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Research report

Transient forebrain ischemia effects interaction of Src, FAK, and PYK2 with the NR2B subunit of *N*-methyl-D-aspartate receptor in gerbil hippocampus

Teresa Zalewska*, Małgorzata Ziemka-Nałęcz, Krystyna Domańska-Janik

NeuroRepair Department, Medical Research Centre, Polish Academy of Sciences, 5 Pawińskiego Street, 02-106 Warsaw, Poland

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Abstract

Two different models of brain ischemia were used to examine the evoked changes in the tyrosine phosphorylation of NMDA receptor subunits 2A and 2B (NR2A and NR2B), as well as their interactions with non-receptor tyrosine kinases (NRTKs: FAK, PYK2 Src), and PSD-95 protein. Only short-term 5 min ischemia followed by 3 h reperfusion resulted in the elevated tyrosine phosphorylation of both investigated NMDA receptor subunits, but in contrast to previously published data, more pronounced in the case of NR2B. Concomitantly, an increased association of NR2B with FAK, PYK2, Src and PSD-95 has been observed. This sharp early reaction to brief ischemia was markedly attenuated during prolonged recovery (72 h) with almost complete return to control values. The initial recruitment of tyrosine kinases to NMDA receptor during the first 3 h of reperfusion is generally consistent with an active postischemic remodeling of PSD and may participate in the induction of the postischemic signal transduction pathway in gerbil hippocampus. In contrast, ischemia of longer duration (up to 30 min) caused an immediate decrease in the protein levels as well as tyrosine phosphorylation of both NR2A and NR2B subunits which was accompanied by the marked attenuation of the association with their investigated molecular partners—PSD-95 and NRTKs. This effect may be mimicked in vitro by Ca^{2+}-dependent activation of endogenous calpains in purified PSD preparation suggesting irreversible deterioration of the synaptic signaling machinery during irreversible long-term ischemia.

Theme: Disorders of the nervous system
Keywords: Ischemia

Keywords: Forebrain ischemia; Hippocampus; NMDA receptor; Protein tyrosine kinases

1. Introduction

Moderate, transient cerebral ischemia sets in motion a series of pathophysiological processes that result ultimately in the death of neurons in specific brain areas at 3–4 days of initially successful reperfusion. The selective vulnerability of neurons to a brief period of brain ischemia is a well-known phenomenon that is particularly evident in the CA1 sector of the hippocampus [20]. However, the exact molecular events that initiate ischemia-induced, delayed cell degeneration in specific hippocampal area are poorly understood. Therefore, knowledge of the processes that occur in the early stages of the postischemic period has become an important objective.

Emerging data indicate that many initial responses to ischemic challenge, such as overactivation of glutamatergic receptors, mainly NMDA, and calcium influx, which can trigger a cascade of secondary events leading to neuronal degeneration, occur at postsynaptic densities (PSD). The rapid remodeling of PSD structure resulting from the early translocation/activation of several protein kinases towards postsynaptic densities [4,8,16,23,38] as well as cytoskeletal protein breakdown (MAP2 and fodrin) largely confined to this compartment [38] might lead to long lasting postische-

* Corresponding author. Fax: +48 22 668 5423.
E-mail address: terezal@cmdik.pan.pl (T. Zalewska).

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mic changes in synaptic function and selective neuronal damage. Thus, the PSD structure may be considered one of the first targets of ischemic challenge.

Since postsynaptic membranes that make up PSD consist of a complex array of proteins participating in signal transduction from the extracellular matrix (ECM) [17], it is therefore logical to anticipate the role of this signaling pathway in regulation of neuronal survival and death. It has been recently suggested that changes in adhesion responses triggered by integrin class matrix receptors are likely to be associated with synaptic conformation and modifications of the cytoskeleton [2]. Consistent with this notion remain our results which show that forebrain ischemia leads to degradation of the ECM component laminin in the CA1 sector of the hippocampus and that this may alter cell-matrix contact and suppress survival signals [36]. The key component of the pathway responsible for the flow of information from the ECM to the cell interior is focal adhesion kinase (pp125FAK), which may interact directly with other non-receptor protein kinases, such as the Src family of tyrosine protein kinases, adapter molecules, and cytoskeletal structural proteins, and perhaps provide a pathway by which the ECM may regulate cell viability [11,18,19].

Although it has been well documented that ischemia performed by four-vessel occlusion in rats induces increase in the association of several tyrosine kinases (Src family kinases and PYK2) with PSD and an elevation in the phosphorylation of several PSD proteins [4,8,26,28], in contrast, there is no information about the FAK kinase modulation which may be expected to affect the linkage between the ECM and the signaling cascade to which it is connected. According to existing data, two members of the FAK kinase family–pp125FAK and PYK2–are in close functional interaction with Src family kinases, which may play a main role in the up-regulation of NMDA receptor activity [14,22,33]. Both kinases–FAK and PYK 2–are thought to be responsible for the activation of Src. However, whereas overexpression of PYK2 precedes apoptosis in non-neuronal cells [31], pp125 FAK seems to be important for cell survival throughout the induction of ERK activity [11,34].

To provide more direct insight into the cascade of ischemia-induced events in hippocampal postsynaptic densities, in this study we have aimed to correlate the interaction of the protein tyrosine kinases FAK, PYK2, and Src with typical PSD-associated proteins, including NMDA receptor and PSD-95, a major protein component of postsynaptic densities. Due to their contradictory function, particular attention has been paid to the possible interplay between FAK and PYK2. In addition, we compare the responses of the above-mentioned kinases in two distinct types of brain ischemia: 30-min ischemia, which causes irreversible depletion of a cellular high-energy reserves and leads to generalized tissue necrosis, and a model of 5-min transient global ischemia in gerbils, which leads to delayed neuronal death in the CA1 region of the hippocampus.

2. Materials and methods

2.1. Ischemic model

Male Mongolian gerbils, weighing 50–70 g, were used to perform brain ischemia by 5- or 30-min bilateral ligation of the common carotid arteries under halothane/N_2O anesthesia in strictly controlled normothermic conditions [7]. Animals subjected to 5-min ischemia were allowed to recover 3 or 72 h after the insult. Sham-operated animals served as controls. Animals were lightly anesthetized and killed by decapitation either after a designated reperfusion time or following 30-min ischemia. Brains were quickly extracted, and in some experiments hippocampi were rapidly dissected and stored at −80 °C until analysis.

The experimental procedure was approved by the Local Commission for Ethics of Experiments on Animals.

2.2. Preparation of PSD

For purified PSD preparation, we used the entire forebrain because of the difficulty of isolating sufficient amounts of material from the hippocampus alone. The preparations of PSD were performed for only sham-operated animals by following the procedure of Cohen [5]. Ten gerbils were employed for PSD preparation and two preparations were analyzed independently. Briefly, the tissue was homogenized in a motor-driven teflon/glass homogenizer in ice-cold buffer A, containing 0.32 M sucrose, 1 mM $NaHCO_3$ pH 7.5, 1 mM $MgCl_2$, 0.5 mM $CaCO_3$, and 100 μM leupeptin. After the removal of nuclei and undisrupted cells, the supernatant (S) was washed two times in buffer A and centrifuged at 17,000 × *g* for 10 min. The pellet (crude particulate fraction, P2) was suspended in buffer B, containing 0.32 M sucrose and 1 mM $NaHCO_3$ pH 7.5, and subjected to a discontinuous sucrose density gradient by centrifugation over 0.85/1.0/1.2 M sucrose for 2 h at 100,000 × *g*. The interface between 1.0 and 1.2 M sucrose was collected (crude synaptoneurosomal fraction, SN1), resuspended in 4 × volume of buffer B, centrifuged at 48,000 × *g* for 20 min (purified synaptonerosomes, SN2), dissolved in 6 mM Tris/HCl pH 8.1 to a protein concentration 4 mg/ml, mixed (1:1 v/v) with 1% Triton X-100 in 6 mM Tris/HCl pH 8.1, and then incubated at 40 °C for 15 min. This fraction was centrifuged at 48,000 × *g* for 20 min. The pellet (synaptic membranes, SM) was resuspended in buffer B and subjected to overnight centrifugation at 275,000 × *g* over a 1.0/1.5/2.0 M sucrose gradient. The interface between the 1.5/2.0 M sucrose layers contained PSD structures. This fraction was collected and treated with 12 ml H_2O and spun down at 275,000 × *g* for 1 h. The final PSD was resuspended in 100 μl 20 mM Tris/HCl, pH 7.5 containing 20% glycerol and 1.0 mM EGTA, and kept frozen at −80 °C. All steps of the PSD purification (except one) were performed at 4 °C.