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NAP protects against cytochrome *c* release: Inhibition of the initiation of apoptosis

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ABSTRACT

NAPVSIPQ (NAP), an 8 amino acid peptide derived from activity-dependent neuroprotective protein (ADNP), provides neuroprotection through interaction with microtubules. Previous results have demonstrated NAP protection against oxygen-glucose deprivation in hippocampal cells in culture. Furthermore, in vivo studies have shown that NAP reduces caspase 3 activation in rats subjected to permanent mid-cerebral artery occlusion (a rat model of stroke). Oxygen-glucose deprivation (ischemia) has been associated with microtubule breakdown and cytochrome *c* release from mitochondria leading to apoptosis. Here, NAP in concentrations ranging from 10^{-14} M to 10^{-8} M completely blocked cytochrome *c* release in cortical neurons subjected to oxygen-glucose deprivation. Furthermore, quantitative microscopy coupled to microtubule immunocytochemistry suggested that NAP prevented microtubule degradation under oxidative stress. As cytochrome *c* release is a known initiator of the apoptotic pathway, it is suggested that NAP inhibits the early events of apoptosis.

1. Introduction

Apoptosis has been shown to play a critical role in neuronal death caused by ischemia (Fujimura et al., 1998; Graham and Chen, 2001; Zhao et al., 2005). Mitochondrial balance is mediated by pro- and anti-apoptotic proteins of the bcl-2 family (Cory and Adams, 2002) which in turn depend on the microtubule network mobility and vice-versa (Longuet et al., 2004). During apoptosis, cytochrome *c* is released from the mitochondria into the cytosol and causes subsequent caspase-9 and caspase-3 activation (Budihardjo et al., 1999; Zhao et al., 2005; Zou et al., 1999).

NAP (NAPVSIPQ) is a potent neuroprotective peptide derived from activity-dependent neuroprotective protein, ADNP (Bassan et al., 1999; Gozes, 2007; Zamostiano et al., 2001). ADNP expression has been shown to be regulated by brain injury associated with head trauma (Gozes et al., 2005b; Zaltzman et al., 2004), the NO-cGMP pathway in the hippocampus during kainic acid-induced seizure (Cosgrave et al., 2008), exposure to alcohol in fetal alcohol syndrome (Pascual and Guerri, 2007; Poggi et al., 2003) and exposure to the anesthetic gas xenon (Cattano et al., 2008).

NAP has shown protection against the consequences of ADNP deficiency/mis-metabolism in ADNP+/− mice (Vulih-Shultzman et al., 2007), in models of closed head injury (Beni-Adani et al., 2001; Zaltzman et al., 2005) and in models of fetal alcohol syndrome (Pascual and Guerri, 2007; Sari and Gozes, 2006; Spong et al., 2001). In the model of prenatal ethanol exposure, it was suggested that NAP could exert its actions by the activation of mitogen-activated protein kinase/extracellular signal-regulated protein kinase, the phosphatidylinositol-3-kinase (PI-3K)/Akt pathways and the transcription factor cAMP response element-binding protein which are important in neuronal growth and differentiation contributing to neuronal plasticity and protection against injury-associated apoptosis (Pascual and Guerri, 2007). Further studies associated NAP neurotrophic function (Gozes and Spivak-Pohis, 2006) and protection against ethanol intoxication with the activation of Fyn kinase (Chen and Charness, 2008), a central molecule in development and cellular regulation. Additionally, ethanol exposure was associated with drug-induced neuro-apoptosis (Olney et al., 2002b) which could be measured by caspase 3 activation (Olney et al., 2002a).

The current research is aimed toward first steps in the elucidation of pathways associated with NAP protection against oxygen-glucose deprivation. Previous studies have shown that NAP protects against oxygen deprivation and ischemia during postnatal development (Rotstein et al., 2006), and against mid-cerebral artery occlusion (a hypertensive rat stroke model) (Gozes et al., 2005a; Leker et al., 2002). In the rat model of stroke, NAP was shown to provide in vivo protection against apoptotic pathways, as it inhibited caspase 3 activation and protected against DNA fragmentation (Leker et al., 2002). In vitro, NAP was shown to be neuroprotective in vitro against

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oxygen-glucose deprivation in cortical and hippocampal cultures and against oxidative stress in neuronal cell lines (Steingart et al., 2000; Zemlyak et al., 2000, 2007).

NAP, which provides protection at femtomolar concentrations, seems to interact with the glial and neuronal microtubules after cellular internalization, a process which does not seem to require a conventional peptide-receptor interaction (Divinski et al., 2004).

As mitochondrial function requires an intact microtubule network which is disrupted by oxidative damage (Santa-Maria et al., 2005) we have investigated if the microtubule-interacting agent, NAP, prevents mitochondrial dysfunction, as measured by cytochrome *c* release in the model of oxygen-glucose deprivation in vitro. Parallel experiments measured the number of cells with intact microtubules (stained with beta III tubulin).

2. Materials and methods

2.1. Tissue culture

Cortical neurons (Brooke et al., 1997) were used for the assessment of NAP protection against cytochrome *c* release following oxygen-glucose deprivation. Briefly, cortical tissue was removed from 18-day-old fetal Sprague–Dawley rats. Cells were dissociated with papain, filtered through an 80-µm cell strainer and resuspended in a modified MEM (Univ. California, San Francisco, Tissue Culture Facility) containing 30 mM glucose and 10% horse serum (HyClone, Logan, UT). Cells were plated at a density of 1.2×10^5 cells/cm^2 on cover slips coated with Poly-D-Lysine (Sigma, St. Louis, Mo). Experiments were conducted on days 10–12 of culturing as follows. Cells on cover slips were treated with various concentrations of NAP (10^{-15}M–10^{-12}M as well as 10^{-8}M, dissolved in MEM). These NAP concentrations were previously shown to be protective against oxygen-glucose deprivation in hippocampal cultures (Zemlyak et al., 2007). Cultures were exposed to oxygen-glucose deprivation by replacement of the cell media with 0 mM glucose MEM (made in-house) and incubation in an anaerobic environment of 90% nitrogen, 5% CO_2 and 5% hydrogen for 3 h followed by 3 h of normoxic/normoglycemic conditions (for cytochrome *c* release assays). Time course experiment (15 min–24 h) suggested that this timed paradigm is optimal to achieve maximal cytochrome *c* release. The cultures were fixed in cold methanol. In control cultures, media was changed to MEM containing 5 mM glucose and left in normoxic conditions.

2.2. Cytochrome c staining

Cold methanol-fixed cultures were treated with Triton X-100 (0.2% in PBS) for 30 min and blocked for another 30 min in 3% BSA in Triton X-100 (0.2% in PBS) before addition of primary anti-cytochrome *c* antibody (1/200 dilution) (Clone 6H2.B4, BD Pharmingen, CA,USA) in 0.1% Triton X-100/1% BSA in PBS for 24 h. Secondary antibody was conjugated to fluoresceine—FITC (1/200 dilution) (Vector, Burlingame, CA, USA) and added in 0.1% Triton X-100/1%BSA in PBS for 18 h. Nuclei were visualized with DAPi staining.

Using a representative sample of 30 images from each coverslip, the amount of cytochrome *c* was detected using an Olympus IX70 Fluorescent Light Microscope and Metamorph Imaging Software (Molecular Devices, Sunnyvale, CA). Data were collected in the form of total antibody (anti-cytochrome *c*)/FITC fluorescence (490 nm). Cultures lacking primary antibody but stained with FITC secondary antibody were used as controls to generate a value for background fluorescence. These values were subtracted from intensity images of cultures stained with anti-cytochrome *c*. From subtracted images, the amount of mitochondrial cytochrome *c* release was determined using in-house automated computer analysis within Metamorph. Intensity values were corrected for the number of cells per field. The intensity value obtained for the oxygen-glucose deprived cells was expressed as a percentage of the intensity value obtained for control-treated cells (normoxia/normoglycemia).

2.3. Tubulin staining

Cortical cultures on the coverslips were treated with various concentrations of NAP (10^{-15}M–10^{-8}M) or with vehicle. The cultures were further exposed to oxygen-glucose deprivation for 3 h and 4.5 h of reperfusion, by restoring glucose levels (adding glucose to a 5 mM final concentration) and replacing the cultures under aerobic conditions (5% CO_2, 95%O_2). The 4.5 h reperfusion was chosen over the 3 h reperfusion used in the case of cytochrome *c* release, as microtubule breakdown was minimal at 3 h reperfusion and reached a maximal oxygen-glucose deprivation-induced disruption (80%) at 4.5 h reperfusion. Control cultures were left in normoxia/normoglycemia the same as in cytochrome *c* assay. Cells were fixed in cold methanol. For tubulin staining the cultures were treated with Triton X-100 (0.2%) for 30 min and blocked in 5% powdered milk in PBS. Primary antibody–anti bIII-tubulin (was originally from Calbiochem. USA and later from Chemicon, MA) (dilution 1/200) was followed by secondary conjugated to Rhodamine (AlexaFluor 594; Calbiochem; dilution1/600). Nuclei were visualized with DAPi staining. Fluorescent imaging was done using Metamorph Imaging Software (Sunnyvale, Ca). Images that were acquired were analyzed by counting cells with intact and disrupted microtubules. The criteria used for disruption of microtubules included beading and the appearance—or lack of microtubule structures (Fig. 3). Approximately three hundred cells per experimental condition were counted. Per each experimental condition, the percentage of cells with completely intact microtubule structures was calculated. The rater was blinded to the experimental condition. For a detailed view, stained cells were visualized by confocal microscopy as before (Divinski et al., 2006).

2.4. Statistics

The results were analyzed by one-way ANOVA with Tukey post-hoc test.

3. Results

Fig. 1 demonstrates representative images from an oxygen-glucose deprived culture and a NAP-treated oxygen-glucose deprived culture, showing reduced cytochrome *c* release (stained by FITC—green) following NAP treatment. Quantitative analysis showed that oxygen-glucose deprivation significantly increased ($P<0.05$) cytochrome *c* release, which was totally reversed by NAP (10^{-13}M–10^{-8}M) treatment ($P<0.05$; Fig. 2). 10^{-15}M NAP showed a trend toward protection, but did not reach a level of significance. The NAP protection seemed to reach higher levels (not significant) as compared to control, which have been observed before and were related to protection against naturally occurring cell death in these culture systems (Bassan et al., 1999).

For staining with anti-tubulin antibodies, cells were fixed as above. Theses tubulin antibodies stain free tubulin as well as assembled microtubules which are easily discernable in the intact cells. Results showed disrupted microtubules in oxygen-glucose deprived neurons (Fig. 3A,B). Treatment with NAP (10^{-14}M–10^{-8}M) resulted in the protection of cellular microtubules as observed by fluorescent microscopy (Fig. 3A). Stained cells were also visualized by confocal microscopy as before (Divinski et al., 2006). Results shown in Fig. 3B demonstrate qualitative NAP protection.

In parallel, fluorescent staining was quantitated using Metamorph Imaging Software followed by neuronal counting (Fig. 4). Results showed complete protection of the microtubular system by NAP treatment. As in the case of cytochrome *c* release, the NAP protection