol ($p < 0.05$, CCH + V vs. CCH + E) (Fig. 3). Furthermore, either vehicle or estradiol treatment that followed chronic cerebral hypoperfusion failed to modulate PARP gene expression when compared to sham-operated animals (Fig. 3).

Assessment of Estradiol Impact on Cell Survival in Hippocampus of the CCH Rats

Fluoro-Jade B staining that specifically marks degenerating somata and their processes was exploited to investigate neuroprotective potential of estradiol at histological level (Fig. 4a–d). Figure 4a–d reveals an apparent increase in the number of Fluoro-Jade B-labeled cells in hippocampus of rats subjected to chronic cerebral hypoperfusion and treated with vehicle, particularly in CA1 subregion. Namely, these animals exhibited a high degree of neuronal cell

death, as shown by the elevated number of stained cells when compared to sham ($p < 0.05$ Sham + V vs. CCH + V, Fig. 4d) while in chronic cerebral hypoperfusion rats treated with estradiol, the number of degenerating neurons was similar to one obtained for sham group resulting in significant difference between both CCH groups ($p < 0.05$, CCH + V vs. CCH + E). Furthermore, to investigate the mode of cell death in these neurons, alongside Fluoro-Jade B staining, sections were marked with Hoechst 33258, a blue fluorescent DNA dye. In rats submitted to chronic cerebral hypoperfusion, many cells contained 1 or more foci of compact Hoechst-stained chromatin, indicating that they were undergoing apoptosis, were detected, while in other, their number was in the range of sham-operated group (data not shown).

The neuroprotective potential of estradiol is also examined by DPA colorimetric assay. Namely, Fig. 4e illustrates the increment about 35 % in content of apoptosis-specific DNA fragments in chronic cerebral hypoperfused rats treated with vehicle when compared to sham ($p < 0.05$, Sham + V vs. CCH + V) and about 33 % in contrast to ones treated with estradiol (p < 0.05).

Discussion

The literature highlights that much of the biochemical machinery implicated in apoptotic cell death can be activated in synaptic terminals by modifying function and endorsing localized degeneration of synapses and neurites and finally transmitting pro-apoptotic signals to cell body and leading to nuclear DNA condensation and fragmentation (Costain et al. 2008; Mattson and Duan 1999). Indeed, time-dependent hippocampal neurodegeneration induced by chronic cerebral hypoperfusion arises along with shifted expression of Bcl-2 family members towards pro-apoptotic Bax, and increased and activated procaspase 3 accompanied by augmented cleaved PARP (89 kDa) in synaptosomal fraction (Stanojlovic et al. 2014a), illustrating that neuroprotective effects of certain compounds should be tested in assertive time frame. One of those substances is estradiol, a potent female steroid hormone that promotes recovery from ischemic injury by supporting neuronal cell proliferation and synaptogenesis and modulating synaptic connectivity, causing axonal sprouting and regeneration (Petrone et al. 2014; Liu et al. 2009; Garcia-Segura et al. 2001) most likely through direct or indirect ER-dependent action on growth factors and components, neurotrophins, synaptic proteins, as well as activation of MAPK/Erk and PKB/Akt signaling cascade (Li et al. 2012) whose downstream effectors, once stimulated, may remain in cytosol or translocate to other compartments of the cell and further phosphorylate and trigger variety of substrates essential for
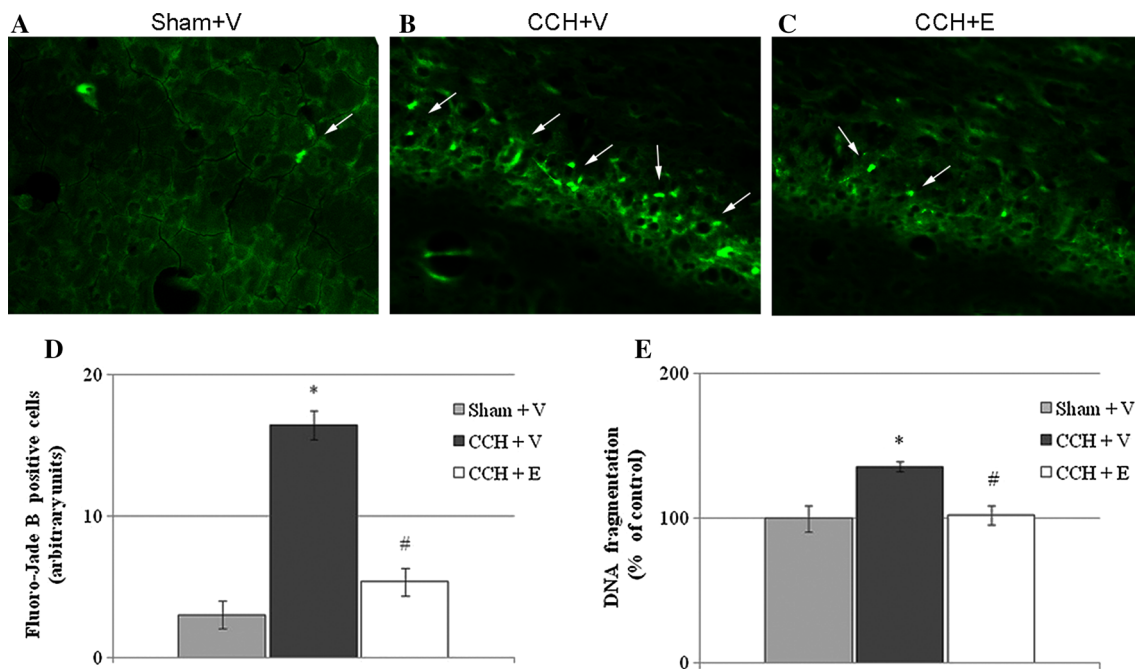
Fig. 4 Estradiol promotes cell survival in hippocampus of rats subjected to chronic cerebral hypoperfusion. Fluoro-Jade B staining of CA1 hippocampal sections of adult male Wistar rats exposed to sham operation and vehicle (commercial flax oil, Sham + V) (**a**), chronic cerebral hypoperfusion and vehicle (CCH + V) (**b**), or chronic cerebral hypoperfusion and estradiol (CCH + E) (**c**). Degenerating neurons are marked with *arrows*. Quantitative analysis of cell survival and prevention of apoptosis (Cargnello and Roux 2011). According to the results of the current study, although expression of other investigated proteins was unaltered, detected effects of estradiol in synaptosomal fraction may be mediated via triggering anti-apoptotic signaling pathways through activation of Akt and regulation of Bcl-2 expression and Bcl-2/Bax protein ratio. Unexpectedly, usually cooperative Akt and Erk response (Tang et al. 2014; Friguls et al. 2002) is not present in this cell compartment, leading to conclusion that due to its high sensitivity to ischemic insult, estradiol treatment in repeated regime of administration is either not sufficient to provoke Erk-dependent response (Hofmeijer and van Putten 2012) and/or that Akt activation is more amenable (Jover-Menguala et al. 2010).

Given that apoptotic signals generated at synapse may be spread to the cell body (Mattson and Duan 1999) while positive effects of estradiol on pro-survival cascade are demonstrated in prefrontal cortex following cerebral ischemia (Stanojlovic et al. 2015), of interest was to establish whether applied estradiol treatment may also prevent activation of pro-apoptotic cascade in the body of hippocampal cells. Interestingly, estradiol treatment elicited an increase in both phospho-Akt and phospho-Erk signals cooperatively in cytosol and nucleus most likely

Fluoro-Jade B staining of adult male CA1 hippocampal subsections (**d**). Measurement of DNA fragments with the diphenylamine (DPA) colorimetric assay (**e**). Data are presented as the mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test (*$p < 0.05$ Sham + V vs. CCH + E, and #$p < 0.05$ CCH + V vs. CCH + E)

through GPR30 or other ER (Tang et al. 2014; Choi et al. 2004; Friguls et al. 2002; Singh 2001; Simpkins et al. 1997) followed by a strong induction of their downstream effector, NF-κB, indicating that detected upregulation of anti-apoptotic gene Bcl-2 and downregulation of pro-apoptotic genes Bax and caspase 3 may be mediated by this transcription factor and investigated kinases. This was also accompanied by significant augmentation in the protein expression of Bcl-2 and Bcl-2/Bax protein ratio as well as stimulated Bcl-2 translocation from cytoplasm to mitochondria and significantly reduced expression of Bax molecule along with reduction of cytochrome C and procaspase 3 levels in cytosol that might be associated with decreased propensity for apoptosis. Namely, overexpression of anti-apoptotic molecule Bcl-2 and downregulation of pro-apoptotic Bax preserves permeability of outer mitochondrial membrane and directly blocks the leakage of cytochrome C pursued by mitigation of procaspase 3 and cleavage of PARP critically involved in programmed cell death (Wang and Youle 2009; Desagher and Martinou 2000). Although initiation of Akt and Erk pathways have been shown to inhibit apoptosis over inhibition of caspase 3 activation (Jover-Menguala et al. 2010; Lebesgue et al. 2009; Terada et al. 2000; Zhou et al. 2000), its immunoreactivity was augmented compared to sham and

similar between both hypoperfused experimental groups. In current experimental setup, the level of intact PARP and its fragment weighed 89 kDa as well as procaspase 3 which are characteristic substrates for caspase 3 cleavage could not be associated with its activity. Obtained results suggest that investigated protein might exert other functions than pro-apoptotic like normal signaling, axon guidance, synaptic plasticity, and even neuroprotection against ischemic and excitotoxic injury (McLaughlin 2004).

Our previous study showed that chronic cerebral hypoperfusion could induce time-dependent neurodegeneration and cell loss by triggering intrinsic mitochondrial apoptosis pathway in hippocampal synaptosomal fraction (Stanojlovic et al. 2014a), while in current experimental setup, estradiol treatment affected previously reported processes and showed protection against injury in rats subjected to chronic cerebral hypoperfusion associated with suppression of apoptosis-positive cells amount and DNA fragmentation. It is likely that observed containment of apoptotic cell death is a result of elevated phosphorylation of Akt in synaptosomal fraction and increased Bcl-2 and shifted Bcl-2/Bax ratio towards anti-apoptotic molecule, as well as cooperative activation of Akt and Erk signaling pathways in other investigated sub-cellular fractions along with decrease of Bax, increase of Bcl-2 and Bcl-2/Bax protein ratio, and reduction of cytochrome C release from mitochondria.

Estradiol, a highly potent female steroid hormone, in both epidemiological and experimental studies has been reported to exert anti-apoptotic, anti-cytotoxic effects and even neuroprotective outcome when administered chronically and acutely before an ischemic insult. However, an area of ambiguity in the therapy is its benefit during post-ischemic period (Petrone et al. 2014; Yang et al. 2000). The current study has started considering this matter by examining the extent of hippocampal neurodegeneration and its association with modulation of the expression, cellular distribution, and activation of various molecules within Akt and Erk signaling pathways like NF-κB, Bcl-2 family members, cytochrome C, caspase 3, and PARP not only in synaptosomal fraction but also in the rest of the cell. According to our findings repeated post-ischemic estradiol treatment reduces neurodegenerative changes induced by chronic cerebral hypoperfusion in hippocampus through the activation of investigated kinases and regulation of their downstream molecules in sub-cellular manner indicating a time window and regime of its administration as a valid therapeutic intervention.

Compliance with Ethical Standards

References

Arevalo MA, Iñigo Azcoitia I, Garcia-Segura LM (2015) The neuroprotective actions of oestradiol and oestrogen receptors. Nat Rev Neurosci 16:17–29

Bingham D, Macrae IM, Carswell HV (2005) Detrimental effects of 17beta-oestradiol after permanent middle cerebral artery occlusion. J Cereb Blood Flow Metab 25:414–420

Bondeau N, Widmann C, Lazdunski M, Heurteaux C (2001) Activation of the nuclear factor-κB is a key event in brain tolerance. J Neurosci 21:4668–4677

Brann D, Raz L, Wang R, Vadlamudi R, Zhang Q (2012) Oestrogen signalling and neuroprotection in cerebral ischaemia. J Neuroendocrinol 24:34–47

Cargnello M, Roux PP (2011) Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. Microbiol Mol Biol Rev 75:50–83

Choi YC, Lee JH, Hong KW, Lee KS (2004) 17 Beta-estradiol prevents focal cerebral ischemic damages via activation of Akt and CREB in association with reduced PTEN phosphorylation in rats. Fundam Clin Pharmacol 18(5):547–557

Costain WJ, Rasquinha I, Sandhu JK, Rippstein P, Zurakowski B, Slinn J, MacManus JP, Stanimirovic DB (2008) Cerebral ischemia causes dysregulation of synaptic adhesion in mouse synaptosomes. J Cereb Blood Flow Metab 28(1):99–110

Desagher S, Martinou JC (2000) Mitochondria as the central control point of apoptosis. Trends Cell Biol 10(9):369–377

Drakulić D, Veličković N, Stanojlović M, Grković I, Mitrović N, Lavrnja I, Horvat A (2013) Low-dose dexamethasone treatment promotes the pro-survival signalling pathway in the adult rat prefrontal cortex. J Neuroendocrinol 25:605–616

Dubal DB, Wise PM (2001) Neuroprotective effects of estradiol in middle-aged female rats. Endocrinology 142(1):43–48

Dubal DB, Kashon ML, Pettigrew LC, Ren JM, Finklestein SP, Rau SW, Wise PM (1998) Estradiol protects against ischemic injury. J Cereb Blood Flow Metab 18(11):1253–1258

Dubal DB, Shughrue PJ, Wilson ME, Merchenthaler I, Wise PM (1999) Estradiol modulates bcl-2 in cerebral ischemia: a potential role for estrogen receptors. J Neurosci 19(15):6385–6393

Friguls B, Petegnief V, Justicia C, Pallàs M, Planas AM (2002) Activation of Erk and Akt signaling in focal cerebral ischemia: modulation by TGF-alpha and involvement of NMDA receptor. Neurobiol Dis 11:443–456

Garcia-Segura LM, Azcoitia I, DonCarlos LL (2001) Neuroprotection by estradiol. Prog Neurobiol 63:29–60

Gibson CL (2013) Cerebral ischemic stroke: is gender important? J Cereb Blood Flow Metab 33:1355–1361

Gordon KB, Macrae IM, Carswell HV (2005) Effects of 17beta-oestradiol on cerebral ischaemic damage and lipid peroxidation. Brain Res 1036:155–162

Grodstein F, Manson JE, Stampfer MJ, Rexrode K (2008) Postmenopausal hormone therapy and stroke: role of time since menopause and age at initiation of hormone therapy. Arch Intern Med 168(8):861–866

Harukuni I, Hurn PD, Crain BJ (2001) Deleterious effect of beta-estradiol in a rat model of transient forebrain ischemia. Brain Res 900:137–142

Hofmeijer J, van Putten MJ (2012) Ischemic cerebral damage: an appraisal of synaptic failure. Stroke 43:607–615

Jia J, Guan D, Zhu W, Alkayed NJ, Wang MM, Hua Z, Xu Y (2009) Estrogen inhibits Fas-mediated apoptosis in experimental stroke. Exp Neurol 215(1):48–52

Jover T, Tanaka H, Calderone A, Oguro K, Bennett MVL, Etgen AM, Zukin RS (2002) Estrogen protects against global ischemia-induced neuronal death and prevents activation of apoptotic signaling cascades in the hippocampal CA1. J Neurosci 22:2115–2124

Jover-Menguala T, Miyawakia T, Latuszeka A, Alborchb E, Zukina RS Suzanne, Etgen AM (2010) Acute estradiol protects CA1 neurons from ischemia-induced apoptotic cell death via the PI3K/Akt pathway. Brain Res 1321C:1–12

Koellhoffer EC, McCullough LD (2013) The effects of estrogen in ischemic stroke. Transl Stroke Res 4:390–401

Lebesgue D, Chevaleyre V, Zukin RS, Etgen AM (2009) Estradiol rescues neurons from global ischemia induced cell death: multiple cellular pathways of neuroprotection. Steroids 74:555–561

Li J, Siegel M, Yuan M, Zeng Z, Finnucan L, Persky R, Hurn PD, McCullough LD (2011) Estrogen enhances neurogenesis and behavioral recovery after stroke. J Cereb Blood Flow Metab 31(2):413–425

Li V, Kong J, Szelemej P, Bi X (2012) Delayed neuronal death in ischemic stroke: molecular pathways. INTECH Open Access Publisher, Rijeka

Liu M, Dziennis S, Hurn PD, Alkayed NJ (2009) Mechanisms of gender-linked ischemic brain injury. Restor Neurol Neurosci 27(3):163–179

Livak KJ, Schmittgen TD (2009) Analysis of relative gene expression data using real-time quantitative PCR and the 2 (-Delta DeltaC(T)) method. Methods 25:402–408

Lobo RA (2013) Where are we 10 years after the Women's Health Initiative? J Clin Endocrinol Metab 98(5):1771–1780

Ma YL, Qin P, Li Y, Shen L, Wang SQ, Dong HL, Hou WG, Xiong LZ (2013) The effects of different doses of estradiol (E2) on cerebral ischemia in an in vitro model of oxygen and glucose deprivation and reperfusion and in a rat model of middle carotid artery occlusion. BMC Neurosci 14:118

Manthey D, Behl C (2006) From structural biochemistry to expression profiling: neuroprotective activities of estrogen. Neuroscience 138(3):845–850

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

Mattson MP, Duan W (1999) "Apoptotic" biochemical cascades in synaptic compartments: roles in adaptive plasticity and neurodegenerative disorders. J Neurosci Res 58:152–166

McCullough LD, Zeng Z, Blizzard KK, Debchoudhury I, Hurn PD (2005) Ischemic nitric oxide and poly (ADP-ribose) polymerase-1 in cerebral ischemia: male toxicity, female protection. J Cereb Blood Flow Metab 25:502–512

McLaughlin B (2004) The kinder side of killer proteases: caspase activation contributes to neuroprotection and CNS remodeling. Apoptosis 9:111–121

Petrone AB, Simpkins JW, Barr TL (2014) 17β-estradiol and inflammation: implications for ischemic stroke. Aging Dis 5(5):340–345

Rutledge RG, Cote C (2003) Mathematics of quantitative kinetic PCR and the application of standard curves. Nucleic Acids Res 31:e93

Sherwin BB (2009) Estrogen therapy: is time of initiation critical for neuroprotection? Nat Rev Endocrinol 5:620–627

Simpkins JW, Rajakumar G, Zhang YQ, Simpkins CE, Greenwald D, Yu CJ, Bodor N, Day AL (1997) Estrogens may reduce mortality and ischemic damage caused by middle cerebral artery occlusion in the female rat. J Neurosurg 87(5):724–730

Singer CA, Rogers KL, Dorsa DM (1998) Modulation of Bcl-2 expression: a potential component of estrogen protection in NT2 neurons. NeuroReport 9(11):2565–2568

Singh M (2001) Ovarian hormones elicit phosphorylation of Akt and extracellular-signal regulated kinase in explants of the cerebral cortex. Endocrine 14:407–415

Stanojlović M, Gušеvac I, Grković I, Mitrović N, Horvat A, Drakulić D (2014a) Time-related sex differences in cerebral-hypoperfusion induced brain injury. Arch Biol Sci Belgrade 66(4):1673–1680

Stanojlović M, Horvat A, Gušеvac I, Grković I, Mitrović N, Buzadžić I, Drakulić D (2014b) Time course of cerebral hypoperfusion-induced neurodegenerative changes in the cortex of male and female rats. Folia Biol 60:123–132

Stanojlović M, Zlatković J, Gušеvac I, Grković I, Mitrović N, Zarić M, Horvat A, Drakulić D (2015) Repeated low-dose 17β-estradiol treatment prevents activation of apoptotic signaling both in the synaptosomal and cellular fraction in rat prefrontal cortex following cerebral ischemia. Neurochem Int. doi:10.1016/j.neuint.2015.03.002

Stoltzner SE, Berchtold NC, Cotman CW, Pike CJ (2001) Estrogen regulates bcl-x expression in rat hippocampus. NeuroReport 12(13):2797–2800

Tang H, Zhang Q, Yang L, Dong Y, Khan M, Yang F, Brann DW, Wang R (2014) GPR30 mediates estrogen rapid signaling and neuroprotection. Mol Cell Endocrinol 387(1–2):52–58

Terada K, Kaziro Y, Satoh T (2000) Analysis of Ras-dependent signals that prevent caspase-3 activation and apoptosis induced by cytokine deprivationin hematopoietic cells. Biochem Biophys Res Commun 267:449–455

Wang C, Youle RJ (2009) The role of mitochondria in apoptosis. Annu Rev Genet 43:95–118

Wang R, Zhang Q-G, Han D, Xu J, Lü Q, Zhang G-Y (2006) Inhibition of MLK3-MKK4/7-JNK1/2 pathway by Akt1 in exogenous estrogen-induced neuroprotection against transient global cerebral ischemia by a non-genomic mechanism in male rats. J Neurochem 99(6):1543–1554

Yang SH, Shi J, Day AL, Simpkins JW (2000) Estradiol exerts neuroprotective effects when administered after ischemic insult. Stroke 31(3):745–749

Yang L, Zhang QG, Zhou C, Yang F, Zhang Y, Wang R, Brann DW (2010) Extranuclear estrogen receptors mediate the neuroprotective effects of estrogen in the rat hippocampus. PLoS One 5(5):e9851

Zhou H, Li XM, Meinkoth J, Pittman RN (2000) Akt regulates cell survival and apoptosis at a postmitochondrial level. J Cell Biol 151:483–494