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BRAIN RESEARCH

Research Report

Neuroprotective effect of the peptides ADNF-9 and NAP on hypoxic–ischemic brain injury in neonatal rats

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ABSTRACT

Perinatal asphyxia is an important cause of neonatal mortality and subsequent serious sequelae such as motor and cognitive deficits and seizures. Recent studies have demonstrated that short peptides derived from activity-dependent neurotrophic factor (ADNF) and activity-dependent neuroprotective protein (ADNP) are neuroprotective at femtomolar concentrations. However, the effect of these peptides on the hypoxic–ischemic brain injury model is unknown. The aim of this study is to investigate the effects of the peptides ADNF-9 and NAP on neurodegeneration and cerebral nitric oxide (NO) production in a neonatal rat model of hypoxic–ischemic brain injury. Seven-day-old Wistar Albino rat pups have been used in the study ($n=42$). Experimental groups in the study were: sham-operated group, ADNF-9-treated hypoxia–ischemia group, NAP-treated hypoxia–ischemia group, ADNF-9+NAP-treated hypoxia–ischemia group, and vehicle-treated group. In hypoxia–ischemia groups, left common carotid artery was ligated permanently on the seventh postnatal day. Two hours after the procedure, hypoxia (92% nitrogen and 8% oxygen) was applied for 2.5 h. ADNF-9, NAP, and ADNF-9+NAP were injected (intraperitoneally; i.p.) as a single dose immediately after the hypoxia period. Brain nitrite levels, neuronal cell death, and apoptosis were evaluated in both hemispheres (carotid ligated or nonligated) 72 h after the hypoxic–ischemic insult. Histopathological evaluation demonstrated that ADNF-9 and NAP significantly diminished number of "apoptotic cells" in the hippocampal CA1, CA2, CA3, and gyrus dentatus regions in both hemispheres (ligated and nonligated). When compared with vehicle-treated group, combination treatment with ADNF-9+NAP did not significantly reduce "apoptotic cell death" in any of the hemispheres. ADNF-9 and NAP, when administered separately, significantly preserved the number of neurons CA1, CA2, CA3, and dentate gyrus regions of the hippocampus, when compared with vehicle-treated group. The density of the CA1, CA2, and dentate gyrus neurons was significantly higher when combination therapy with ADNF-9+NAP was used in the carotid ligated hemispheres. In the nonligated hemispheres, combination therapy preserved the number of neurons only in the CA1 and dentate gyrus regions. Brain nitrite levels were evaluated by Griess reagent and showed that hypoxic–ischemic injury caused a significant increase in NO production. Brain nitrite levels in ADNF-9+NAP-treated animals were not

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different in carotid ligated or nonligated hemispheres. The peptides ADNF-9 and NAP significantly decreased NO overproduction in the hypoxic–ischemic hemisphere, whereas no significant change appeared in hypoxia alone and also in the sham-operated group. These results suggest the beneficial neuroprotective effect of ADNF-9 and NAP in this model of neonatal hypoxic–ischemic brain injury. To our knowledge, this is the first study that demonstrates a protective effect of these peptides against hypoxia–ischemia in the developing brain.

1. Introduction

Cerebral hypoxia–ischemia is a major cause of perinatal brain injury and is an important cause of neonatal mortality and leading ultimately by neurological sequelae such as cerebral palsy, mental retardation, learning disability, and epilepsy (Berger and Garnier, 1999; Tan et al., 1998; Tomfighi et al., 1997). Despite the fact that perinatal asphyxia closely corresponds to experimental models of cerebral hypoxia–ischemia, where successful neuroprotective interventions were introduced, currently no agent has been proven valuable to improve the chronic sequelae of perinatal asphyxia in the clinical setting (Tomfighi et al., 1997; Vannucci and Perlman, 1997). It is obvious that destructive processes such as glutamate and nitric oxide (NO) neurotoxicity, free radical formation, intracellular calcium accumulation, and immune/inflammatory activation continue to damage the brain for many hours after oxygenation and circulation have been restored (Delivoria-Papadopoulos and Mishra, 1998; Fellman and Raivio, 1997; Higuchi et al., 1998; Tan et al., 1998). Pharmacological agents may provide neuroprotection in this condition interfering with any of these processes.

The most accepted and widely used animal model of perinatal asphyxia is a modification of the Levine preparation described by Rice et al. (Vannucci, 2000), which includes combination of ischemia, obtained by unilateral occlusion of carotid artery, followed by exposure to hypoxia in 7-day-old rats. Neurodevelopmental stage of 7-day-old rats corresponds to that of newborn infants (Romijn et al., 1991; Tomfighi et al., 1997). This animal model represents a useful tool for studying potential neuroprotective strategies capable of preventing or limiting the perinatal hypoxic–ischemic injury in humans.

Two peptides NAP and ADNF-9 are associated with novel glial proteins regulated by vasoactive intestinal peptide and may provide protective intervention in a model of hypoxic–ischemic brain injury in newborn rats. Neuropeptides exhibit multiple roles in the maintenance of homeostasis. They were found to exert neurohormonal and neurotransmitter effects in the central and peripheral nervous system. They also act as regulators of cell division, differentiation, and survival (Gozes et al., 1999; Said, 1996b). VIP is extensively distributed in the nervous system (Gozes et al., 1987). Its neuroprotective properties were first described in spinal cord cell cultures with a maximum effect at concentrations as low as 0.1 nM (Brenneman and Eiden, 1986). It has been shown that some of the neuroprotective action of vasoactive intestinal peptide (VIP) is mediated by two different glial derived proteins: activity-dependent neurotrophic factor (ADNF) (Brenneman and Gozes, 1996) and activity-dependent neuroprotective protein (ADNP) (Bassan et al., 1999). Their active sites have been identified and synthesized as short peptides ADNF-9 (16), a 9-amino acid peptide, and NAP, an 8-amino acid peptide (Bassan et al., 1999). Both ADNF-9 and NAP are protective at femtomolar concentrations in vitro against the neural toxicity of a wide range of compounds and cellular insults (Brenneman et al., 1998; Divinski et al., 2004; Glazner et al., 1999; Gozes and Divinski, 2004; Holtser-Cochav et al., 2006; Zamostiano et al., 1999). The peptides are also neuroprotective in vivo against diverse neuronal insults, including excitotoxicity (Gressens et al., 1997), closed head injury (Beni-Adani et al., 2001), ischemic brain injury (Leker et al., 2002), apoplipoprotein E deficiency (Bassan et al., 1999), exposure to the cholinotoxin ethylcholine aziridium (Gozes et al., 2000), and prenatal ethanol exposure (Spong et al., 2001). Therefore, the aim of this study was to investigate the possible neuroprotective and ameliorating effect of ADNF-9 and NAP treatment immediately after hypoxic–ischemic injury induced neuronal cell death, apoptosis, and NO formation in a neonatal rat model.

2. Results

2.1. *Effects of ADNF-9 and NAP treatment on neuronal density*

Seventy-two hours after hypoxic–ischemic brain injury, treatment with ADNF-9 and NAP significantly preserved the number of neurons CA1, CA2, and CA3 regions of hippocampus and dentate gyrus in the right and left hemispheres when compared with vehicle-treated group ($p<0.05$) (Tables 1–2). The densities of the CA1, CA2, and dentate gyrus neurons were significantly higher in the ADNF-9+NAP combination treatment group when compared with vehicle-treated group ($p<0.05$) in the left hemispheres. The neuronal densities of CA1 and gyrus dentatus were significantly higher than vehicle-treated groups in the right hemispheres in the brains of these animals (Table 3). The neuronal densities of CA3 region in the left hemispheres and CA2 and CA3 regions of the right hemispheres were not significantly different between ADNF-9+NAP and vehicle-treated groups ($p>0.05$) (Table 3) (Fig. 1).

2.2. *Effects of ADNF-9 and NAP treatment on apoptosis*

TUNEL-positive cells showed the typical morphological features of apoptosis such as the chromatin condensation, cytoplasmic budding, and apoptotic bodies. Seventy-two hours after hypoxic–ischemic brain injury, increased number