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EFFECTS OF CHRONIC CEREBRAL HYPOPERFUSION AND LOW-DOSE PROGESTERONE TREATMENT ON APOPTOTIC PROCESSES, EXPRESSION AND SUBCELLULAR LOCALIZATION OF KEY ELEMENTS WITHIN Akt AND Erk SIGNALING PATHWAYS IN RAT HIPPOCAMPUS

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Abstract—The present study attempted to investigate how chronic cerebral hypoperfusion (CCH) and repeated low-dose progesterone (P) treatment affect gene and protein expression, subcellular distribution of key apoptotic elements within protein kinase B (Akt) and extracellular signal-regulated kinases (Erk) signal transduction pathways, as well as neurodegenerative processes and behavior. The results revealed the absence of Erk activation in CCH in cytosolic and synaptosomal fractions, indicating a lower threshold of Akt activation in brain ischemia, while P increased their levels above control values. CCH induced an increase in caspase 3 (Casp 3) and poly (ADP-ribose) polymerase (PARP) gene and protein expression. However, P restored expression of examined molecules in all observed fractions, except for the levels of Casp 3 in synapses which highlighted its possible non-apoptotic or even protective function. Our study showed the absence of nuclear factor kappa-light-chain-enhancer of activated b cells (NF-κB) response to this type of ischemic condition and its strong activation under the influence of P. Further, the initial increase in the number of apoptotic cells and amount of DNA fragmentation induced by CCH was significantly reduced by P. Finally, P reversed the CCH-induced reduction in locomotor activity, while promoting a substantial decrease in anxiety-related behavior. Our findings support the concept that repeated low-dose post-ischemic P treatment reduces CCH-induced neurodegeneration in the hippocampus. Neuroprotection is initiated through the activation of investigated kinases and regulation of their downstream molecules in subcellular specific manner, indicating that this treatment may be a promising therapy for alleviation of CCH-induced pathologies.

Key words: progesterone, chronic cerebral hypoperfusion, apoptosis, neurodegeneration.

INTRODUCTION

Chronic cerebral hypoperfusion (CCH), a common pathophysiological state characterized as a long-term, mild ischemic brain insult, is one of the leading factors for the development of Alzheimer's disease, vascular and aging dementia (Farkas et al., 2007). Due to the specificity of ischemic insult induced by CCH apoptosis is considered the dominant cell death mechanism under this condition. It is activated through the intrinsic mitochondrial pathway and partially regulated by activity of protein kinase B (Akt) and extracellular signal-regulated kinases (Erk). Akt and Erk signaling pathways have important roles in the development and progression of cerebrovascular and neurodegenerative disorders (Lee et al., 2009; Kim and Choi, 2010). These kinases are critical factors in the ischemic brain, since cell destiny is significantly determined by their activation and performance (Friguls et al., 2002). One of the most important downstream targets of Akt and Erk is nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). This transcriptional factor is involved in plasticity, learning, memory, cell survival and inflammation (Meffert and Baltimore, 2005). Although studies have shown that NF-κB may have an anti-apoptotic role in the brain, it contributes to neuronal cell death under severe ischemic conditions (Zhang et al., 2005). Together, Akt and Erk are involved in the regulation of apoptosis through modulation of Bcl-2 family member expression (Zhuang and Schnellmann, 2006), proteins that play a central role in the initiation of intrinsic mitochondrial-dependent apoptosis. Under ischemic conditions Bcl-2/Bax interaction and translocation leads to the release of cytochrome C (Cyt C), apoptosome formation and subsequent transition of caspase 3 (Casp 3) from pro-enzyme form (pCasp 3) to active form (aCasp3) (Love, 2003). Casp 3, an executor

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Abbreviations: 2VO, bilateral common carotid artery occlusion; Akt, protein kinase B; Casp 3, caspase 3; CCH, chronic cerebral hypoperfusion; Cyt C, cytochrome c; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; EPM, elevated plus maze; Erk, extracellular signal-regulated kinases; NF-κB, nuclear factor kappa-light-chain-enhancer of activated b cells; OF, open field test; P, progesterone; PARP, poly (ADP-ribose) polymerase.

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of apoptosis cleaves large numbers of different target proteins including poly (ADP-ribose) polymerase (PARP) whose primary function is DNA repair. However, some studies showed that this protein has an active role in apoptotic processes and that its activation can be considered a pro-apoptotic factor (McCullough et al., 2005).

It is well known that progesterone (P) exerts neuroprotective properties in various types of neuronal injury (Ishrat et al., 2012) as well as neurodegenerative diseases (Singh and Su, 2013). P acts through genomic and non-genomic mechanisms and modulates target gene expression and activation of different signaling pathways and proteins (Brinton et al., 2008). In complete brain ischemia, this potent neurosteroid induces the activation of Akt and Erk (Ishrat et al., 2012), transcriptional activation by NF-κB (Liu et al., 2009). Further P alters apoptotic processes by regulating Bcl-2 family expression and modulating Casp 3 activity (Espinosa-Garcia et al., 2013). Furthermore, P treatment induces behavioral changes in intact (Auger and Forbes-Lorman, 2008) as well as ischemic animals (Yousuf et al., 2014). However, molecular mechanisms of P action involved in CCH remain unknown and, therefore, interesting for investigation.

In the following study, we mimicked the rapid reduction of cerebral blood flow that results in lasting ischemic insult. This reduction in cerebral circulation usually occurs as a consequence of heart failure, major surgeries, stenosis or occlusion of blood vessels, leading to CCH, the major factor in the development of neurodegenerative diseases. Alongside CCH effects, we tried to estimate whether and how repeated low-dose P treatment affects complex Akt/Erk and mitochondrial-dependent apoptotic signaling, gene expression, neurodegeneration and behavior.

EXPERIMENTAL PROCEDURES

Animals

All animal procedures were approved by the Ethics Committee for the Use of Laboratory Animals of "Vinča" Institute of Nuclear Sciences, University of Belgrade, Belgrade, Serbia according to the guidelines of the EU registered Serbian Laboratory Animal Science Association (SLASA). Adult male Wistar rats (300–350 g) were maintained under the standard conditions: group-housed (four per cage) with *ad libitum* access to commercial rat pellet and tap water; regular 12-h light/12-h dark cycle; constant temperature (21 ± 2°C) and humidity.

Surgical procedure

To induce CCH in rats, bilateral common carotid artery occlusion (2VO) was performed as previously described (Stanojlovic et al., 2015). Rats were anesthetized with chloral-hydrate (5%, 400 mg/kg). Both common carotid arteries were exposed through a ventral midline incision. The arteries were carefully separated from the carotid sheath, cervical sympathetic and vagus nerve and each carotid artery was double-ligated with 5–0 silk suture. Sham animals underwent the same surgical procedure but without the actual ligation. After surgery, all rats were closely monitored on their physical health condition (breathing, body temperature, body weight) during post-operative recovery. Breathing was closely monitored until animals regained their consciousness. Body temperature was measured hourly and strictly controlled between 36.5 and 37.5 by incandescent lamp 3 h following the procedure. Body mass was observed daily. Mortality rate of 2VO animals was ≈25%, and for sham animals was ≈10%, which is in concordance with previous studies (Farkas et al., 2005; Farkas et al., 2006; Institoris et al., 2007; Cechetti et al., 2010).

Treatment

Following procedure, animals were randomly assigned in three groups (I) Sham-operated animals + vehicle treatment (Cont); (II) 2VO animals + vehicle treatment (2VO); (III) 2VO animals + progesterone (4-Pregnene-3,20-dione, Sigma, St. Louis, MO, USA) treatment (2VO + P). Rats were s.c. injected either with P, dissolved in flax oil (1.7 mg/kg/day), or with equivalent volume of vehicle (commercial flax oil, 1 ml/kg). Treatments were administrated from 09.00 to 10.00 AM for seven consecutive days.

Elevated plus maze (EPM)

Anxiety-related behaviors as well as general activity were measured using the EPM test (Pellow et al., 1985; Hogg, 1996). Tests were conducted 7 days following 2VO procedure, between 8:00 and 11:00 AM. Animals were recorded for a period of 10 min.

The apparatus consisted of a central platform (10 × 10 cm), with two opposite open arms and enclosed arms (50 × 10 cm), elevated 50 cm above the floor. Each animal was placed on the central platform with its head facing an open arm and recorded. The parameters recorded were the numbers of open or enclosed arm entries, and the total time each animal spent in open arms. The number of total arm entries was considered as index of general locomotive activity, while the percentage of open arm entries and percentage of time spent in the open arms were regarded as anxiety-related behavior (Kakehata et al., 2010). Each test was assessed by three examiners individually. Total of 10 animals per group were subjected to EPM following experimental procedure.

Open field (OF)

Rats were assessed for locomotive, exploratory activity and anxiety in the OF test as previously described (Wang et al., 2008). Tests were conducted 7 days following 2VO procedure, between 8:00 and 11:00 AM. Following 30 s of adaptation, animals were recorded for 10 min.

OF testing was conducted in a square arena 100 × 100 × 40 cm divided into 25 × 25-cm squares. A single rat was placed at the center of the arena and recorded. The behaviors measured included locomotive activity, assessed by the number of grid crosses and defined as crossing all four paws across one of the grid