A femtomolar-acting neuroprotective peptide induces increased levels of heat shock protein 60 in rat cortical neurons: a potential neuroprotective mechanism

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Abstract

Activity-dependent neurotrophic factor (ADNF) was recently isolated from conditioned media of astrocytes stimulated with vasoactive intestinal peptide (VIP). ADNF provided neuroprotection at femtomolar concentration against a wide variety of toxic insults. A nine amino acid peptide (ADNF-9) captured with even greater potency the neuroprotective activity exhibited by the parent protein. Utilizing Northern and Western blot analyses, it was now shown that ADNF-9 increased the expression of heat shock protein 60 (hsp60) in rat cerebral cortical cultures. In contrast, treatment with the Alzheimer’s toxin, the β-amyloid peptide, reduced the amount of intracellular hsp60. Treatment with ADNF-9 prevented the reduction in hsp60 produced by the β-amyloid peptide. The protection against the β-amyloid peptide-associated cell death provided by ADNF-9 may be mediated in part by intracellular increases in hsp60. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

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Activity-dependent neurotrophic factor (ADNF) [3] is unique among the neuroprotective compounds in that it acts at femtomolar concentration [3], has structural similarity to heat shock protein 60 (hsp60) [3,7], and the entire protein’s neuroprotective activity is mimicked by short peptides derived from it [3,5,7,10].

ADNF constitutes a part of the neuroprotective milieu secreted from astroglial cells in the presence of the neuro-peptide vasoactive intestinal peptide (VIP) [4,17]. The active site of ADNF has recently been shown to be associated with a stretch of nine amino acids, ADNF-9. ADNF-9 protected neurons against death associated with electrical blockade (tetrodotoxin), excitotoxicity (N-methyl-D-aspartate, NMDA) and the Alzheimer’s neurotoxin (the β-amyloid peptide [2,6], amino acids 25–35 [5]).

Hsp60 is a stress protein associated with protein folding and hence supports cell survival [12]. Exposure of neurons to an elevated temperature is sufficient to induce the heat shock response and to provide neuroprotection [14]. The current study investigated whether part of the activity of ADNF-9 is mediated through intracellular increases in hsp60.

Peptide synthesis and purification of ADNF-9 (SAL-LRSIPA) was conducted as previously described [5]. Cerebral cortical cultures from newborn rats were used [3,5,8,10]. Neurons were plated on a bed of astrocytes and cultures were maintained for 1 week. Peptides ADNF-9 (10–15 M, dissolved in DMSO, 1 mg/10 ml, followed by serial dilutions in saline) and β-amyloid (amino acids 25–35, 25 μM) were added for a 3-h incubation period at 37°C/10% CO₂ RNA was extracted from the cultures utilizing RNAzolB (Biotex, Friendswood, TX) and subjected to Northern blot hybridizations [9]. The hsp60 mRNA was detected by Northern blot hybridization utilizing a radioactive probe. The probe was obtained by reverse transcript-
tion of total rat brain RNA followed by polymerase chain reaction (RT-PCR) with the hsp60-specific primers: 5'-CGAGCCTTAATGTCAAGG-3' and 5'-GTGATGACACCTCTTCTCC-3' using AmpliTaq DNA Polymerase (Perkin Elmer, Branchburg, NJ) and α-32P-dCTP (Amersham, Little Chalfont, UK; 3000 Ci/mmol).

To obtain glia-depleted, neuronal cultures [5], 35 mm culture dishes were coated with 10 mg/ml of poly-L-lysine. Dissociated cerebral cortical cells (3 x 10^6 cells) were added to the culture dish with 5% horse serum in MEM supplemented with defined medium components. The cultures grown on poly-L-lysine were not totally free of astrocytes as determined by immunocytochemical analysis with antiserum to glial fibrillary acidic protein; rather, astrocytes comprised < 10% of the total cells in the cultures. Seven days after adding the cerebral cortical suspension to the dishes, the culture preparations were given a complete change of medium (prior to peptide treatment). The β-amyloid peptide and increasing concentrations of ADNF-9 were added as above. Cultures were given their respective treatment once and cell extracts were subjected to Western blot analysis after a 24-h incubation period.

To obtain cell lysates, cultures were harvested (1 ml PBS/dish) and cell pellets (1 min, 10 000 g) were extracted (15 min at 4°C) in lysis buffer (100 μl/dish: 150 mM NaCl, 1 mM EDTA, 50 mM Tris–HCl pH 8.5, 1 mM MgCl₂, 0.1 mM ZnCl₂, 0.1% Triton X-100 and 0.1% SDS) and supernatants (10 min, 10 000 g) were collected and frozen for further analysis.

Following Bradford protein determination (BioRad, Hercules, CA), 10 μg protein/lane were separated by electrophoresis on 10% (w/v) polyacrylamide (BioRad) slab gel containing 0.1% SDS as before [1]. A portion of the gel was stained with Coomassie Brilliant Blue (CBB) and remaining proteins were transferred to nitrocellulose filters. After transfer, the nitrocellulose filter was treated with 10% low fat milk (w/v) to block unoccupied sites. Antigen detection was performed with three different antibodies against hsp60 (StressGen Biotechnologies, Victoria, Canada) as follows. (i) Mouse monoclonal antibodies (antibody 806, recognizing the epitope between residues 383–447 of human hsp60); (ii) polyclonal antibody 804; and (iii) polyclonal antibody 805. Incubation with the primary antibody was performed for 12 h at 4°C. For detection, the secondary antibody (peroxidase conjugate; Sigma) was used. As a positive control, purified recombinant hsp60 (StressGen) was utilized. Antibody-antigen complexes were visualized by the ECL-Western blotting detection system (Amersham) which enables detection of the specific reaction on an ECL-Hyperfilm by chemical luminescence. Relative levels of proteins were quantified by scanning densitometry (BIS 202D, Dinzco & Rheumien, Jerusalem, Israel). Further specificity was determined by actin immunoreactivity (Sigma, Rehovot, Israel).

All statistical comparisons were made with an analysis of variance (ANOVA) followed by the Student–Newman–Keuls multiple comparison of means test.

Previous studies have shown that a 2-h exposure to 10^{-15} M ADNF-9 protected against β-amyloid (amino acids 25–35) toxicity in mixed neuron-glial preparations [5]. To assess rapid changes in hsp60 induction following ADNF-9 treatment in the same cell system, the mRNA levels of hsp60 were determined by Northern blot hybridization (Fig. 1). Densitometric scanning showed a 28–31% reduction in hsp60 mRNA following a 3-h incubation with β-amyloid. Co-treatment with 10^{-15} M ADNF-9 attenuated this decrease (only a 7–19% reduction). Similar treatment with 10^{-15} M ADNF-9 alone increased hsp60 mRNA by 20%. Five minutes heat shock (42°C) resulted in 46–54% increase in hsp60 mRNA. Calibration of the results in comparison with rRNA (ethidium bromide staining, Fig. 1) or to actin hybridization performed as before [9] indicated a similar trend.

To further identify whether the changes in hsp60 expression (i) can be detected in a cell population enriched in neuronal cells; (ii) can also be measured at the protein level, and (iii) can be maintained after a 24-h treatment; glia-depleted neuronal cultures were utilized. In this paradigm, treatment with the β-amyloid peptide decreased the hsp60-immunoreactive protein-band, while ADNF-9 (10^{-15} M) prevented this attenuation (Fig. 2a). The same blots were subjected to actin antibodies, indicating no change in actin contents upon different manipulations (Fig. 2b). Densitometric scanning (Fig. 3) estimated the reduction to be 46 ± 12% (n = 6, P < 0.01). The specificity of the three antibodies used was determined, in that only one protein band was detected, corresponding in size to the hsp60 positive control [1]. It is hypothesized that the changes in the hsp60 mRNA observed after 3 h incubation are sustained at the protein level for at least 24 h.

![Fig. 1. Effects of β-amyloid and ADNF-9 on hsp60 mRNA in mixed glia-neuronal cultures. RNA was extracted from cerebral cortical cultures derived from newborn rats [3,5,8–10]. RNA (10–12 μg/lane) was then subjected to Northern blot hybridization with a PCR-labeled hsp60 specific probe. Results are shown in comparison with ethidium bromide staining of the 18S ribosomal RNA.](image-url)
Concentration-effect studies of ADNF-9 indicated an EC50 of $10^{-15}$ M, with $10^{-14}$ M ADNF-9 providing a complete protection against $\beta$-amyloid-associated hsp60 reduction (Fig. 3, $P < 0.01$, $n = 4$). Furthermore, treatment of cultures with ADNF-9 alone resulted in a dose-dependent increase in hsp60-immunoreactive protein band (Fig. 2 and Fig. 3, $P < 0.014$, $n = 3$). These increases may occur in both neurons and glial cells, as even in the enriched neuronal preparations there is still a significant amount of astrocytes (10%). In this system, neuronal cell counts [5,8] indicated a 40 ± 8% reduction in the presence of the $\beta$-amyloid peptide. Neuroprotection was obtained at $10^{-16}$ M and at $10^{-15}$ M ADNF-9.

The interactions of the $\beta$-amyloid peptide and heat shock proteins can be of a direct nature. Small heat shock proteins can be secreted from cells and protect against amyloidogenesis [11]. In this respect, hsp60 has recently been shown to be a secreted protein [1] and it may also interact either in the intracellular milieu or extracellularly with other proteins to help maintain the proper architecture of proteins and peptides [12,13].

Heat shock induction prior to exposure to toxic injury has been demonstrated to protect neurons against glutamate toxicity [16] and apoptosis [14]. Antibodies to ADNF induce apoptosis that can be prevented by ADNF [10]. It is hypothesized that VIP causes a rapid release of intracellular hsp60 [1] and ADNF [3,5,7], thereby enhancing the protective extracellular milieu. Extracellular ADNF-9 consequently stimulates increases in hsp60 in neurons that may render them more resistant to toxic insults.

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