Neuroscience 187 (2011) 93–102

RELA/P65-SERINE 536 NUCLEAR FACTOR-KAPPA B PHOSPHORYLATION IS RELATED TO VULNERABILITY TO STATUS EPILEPTICUS IN THE RAT HIPPOCAMPUS

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Abstract—Although nuclear factor-kappa B (NF-κB) is essential for neuron survival and its activation may protect neuron against oxidative-stresses or ischemia-induced neurodegeneration, NF-κB activation can contribute to inflammatory reaction and apoptotic cell death after brain injury and stroke. However, there are little data concerning the specific pattern of NF-κB phosphorylations in neuronal damage/survival induced by status epilepticus (SE). In the present study, NF-κB phosphorylation showed the cellular specific pattern in responses to SE. p52-S865, p52-Ser869, p65-Ser276, p65-Ser311, p65-Ser468, and p65-Ser529 NF-κB phosphorylation was significantly decreased in the CA1 and CA3 pyramidal cells vulnerable to SE, although neuronal specific nuclear antigen immunoreactivity was strongly detected. In contrast, p65-Ser536 NF-κB phosphorylation was enhanced in these neurons accompanied by TUNEL- and Fluoro-Jade B 244signals. These findings serve as the first comprehensive description of the cellular specific distribution of NF-κB phosphorylation in response to pilocarpine-induced SE in the rat hippocampus, and suggest that enhancement in p65-Ser536 NF-κB phosphorylation may be closely relevant to neuronal vulnerability to SE, while others may be involved in neuronal survival. © 2011 Published by Elsevier Ltd on behalf of IBRO.

Key words: NF-κB, phosphorylation, status epilepticus, neuronal damage, neuronal survival.

Epilepsy is a chronic neurological disease that is characterized by the periodic occurrence of seizures. In epilepsy studies, neurodegeneration has been the main focus, since neuronal loss occurs both in principal neurons and in interneurons (Mathern et al., 1995; Wittner et al., 2001). The cause of epileptogenesis is mainly related to the failure of inhibitory control, which includes insufficient GABA, the loss of GABAergic interneurons (De Lanerolle et al., 1989; Obenaus et al., 1993), change in circuits involving GABAergic interneurons (Bekenstein and Lothman, 1993; Sloviter, 1987), and altered expression of $GABA_A$ receptors (Brooks-Kayal et al., 1998; Buhl et al., 1996). Recently, it has been reported that seizure or epilepsy is also emphasized as an inflammatory condition, and that seizure activity rapidly increases the synthesis and release of various cytokines/chemokines in rodent brain area involved in seizure generalization (Ravizza and Vezzani, 2006; Ravizza et al., 2008; Boer et al., 2007; Vezzani et al., 2008). Indeed, proinflammatory cytokine expressions are increased in the rat hippocampus within the first hour—several days after status epilepticus (SE) (Eriksson et al., 1999; De Simoni et al., 2000; Vezzani et al., 2008; Ravizza et al., 2008; Turrin and Rivest, 2004; Minami et al., 1991). Furthermore, proinflammatory cytokines are involved in the neurotransmission (Bezzi et al., 2001; Kovács et al., 2006; Rizzi et al., 2003; Bartfai et al., 2007), since proinflammatory cytokines regulate both synaptic glutamate release and postsynaptic glutamate receptor expression (Vezzani et al., 2008; Zhu et al., 2006).

Nuclear factor-kappa B (NF-κB) pathway is involved in signal transduction in inflammatory processes (Tartaglia and Goeddel, 1992). NF-κB is a dimeric transcription factor composed of five members, p50, RelA/p65, c-Rel, RelB, and p52 that can diversely combine to form the active transcriptional dimer. NF-κB is present in the cytoplasm and serves as a critical regulator of the inducible expression of genes involved in immunity, inflammation, and cell adhesion, as well as in cell growth and death (Barnes and Karin, 1997; Li and Verma, 2002). NF-κB is normally sequestered in the cytoplasm where it is bound to the inhibitor of NF-κB (IκB). Stimuli activate upstream kinases to phosphorylate IκB leading to its ubiquitination and proteasomal degradation. IκB degradation liberates NF-κB to enter the nucleus and induce gene expression (Rothwarf and Karin, 1999). Phosphorylations of NF-κB proteins themselves are also required for optimal NF-κB activations. These NF-κB phosphorylations occur in the cytoplasm or in the nucleus, and are stimuli-/cell type-specific. Furthermore, the distinct phosphorylation site of NF-κB differently shows the differential effect of NF-κB on gene activation or repression upon various stimuli (Viatour et al., 2005).

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Abbreviations: FJB, fluoro-jade B; IκB, inhibitor of NF-κB; NeuN, neuronal specific nuclear antigen; NF-κB, nuclear factor-kappa B; PB, phosphate buffer; PBS, phosphate-buffered saline; SE, status epilepticus.

0306-4522/11 $ - see front matter © 2011 Published by Elsevier Ltd on behalf of IBRO.
doi:10.1016/j.neuroscience.2011.04.048

Although NF-κB is essential for neuron survival and its activation may protect neuron against oxidative-stress or ischemic insults, NF-κB activation can contribute to inflammatory reaction and apoptotic cell death after brain injury and stroke (Sarnico et al., 2009). With respect to the differential effect of NF-κB by phosphorylation site (Viatour et al., 2005), it is noteworthy that the specific pattern of NF-κB phosphorylation site would contribute to neuronal vulnerability in response to SE. Therefore, during the course of this study, we addressed the question of whether the distinct pattern of NF-κB phosphorylation in neuron represents the vulnerability to SE insult in the rat hippocampus in an effort to better understand the role of NF-κB phosphorylation in epileptogenic insult.

EXPERIMENTAL PROCEDURES

Experimental animals and chemicals

This study utilized the progeny of male Sprague-Dawley rats (7 weeks old) obtained from Experimental Animal Center, Hallym University, Chunchon, South Korea. The animals were provided with a commercial diet and water *ad libitum* under controlled temperature, humidity, and lighting conditions (22±2 °C, 55±5% and a 12:12hrs light/dark cycle with lights). Procedures involving animals and their care were conducted in accord with our institutional guidelines that comply with NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, 1996). In addition, all possible efforts were taken to avoid animals' suffering and to minimize the number of animals used at each stage of the experiment. All reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA), except as noted.

SE induction and drug treatments

Animals were given LiCl (3 mEq/kg, i.p.) 24 h before the pilocarpine treatment. Animals were i.p. treated with pilocarpine (30 mg/kg) 20 min after atropine methylbromide (5 mg/kg, i.p.) and were placed in individual observation chambers where seizure activity was scored according to the system of Racine (1972). Animals that entered SE typically did so within 20–30 min of the administration of pilocarpine and exhibited continuous seizure activity between 2 and 5 on the Racine scale (including akinesia, facial automatisms, limbic seizures consisting of forelimb clonus with rearing, salivation, masticatory jaw movements, and falling). Diazepam (Valium, Hoffman la Roche, Neuilly sur-Seine, France; 10 mg/kg, i.p.) was administered 2 h after onset of SE and repeated, as needed. Age-matched animals were used as non-SE experienced controls (non-SE animals, $n=7$). Non-SE animals received saline in place of pilocarpine.

Tissue processing

In our previous studies (Kang et al., 2006; Ryu et al., 2010), neuronal damage in the hippocampus was noticeable at 3–4 days after SE. Therefore, we determined 4 days after SE as the best time point to evaluate neuronal damage induced by SE. SE-induced animals ($n=15$) were perfused transcardially with phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) under urethane anesthesia (1.5 g/kg, i.p.). Control animals were also perfused by the same methods. The brains were removed, postfixed in the same fixative for 4 h and rinsed in PB containing 30% sucrose at 4 °C for 2 days. Thereafter the tissues were frozen and sectioned with a cryostat at 30 μm.

Fluoro-Jade B staining

In each animal, brain sections were rinsed in distilled water, and mounted onto gelatin-coated slides and then dried on a slide warmer. The slides were immersed in 100% ethanol for 3 min, followed by 70% ethanol for 2 min and distilled water for 2 min. The slides were then transferred to 0.06% potassium permanganate for 15 min and gently agitated. After rinsing in distilled water for 2 min, the slides were incubated for 30 min in 0.001% Fluoro-Jade B (FJB, Histo-Chem Inc., Jefferson, AR, USA), freshly prepared by adding 20 ml of a 0.01% stock FJB solution to 180 ml of 0.1% acetic acid, with gentle shaking in the dark. After rinsing for 1 min in each of three changes of distilled water, the slides were dried, dehydrated in xylene, and coverslipped with DPX.

Immunohistochemistry

In each animal, the free-floating sections were first incubated with 10% normal goat (Vector, Burlingame, CA, USA) serum for 30 min at room temperature. Some sections were incubated in each antibody, respectively: rabbit anti-p52 phospho-serine 865 NF-κB (p52-S865), p52 phospho-serine 869 NF-κB (p52-Ser869), p65 phospho-serine 276 NF-κB (p65-Ser276), p65 phospho-serine 311 NF-κB (p65-Ser311), p65 phospho-serine 468 NF-κB (p65-Ser468), p65 phospho-serine 529 NF-κB (p65-Ser529), and p65 phospho-serine 536 NF-κB (p65-S536) antibody (Abcam, Cambridge, UK, diluted 1:100) in PBS containing 0.3% Triton X-100 and 2% normal goat serum (Vector) overnight at room temperature. After washing three times for 10 min with PBS, sections were incubated sequentially, in rabbit anti-goat IgG (Vector) and ABC complex (Vector), diluted 1:200 in the same solution as the primary antiserum. Between the incubations, the tissues were washed with PBS three times for 10 min each. The sections were visualized with 3,3′-diaminobenzidine in 0.1 M Tris buffer and mounted on the gelatin-coated slides. The immunoreactions were observed under the Axioscope microscope (Carl Zeiss, Munchen-Hallbergmoos, Germany). For double immunofluorescent study, some sections were incubated in each mixture of antibodies: each NF-κB (p52-S865, p52-Ser869, p65-Ser276, p65-Ser311, p65-Ser468, p65-Ser529, p65-S536) antibody (Abcam, Cambridge, UK, diluted 1:50, respectively)/mouse anti-NeuN antibody (a neuronal marker, Millipore Corporation, Billerica, MA, USA, diluted 1:1000), p52-Ser869 antibody (Abcam, Cambridge, UK, diluted 1:50)/mouse anti-GFAP antibody (an astroglial marker, Millipore Corporation, Billerica, MA, USA, diluted 1:1000), and p65-Ser536 antibody (Abcam, Cambridge, UK, diluted 1:50)/mouse anti-somatostatin (SOM) antibody (Millipore Corporation, Billerica, MA, USA, diluted 1:1000) in PBS containing 0.3% triton X-100 overnight at room temperature. After washing three times for 10 min with PBS, sections were also incubated in a mixture of FITC-conjugated secondary antiserum and Cy3-conjugated secondary antiserum (Amersham, San Francisco, CA, USA, diluted 1:200) for 1 h at room temperature. The sections were washed three times for 10 min with PBS, and mounted on gelatin-coated slides. For nuclei counterstaining, we used Vectashield mounting medium with DAPI (Vector). For negative control, the rat hippocampal tissues were incubated with 1 μg of the antibody that was preincubated with 1 μg of purified peptide for 1 h at room temperature (for SOM) or incubated with preimmune serum instead of primary antibody (for NF-κB, NeuN, and GFAP antibody). The negative control resulted in the absence of immunoreactivity in any structures.