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Research Report

NAPOR-3 RNA binding protein is required for apoptosis in hippocampus

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Abstract

NAPOR-3 is a central nervous system RNA binding protein that is associated with downstream mRNA targets and has been demonstrated to be selectively overexpressed during apoptotic cell death. In this study, we first examined the regional distribution of NAPOR-3 mRNA in the adult rat brain by in situ hybridization: the transcript was abundantly expressed in many brain regions, mostly in gray matter, including the CA1–CA4 regions and dentate gyrus of the hippocampus, the piriform cortex and the cerebellar granule cell layer. We then investigated the role of NAPOR-3 in neuronal cell death by monitoring its mRNA and protein expression levels using semiquantitative RT-PCR and Western blotting, respectively. NAPOR-3 was overexpressed in rat organotypic slices exposed to staurosporine and to oxygen–glucose deprivation (OGD), an in vitro model of apoptotic cerebral ischemia, but not when exposed to glutamate toxicity. Our results also demonstrate that NAPOR-3 gene overexpression is an early step in the chain of signaling events leading to apoptosis, taking place upstream of caspase-3 activation. Finally, antisense-mediated downregulation of NAPOR-3 gene expression protected hippocampal cultures against OGD-induced apoptosis and prevented caspase-3 activation. Our results demonstrate that NAPOR-3 gene overexpression is necessary for the execution of OGD-induced programmed cell death.

Theme: Development and regeneration
Topic: Neuronal death

Keywords: Ischemic injury; Apoptosis; Rat organotypic hippocampal slice; Antisense oligonucleotide

1. Introduction

Apoptotic cell death, as other major cell programs like proliferation and differentiation, is a highly regulated active process which determines profound biochemical and structural changes due to modulation of the gene expression profile. The view that transcriptional regulation is the predominant regulatory mechanism, however, has been increasingly challenged by the discovery of ever-increasing examples of post-transcriptional mechanisms for regulating gene expression. Post-transcriptional regulation of gene expression can involve the on/off regulation of particular gene products in a temporally and spatially regulated manner, allowing cells to fine tune their pattern of expression. In fact, many important events in development are regulated by an array of post-transcriptional mechanisms, controlling mRNA stability, localization and translation [34].

RNA-binding proteins play central roles in the post-transcriptional regulation of gene expression. These proteins contain regions which function as RNA-binding domain and auxiliary domains that mediate protein–protein interaction and subcellular targeting [5]. In the past several years, many RNA-binding motifs have been identified [23], and one of

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the best-studied is the RNP (ribonucleoprotein) motif, also referred as to RBD (RNA-binding proteins) or RRM (RNA recognition motif) [5,23].

At present, there are nearly 300 known RNP motif proteins, and, among RNP-containing proteins, the *e*mbryonic *l*ethal *a*bnormal *v*ision (ELAV), discovered in a genetic screen of the fruit fly *Drosophila melanogaster*, is a member of an evolutionarily conserved family of neural RNA-binding proteins. The structures and expression patterns of the ELAV family members are highly conserved from *Drosophila* [31,32,45] to mammals [26,37]. In *Drosophila*, the ELAV RNA-binding protein exhibits a neuron-specific expression pattern and is believed to play an important role in the regulation of alternative splicing of neuroglian [15]. In mammals, the ELAV-related Hu-type RNA-binding proteins, HuB, HuC and HuD, have a neural-specific expression pattern and associate with a distinct subset of mRNAs [14]. Their characterized functions involve the control of mRNA stability and translation [2,18].

Other members of the RBP family are provided by the *Drosophila* Bruno factors, which have been functionally characterized as translational regulators [12]. The mammalian ortholog of this RNA-binding protein family contain many members: NAPOR, CUG-BP, ETR3, BRUNOL, CELF and EDEN-BP, highly related to each other at the level of sequence and domain organization. In mammals, CUG-BP (CUG-binding protein) was initially identified as an RNA-binding activity having a preference for the CUG triplet repeats associated with CUG expansions in myotonic dystrophy [41]. The human ETR-3 factors (also called CELF, for CUG-BP and ETR3-like factors) show a behavior similar to CUG-BP in regulating cell-specific alternative splicing during normal development and disease [17]. EDEN-BP (also called deadenylation factor), the embryo deadenylation element-binding protein, is a *Xenopus* CUG-BP believed to function in embryonic development, and evidence for its role in the deadenylation of c-Jun mRNA has recently been shown [28].

Human NAPOR contains three RRM-type RNA-binding domains, highly related to human CUG-BP and ETR3, and is encoded by a single gene, which gives rise to three mRNA isoforms by alternative splicing, NAPOR-1, -2 and -3 [20]. Interestingly, these RNA-binding proteins appear to be involved in post-transcriptional control mechanisms important for cell differentiation and development. In particular, NAPOR-3 is highly expressed in differentiating cells throughout embryonic and early postnatal development of forebrain, and it has been suggested that its spatio-temporal expression is coincident with the occurrence of programmed neuronal cell death [8,19]. Notably, the expression of NAPOR-3 is induced in many neuronal apoptosis systems [7], and several RNA-binding proteins have been known to play a crucial role in other apoptotic pathways [38,40]. It is thus conceivable that NAPOR-3 may also be involved in the process of programmed neuronal cell death that may, at least in part, account for ischemic injury to the brain [9,25]. Here, we show that NAPOR-3 gene expression undergoes dramatic changes during apoptotic cell death and that it plays a critical role in dictating the cellular outcome following OGD, a stress that mimics in vivo ischemia.

2. Materials and methods

2.1. Preparation and digoxigenin (DIG) labeling of the NAPOR-3 RNA probe

To obtain the RNA probe for NAPOR-3 in situ hybridization, a 253-bp fragment was amplified by RT-PCR from rat brain total RNA using the following primers: forward primer: 5′-GCCACTGATTCTCCTTGGTT-3′; reverse primer: 5′-CCACGCCATCTCTAATCGT-3′. The PCR-amplified fragment, encompassing nucleotides 1935–2188 in the NAPOR-3 UTR sequence (GenBank accession number NM_017197), was obtained with the SuperScript One-Step RT-PCR kit (Invitrogen, Milan, Italy), subcloned into plasmid vector pCR4-TOPO (Invitrogen) and the identity and orientation of the insert verified by sequencing. The recombinant plasmid was then linearized and transcribed with T7 RNA polymerase. The labeling of the probe was performed with the DIG labeling kit (Roche, Milan, Italy), according to the manufacturer's protocol, as follows: each template was incubated 2 h at 37 °C in the presence of a transcription buffer made of ATP, GTP and CTP at 1 mM each, UTP 0.7 mM, DIG-UTP 0.3 mM, DTT 10 mM, RNase inhibitor (1 unit/μl of transcription mix) and 1 U/μl T7 RNA polymerase for the antisense or T3 polymerase for the sense probe. Transcripts were purified using the High Pure PCR product purification kit (Roche). The DIG incorporation into the probe was controlled by dot spots where DIG was visualized with an anti-DIG antibody coupled to alkaline phosphatase (anti-DIG-AP).

2.2. Non-isotopic in situ hybridization (ISH)

To examine the region-specific expression of NAPOR-3 mRNA in the rat brain, serial coronal cryostat sections of 20 μm were thawed onto 3-aminopropyl ethoxysilane-coated slides for in situ hybridization with DIG-labeled probe as follows. After fixation in 4% paraformaldehyde (PFA) for 15 min, slides were rinsed twice in PBS and once in distilled water. Sections were permeabilized for 30 min at 37 °C with TE buffer (100 mM Tris–HCl pH 8.0, 50 mM EDTA) containing 1 μg/ml RNase-free proteinase K and then postfixed with 4% PFA-PBS for 5 min at 4 °C. Sections were prehybridized for 10 min at 37 °C in 5× SSC (NaCl 0.75 M, Na-citrate 0.075 M) and 50% formamide. The hybridization reaction was carried out at 42 °C for 16 h in a humidified chamber with 30 μl/section of hybridization mix (40% formamide, 10% dextran sulfate, 1× Denhardt's solution, 4× SSC, DTT 10 mM, polyadenylic acid 0.5