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Recombinant activity-dependent neuroprotective protein protects cells against oxidative stress

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Abstract

Activity-dependent neuroprotective protein (ADNP) is essential for brain formation. Here, we investigated the potential neuroprotective effects of recombinant ADNP under stress conditions. The human ADNP cDNA was sub-cloned into a vector that contains VP22, a Herpes virus protein that may allow penetration of fused proteins through cellular membranes. When incubated with pheochromocytoma (PC12) cells, a neuronal model, VP22-ADNP was associated with the cells after a 25-min incubation period. Pre-incubation with VP22-ADNP enriched protein fractions protected against β amyloid peptide toxicity and oxidative stress (H_2O_2) in PC12 cells. VP22 by itself was devoid of protective activity. Furthermore, the pro-apoptotic protein p53 increased by 3.5-fold from control levels in the presence of H_2O_2, while treatment with VP22-ADNP prior to H_2O_2 exposure significantly reduced the p53 protein levels. ADNP expression was previously shown to oscillate as a function of the estrus cycle in the mouse arcuate nucleus, these oscillations are now correlated with increased cellular protection.

Keywords: Activity-dependent neuroprotective protein (ADNP); β Amyloid 25–35; Pheochromocytoma (PC12); Neuroprotection; P53; Oxidative stress

1. Introduction

Activity-dependent neuroprotective protein (ADNP) (Bassan et al., 1999) was cloned and characterized as a vasoactive intestinal peptide (VIP) responsive gene (Bassan et al., 1999; Zamostiano et al., 2001). ADNP mRNA is highly expressed in the cerebellum, cortex, hippocampus medulla and the midbrain in humans and in rodents. ADNP expression level increases in mouse embryo during brain and central nerve system formation (Poggi et al., 2002; Pinhasov et al., 2003). Gene knock-out experiments resulted in neural tube closure failure and embryonic death, implicating ADNP as an essential protein for brain formation (Pinhasov et al., 2003). The estimated human ADNP gene size is 40,647 bp, consisting of five axons and located on human chromosome 20q12–13.2. The putative ADNP protein [estimated protein size 123562.8 Da and apparent molecular weight on sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis ∼114,000 Da] contains nine zinc fingers, a proline rich region and a homeobox profile that includes a nuclear transport domain (Zamostiano et al., 2001; Gozes et al., 2000).

ADNP includes an active neuroprotective site of eight amino acid, NAPSVIPQ (NAP, Bassan et al., 1999). In vitro NAP has shown neuroprotective activity in an Alzheimer's disease model (β amyloid toxicity) (Gozes et al., 1999; Zemlyak et al., 2000), Parkinson's disease model (6-hydroxydopamine toxicity) (Offen et al., 2000) and oxidative stress models (H_2O_2) (Steingart et al., 2000) in rat cerebral cortex primary neuronal cultures and neuronal cell models such as pheochromocytoma (PC12) and neuroblastoma. In vivo injection of NAP 15 min after closed head injury caused significant reduction in the severity of the injury outcome as examined by mortality rate, edema level, and neurobehavioral functions (Beni-Adani et al., 2001; Romano et al., 2002; Zaltzman et al., 2005).

P53 is known as a pro-apoptotic protein that is involved in neuronal cell death (Morrison et al., 2003). The level of the p53 protein increases in neurons after acute injury such as ischemia or oxidative stress (Chopp et al., 1992; Napieralski et al., 1999) as well as in the brain of patients or animals with chronic neurodegeneration like in Alzheimer's disease (de la Monte et al., 1997). In addition, it has been showed that a reduction in p53 expression can protect neurons from ischemia or the β amyloid toxin (Culmsee et al., 2001). NAP was recently shown to protect against increases in p53 that are associated with oxidative stress. Here, ADNP was shown to protect PC12 cells against oxidative stress induced by H_2O_2 as

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well as against β amyloid toxicity. Further studies demonstrated changes in the expression levels of p53 following treatment with ADNP.

2. Materials and methods

2.1. Expression and purification of VP22-ADNP fusion protein

The human ADNP (hADNP, Zamostiano et al., 2001) was cloned from human brain cDNA library (Stratagene, La Jolla, CA, USA) by polymerase chain reaction (PCR) using the following primers, 5′-ATGTTCAACTTCCT-3′ (forward) and 5′-AGGGAACCTGGCACTTAG-3′ (reverse) (Steingart et al., 2002). The expected PCR product of 3.3 kb was sub-cloned into the Voyager vector (Invitrogen, Ghroningen, Netherlands). This vector includes the Herpes virus protein VP22 upstream to the insert cDNA and both a c-myc and a His tag downstream to the insert.

The voyager vector that included the ADNP insert was expressed in BL21 cells (Invitrogen). The expression of the protein was induced by incubation with 1 mM IPTG for 3 h and the cells were collected (according to the Invitrogen protocol). The fusion protein, VP22-ADNP was purified on a Nickel column (Ni-AT, Qiagen, Hilden, Germany). The VP22 protein is able to penetrate through cell membranes, and by fusion to another protein, it may enable the foreign protein's penetration. The purified VP22-ADNP was used in cell culture experiments.

2.2. Cell cultures

Rat pheochromocytoma (PC12) cells (Steingart et al., 2000) were grown in 75 mm flasks in Dulbecco's Modified Eagle's Medium (DMEM) containing 8% fetal calf serum, 8% donor horse serum, 2 mM L-glutamine and penicillin–streptomycin solution (Biological Industries, Beit-Haemek, Israel). For survival assays, cells were seeded in 24 well plates (2×10^5 cells/well), that were pre-coated with collagen (Sigma, Rehovot, Israel) in the above medium supplemented with 50 ng/ml nerve growth factor (NGF). After 3 incubation days, the VP22-ADNP fusion protein or the VP22 protein (control) were added for an additional pre-incubation period of 25–35 min. This was followed by the addition of 300 μM H_2O_2 (Steingart et al., 2000) or the 75 μM β amyloid peptide (amino acids 25–35); for 16 or 48 h, respectively. Cell survival was measured by mitochondrial activity using a tetrazolium compound, [3-(4,5-dimethylthiazol)-2-yl-3-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl) 2H tetrazolium (MTS)] (Promega, Madison, WI, USA; Steingart et al., 2000).

2.3. Western analysis

Protein samples from treated cells or Ni column purification were separated by 10% polyacrylamide SDS gel electrophoresis, and transferred to PolyScreen, PVDF transfer membranes (NEN, Boston, MA, USA). The VP22-ADNP fusion protein level was determined by: (1) rabbit polyclonal affinity purified antibody against a peptide derived from ADNP (989 CEMKPGTWS-DESSQSEDARSSKPAAKK 1015; Zamostiano et al., 2001); (2) mouse monoclonal VP22 antibodies (at a 1:5000 dilution) or (3) rabbit polyclonal poly-His antibody (at a dilution of 1:1000; Sigma, Rehovot, Israel). P53 protein level was assessed by mouse monoclonal P53 antibody (at a 1:300 dilution; Santa Cruz, Santa Cruz, CA, USA). The level of β actin was detected by rabbit polyclonal antibodies (at 1:2500 dilution; Sigma) and served as an internal standard. Detection was performed using the appropriate horse radish peroxidase conjugated secondary antibody (1:25,000 dilution; Sigma or Jackson, ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and ECL+ Western blotting detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK). Proteins on the gels were stained by GelCode blue gel staining reagent (Pierce, Rockford, IL, USA).

2.4. RNA purification

RNA was obtained using the Tri-Pure reagent (Roche, Indianapolis, IN, USA) following the manufacturer's instructions. RNA samples were then purified from residual DNA using the DNA Free kit (Ambion, Austin, TX, USA).

2.5. Statistical analysis

Each experimental point was performed in duplicates or triplicates. Each value presented is the mean + S.E.M of 3–4 independent experiments. Statistical analysis was performed using one-way ANOVA with post-hoc Kruskal–Wallis analysis of variance.

3. Results

3.1. Expression and purification of the VP22-ADNP fusion protein in bacteria

Fig. 1A demonstrates the different stages of purification of VP22-ADNP and VP-22 on a Ni column that binds the poly-histidine chain of recombinant proteins. Fig. 1B shows the identification of the purified proteins with a VP22 antibody on western blots (VP22 by itself, 22 kDa, lane 6). The fusion protein was also detected when a specific antibody to ADNP was used (Fig. 1C). The purification of the VP22-ADNP fusion

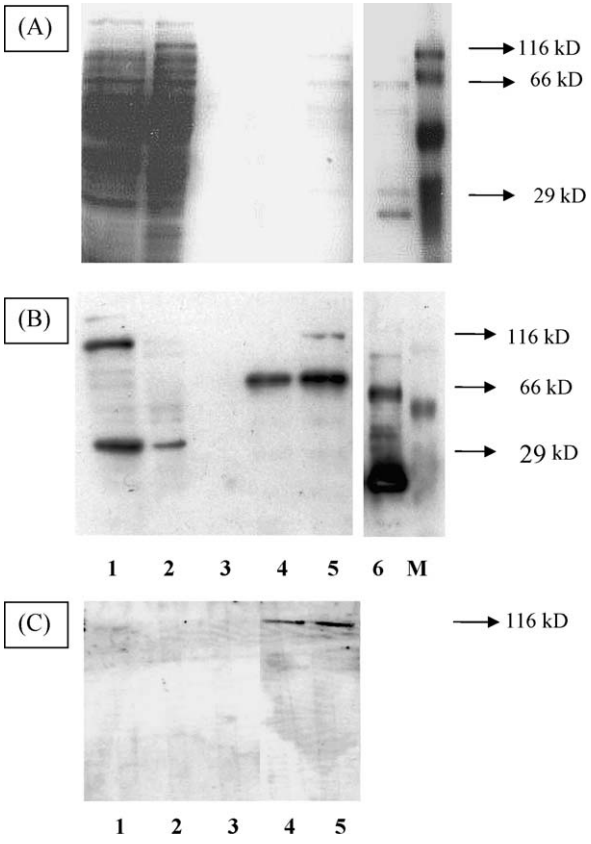

Fig. 1. Purification of VP22 and the VP22-ADNP fusion protein on Ni column. (A) GelCode blue stain reagent (Pierce) staining of SDS-10% polyacrylamide gel with samples from the different purification stages of the Ni column. (B) Western analysis with antibodies directed against VP22. (C) Western analysis with antibodies directed against ADNP (anti CEM, Zamostiano et al., 2001). Gel lanes are as follows: 1, crude extract (VP-22-ADNP fusion protein) in 20 mM Tris, pH 7.5, 5 mM imidazole, 500 mM NaCl; 2, column wash-through (VP-22-ADNP fusion protein); 3, column wash (VP-22-ADNP fusion protein): 20 mM Tris, pH 7.0, 20 mM imidazole, 500 mM NaCl, 10% glycerol; 4 and 5, VP-22-ADNP fusion-protein elution; 6, VP-22 elution off a column similar to the one described in lanes 1–5; M, Markers: Bio-Rad: Precision Plus Protein Dual Color Standards.