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Research Report

Identification of protein kinase C isoforms involved in cerebral hypoxic preconditioning of mice

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Abstract

Recently, accumulated studies have suggested that protein kinases C (PKC) play a central role in the development of ischemic–hypoxic preconditioning (I/HPC) in the brain. However, which types of PKC isoforms might be responsible for neuroprotection is still not clear, especially when the systematic investigation of PKC isoform-specific changes in brain regions was rare in animals with ischemic–hypoxic preconditioning. By using Western blot, we have demonstrated that the levels of cPKC βII and γ membrane translocation were increased in the early phase of cerebral hypoxic preconditioning. In this study, we combined the Western blot and immunostaining methods to investigate the effects of repetitive hypoxic exposure (H1–H4, $n = 6$ for each group) on membrane translocation and protein expression of several types of PKC isoforms, both in the cortex and hippocampus of mice. We found that the increased membrane translocation of nPKCε ($P < 0.05$, versus normoxic H0) but not its protein expression levels in both the cortex and hippocampus during development of cerebral HPC in mice. However, there were no significant changes in both membrane translocation and protein expression levels of nPKCδ, θ, η, μ, and aPKC ι/λ, ζ in these brain areas after hypoxic preconditioning. Similarly, an extensive subcellular redistribution of cPKCβII, γ, and nPKCε was observed by immunostaining in the cortex after three series of hypoxic exposures (H3). These results indicate that activation of cPKCβII, γ, and nPKCε might be involved in the development of cerebral hypoxic preconditioning of mice.
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1. Introduction

Ischemic–hypoxic preconditioning (I/HPC) is an endogenous strategy in which sublethal ischemic–hypoxic exposure protects the tissue from damage caused by a severe ischemic–hypoxic insult. This phenomenon was first observed in the heart [37] and later in gerbil brain [21]. The profound protection mechanism induced by I/HPC makes it an attractive target for a potential clinical therapeutic approach. Recently, the molecular mechanisms of intracellular signals have been reported to be involved in the development of various types of hypoxic preconditioning. These important cellular factors include adenosine and A1 receptors [17,41], ATP-sensitive potassium channels [17],

Abbreviations: I/HPC, ischemic–hypoxic preconditioning; PKC, protein kinases C; cPKC, conventional PKCs; nPKC, novel PKCs; aPKC, atypical PKCs

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nitric oxide synthase system (NOS) [15,26], hypoxia-inducible factor (HIF) [3,4], *N*-methyl-D-aspartate (NMDA) receptors [7], and superoxide dismutase (SOD) system [12]. However, this array of cellular molecules does not explain the mechanism underlying the protective effect of I/HPC. Recently, the concept of two proposed types of ischemic or hypoxic tolerance [52] was widely reported. One type reported was the tolerance induced at an early phase starting from several minutes to hours post-ischemia or hypoxia loading (classic or early preconditioning). The other type was observed at a delayed phase, which occurred several days post-ischemia (second window of protection or delayed preconditioning). The two types differed in the induction mechanism and duration [34]. The early preconditioning disappeared within 2 h thereafter and was reported to be related to changes in the intracellular signal transduction pathways, such as protein kinases and protein phosphatase. However, the delayed preconditioning reappeared 24 to 72 h after the initial preconditioning stimulation, and its mechanism was reported to be associated with the activation of cellular genome changes and a novel protein synthesis process. To understand the defense mechanism that may contribute to the accommodation of ischemia–hypoxia, it is necessary to detect the intracellular signal transduction system as both early and delayed preconditioning.

It has been demonstrated that the activation or translocation of protein kinases C (PKC) in the central nervous system may play a key role in mediating both classic and delayed preconditioning [18,19,36,44,47,48,59]. However, information regarding which types of PKC isoforms are responsible for neuroprotection is unclear, especially changes in active PKC isoforms in specific brain regions in mice with a systematic intact HPC exposure. It is well known that PKC family members catalyze serine/threonine phosphorylation of target proteins and have been implicated in regulating the expression or activation of several neurotransmitters, such as growth factors and tumor promoters. Currently, at least 11 PKC isoforms have been identified in in vivo experiments. According to the forms of their activation, PKC isoforms were included in the following groups: (1) conventional PKCs (cPKCα, βI, βII, and γ) which require diacylglycerol, phosphatidylserine, and Ca^{2+} influx; (2) novel PKCs (nPKCδ, ε, η, θ, and μ) which are not dependent on Ca^{2+} influx but do require diacylglycerol; and (3) atypical PKCs (aPKCζ and ι/λ) whose regulation are not fully understood and may not require either Ca^{2+} influx or diacylglycerol. Therefore, it is important to identify the PKC isoforms responsible for cerebral HPC at the level of the intact animal. This study may lead to a better understanding of the signal transduction mechanisms underlying early and late I/HP conditioning and the PKC family members involved.

To investigate the molecular mechanism of I/HPC in the brain, we have developed a unique HPC-induced "auto-hypoxia" mouse model that mimics clinical asphyxia [32]. Using this model, we have demonstrated that the survival time in hypoxic chamber, cyanide toxification, mandibular respiration, and spinal reflex in vitro was increased, as well as the enhanced SOD and adenosine with decreased expression of lipid peroxidase, glutamate (GLU), and nitric oxide [12,31,32]. The cerebral cortex and hippocampus neurons are particularly vulnerable to ischemia–hypoxia insult [6], and changes in the cellular membrane translocation, as well as changes in their plasma membrane, cytoskeletal elements, and perinuclear structures, are the key targets that may be involved in the activation of PKC [27,42,56], especially since there is currently no information available regarding the effects of HPC on individual PKC isoforms in brain using an in vivo animal model. The main goal of this study is to identify which PKC isoforms are responsible for cerebral HPC. Additionally, the membrane translocation and protein expression of all 11 PKC isoforms in the cortex and hippocampus areas have been investigated in mice with induced cerebral HPC after repetitive hypoxic exposure.

2. Materials

The following materials were obtained from the indicated sources: proteinase inhibitors (leupeptin, aprotinin, pepstatin A, and chymostatin); phosphatase inhibitors (okadaic acid, sodium pyrophosphate, and potassium fluoride); and monoclonal anti-β-actin antibody, as well as other reagents, such as dithiothreitol (DTT), Nonidet P-40, EDTA, EGTA, and SDS, were purchased from Sigma Company (St. Louis, MO, USA). Protein assay reagent, horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG was purchased from Bio-Rad Company (Hercules, CA, USA).

2.1. Hypoxic preconditioned mouse model

Experiments were conducted at room temperature (18–22 °C) on adult BALB/c mice (weighing 18–20 g) of both sexes. All experimental protocols were approved by the University Animal Care and Use Committee of Capital University of Medical Sciences and were consistent with the NIH policy on the use of experimental animals. The hypoxic preconditioned mouse model was adapted with minor modifications as previously discussed [12,31,32]. Briefly, the mice were placed individually in a 125-ml airtight jar with fresh air and sealed with a rubber plug to duplicate a progressive auto-hypoxia environment. This procedure is believed to closely simulate the clinical conditions of "asphyxia". The mice were removed from the sealed jars as soon as the first gasping appeared (an indicator of end hypoxic exposure) and the tolerant time recorded. A minimum of 30 min was allowed for recovery under normoxic conditions. Next, the mice were switched to another hermetically sealed jar with the same volume of fresh air after recovery from the previous hypoxic exposure. The hypoxic precondition was evaluated by the overall behavior changes and several physiological parameters such

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