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Organelles do not Colocalize with mRNA Granules in Post-Ischemic Neurons

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Abstract

Following global brain ischemia and reperfusion, it is well-established that neurons undergo a translation arrest that is reversible in surviving neurons, but irreversible in vulnerable neurons. We previously showed a correlation between translation arrest in reperfused neurons and the presence of granular mRNA-containing structures we termed “mRNA granules.” Here we further characterized the mRNA granules in reperfused neurons by performing colocalization studies using fluorescent in situ hybridization for poly(A) mRNAs and immunofluorescence histochemistry for markers of organelles and mRNA binding proteins. There was no colocalization between the mRNA granules and markers of endoplasmic reticulum, cis- or trans-Golgi apparatus, mitochondria, microtubules, intermediate filaments, 60S ribosomal subunits, or the HuR ligands APRIL and pp32. The mRNA granules colocalized with the neuronal marker NeuN regardless of the relative vulnerability of the neuron type. RNA immunoprecipitation of HuR from the cytoplasmic fraction of 8 hr reperfused forebrains selectively isolated *hsp70* mRNA suggesting the mRNA granules are soluble structures. Together, these results rule out several organelle systems and a known HuR pathway as being directly involved in mRNA granule function.

Keywords

brain ischemia and reperfusion; hippocampus; HuR; mRNA granules; translation arrest

INTRODUCTION

It is well-known that all post-ischemic neurons display a translation arrest (TA) which is transient in neurons that will survive the insult. However, the persistence of TA in post-ischemic neurons, such as the hippocampal CA1 region, is one of the strongest predictors of inevitable cell death following brain ischemia and reperfusion (I/R) (Hossmann, 1993). We previously showed that polyadenylated mRNAs [poly(A)] formed granular structures in post-ischemic neurons, which we named “mRNA granules” (Jamison et al. 2008). The presence of mRNA granules correlated precisely with decreased protein synthesis rates in

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vivo, and hence with the selective vulnerability of CA1 neurons following brain I/R. The mRNA granules colocalized with the mRNA binding proteins poly-A binding protein (PABP) and eukaryotic initiation factor 4G, but did not colocalize with the 40S small ribosomal subunit protein S6. Additionally, the mRNA binding protein HuR colocalized with mRNA granules in resistant CA3 neurons, but not vulnerable CA1 neurons, at early (< 16 hr) reperfusion durations, and this correlated with translation of *hsp70* mRNA.

To further ascertain the molecular composition of the mRNA granules in vivo, we here describe additional colocalization studies between poly(A) mRNA and markers of intracellular organelles and mRNA regulatory systems. Of all the markers tested, only the neuronal marker NeuN showed colocalization in the form of extra-nuclear granules in post-ischemic neurons. Additionally we show that RNA immunoprecipitation of HuR but not PABP from homogenates of 8 hr reperfused forebrain selectively isolated *hsp70* mRNA. Together, these results shed additional light on the identity of the mRNA granule by ruling out a direct involvement with the organelle and mRNA processing systems we tested here.

MATERIALS AND METHODS

Materials

Antisera for α -tubulin (T6199) and neurofilament (NF) H/M (N2912) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antisera for acidic protein rich in leucine, a HuR accessory protein, (APRIL; ab4224), and cytochrome C oxidase subunit 4, a mitochondrial marker, (COX IV; ab16056) were purchased from Abcam (San Francisco, CA, USA). Antisera for protein disulfide isomerase, a marker of the endoplasmic reticulum (PDI; MA3-019) and the trans-Golgi marker TGN38 (MA3-063) were purchased from Thermo Scientific (Rockford, IL, USA). A marker of the cis-Golgi, GM130 (610822), was purchased from BD Biosciences (Sparks, MD, USA). Anti-HuR (sc-5261) was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). Anti-NeuN, used here as a marker for neuronal nuclei, (MAB377) was purchased from Millipore (Billerica, MA, USA). Antisera for pp32, another HuR accessory protein (ADI-905-234-100) was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Ribosomal P antigen (RPA; HPO-0100), a marker of the 60S ribosomal subunit (Bonfa et al., 1988), was purchased from ImmunoVision (Springdale, Arkansas, USA). All other chemicals were reagent grade.

Animal model

All animal experiments were approved by the Wayne State University Animal Investigation Committee and were conducted following the *Guide for the Care and Use of Laboratory Animals* (National Research Council, revised 2011). All efforts were made to reduce animal suffering and minimize the total number of animals used. Normothermic global forebrain ischemia of 10 min duration was induced in male Long Evans rats using the bilateral carotid artery (two-vessel) occlusion and hypovolemic hypotension (2VO/HT) model of Smith et al., (1984), as we have previously described (DeGracia et al., 2007; Roberts et al., 2007; Jamison et al., 2008). Exclusion criteria and survival rates were as previously reported (Jamison et al., 2008). Experimental groups (n = 5/group) were: sham-operated, nonischemic controls (NIC), 10 min ischemia and reperfusion durations of: 1 hr (1hR), 8 hr (8hR), 16 hr (16hR), 36 hr (36hR) and 48 hr (48hR).

Tissue Slice Preparation and Double Immunofluorescence/Fluorescent in situ hybridization

At appropriate times, animals were transcardially perfused, brains dissected, and 50 micron slices through the dorsal hippocampus were obtained via vibratome and stored at -20°C in cryostat solution until used, as previously described (Kayali et al., 2005). Double

immunofluorescence (IF)/fluorescent in situ hybridization (FISH) was performed exactly as previously described (Jamison et al., 2008), using 50 ng/ml of a 5'-biotinylated 50-mer oligo-dT probe (Integrated DNA Technologies, Inc., Coralville, IA). Antisera dilutions were: α -tubulin, (1:100); APRIL, (1:100); COX IV, (1:50); GM130, (1:100); NeuN, (1:500); NF H/M, (1:300); PDI, (1:200); pp32, (1:250); RPA, (1:5000); TGN38, (1:200).

Validation of antisera stainings included (not shown): (1) loss of signal with omission of primary antisera, (2) graded loss of signal with antisera dilution, and (3) agreement with published descriptions of antisera neuronal staining patterns where available [e.g. endoplasmic reticulum and Golgi (Takeda et al., 2001); NeuN and alpha tubulin (Gu et al., 2009); neurofilament M (Kim et al., 2002); poly(A) mRNAs (Martone et al., 1996)]. We previously validated the specificity of RPA staining (Kayali et al., 2005).

Slides were examined on an Axioplan 2 Imaging System (Carl Zeiss, Oberkochen, Germany) equipped with an ApoTome. Excitation at 488 nm and 568 nm, and emission at 518 nm and 600 nm were used for Alexa 488 (green) and Alexa 555 (red), respectively. Optical sectioning was performed using the X63 oil immersion objective to generate z-stacks as previously described (Jamison et al., 2008). Fluorescent micrographs shown in the figures are orthographic projections of 3.5 micron z-stacks (10×0.35 micron optical sections), unless otherwise stated.

Western blot validation of antisera

To further validate antibody specificity, Western blots were performed for each antigen on clarified homogenates of non-ischemic brain. SDS-PAGE gels contained 50 μ g protein per lane, determined by Lowry assay. Conditions for each Western blot are listed in Table 1, which includes primary antisera: dilution, incubation buffer, incubation time, incubation temperature, and type of membrane for electroblot transfer, (NC, nitrocellulose; PDVF, polydivinylfluoride). The base buffer for all primary antisera incubations was TTBS. Other than the primary antisera incubation conditions, Western blots were performed as previously described (Jamison, 2008).

RNA Immunoprecipitation (RIP)

RIP was based on the method of Keene et al. (2006) and Baroni et al. (2008). 8hR or NIC animals were sacrificed and brains rapidly removed. Whole forebrain was dissected at 4°C and homogenized on ice in 5:1 (w/v) of 50 mM HEPES pH 7.5, 150 mM NaCl, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 1 mM MgCl₂, 1 mM EGTA, 80 U/mL RNase inhibitor (Ambion, Austin, TX, USA), 0.2% ribonucleoside vanadyl complexes (Sigma, St. Louis, MO, USA), and 1:85 protease inhibitor cocktail (Sigma). Centrifugation of the 2500 g post-nuclear supernatant at 25,000 g generated a cytoplasmic supernatant, and 600 μ g of supernatant protein was precleared with 1 μ L of an unrelated antibody (Lamin A/C, Santa Cruz Biotech, Santa Cruz, CA, USA) plus 20 μ L Protein A-Sepharose beads (Invitrogen, Carlsbad, CA, USA). Precleared supernatants were rotated 16 hr, 4°C in 15 μ L Protein A-Sepharose prebound with 16 μ g HuR antiserum (Santa Cruz). Beads were washed $\times 3$ in sterile phosphate buffered saline. RNA was extracted from precipitated protein using TRIzol reagent (Invitrogen) and reverse transcriptase PCR was performed for *gapdh* (5'-ACAAGATGGTGAAGGTCGGTGTGA-3', 5'-TTGTCATTGAGAGCAATGCCAGCC-3'; 1.0 kb product) and *hsp70* mRNA (5'-TCTTGTTGCCAACACCCAAATCC-3', 5'-AAAGGTCAGTCTAGCTCCGTGTT-3'; 0.5 kb product) using 2 μ g total RNA according to vendor instructions (Roche, Boulder, CO, USA). Amplification products were run on Tris-acetic acid- EDTA-1% agarose gels and visualized by SYBR gold (Invitrogen). For some IP reactions, RNA was not extracted and beads were boiled in Laemmli buffer, run on

SDS-PAGE gels, and western blotted for HuR or PABP using methods previously described (Jamison, et al., 2008).

RESULTS

Double IF/FISH

We previously showed that 10 min of 2VO/HT forebrain ischemia in rat caused selective CA1 cell death at 3 days reperfusion and that mRNA granules were present in CA1 neurons to 48 hr reperfusion and in CA3 neurons to 16 hr of reperfusion (Jamison et al., 2008). Identical results were obtained in the present study across the reperfusion time course (data not shown). Figures 1 and 2 illustrate representative photomicrographs of double IF/FISH staining in NIC and 1hR CA1 and CA3. We show only 1hR samples because the qualitative pattern of colocalization (or lack thereof) for a given antigen with the mRNA granules did not change over the reperfusion time course. Additionally, similar colocalization patterns were seen in cerebral cortical, thalamic and hilar neurons that evidenced mRNA granules following reperfusion (data not shown).

Figure 1 shows double-labeling of poly(A) mRNAs and antisera detecting intracellular organelles. Only the antisera for PDI showed slight colocalization with poly(A) in the NIC samples as indicated by the slight yellowish hue of the NIC cytoplasm (Figure 1A, NIC CA1 and CA3), likely indicative of endoplasmic reticulum-localized translation. However, at 1hR, the mRNA granules were distinctly green against the red PDI staining, thus the mRNA granules did not colocalize with endoplasmic reticulum (Figure 1A, 1hR CA1 and CA3). Markers of cis and trans- Golgi apparatus, GM130 and TGN38, respectively, showed no colocalization in NICs or at 1hR (Figures 1B and 1C). A lack of colocalization also held for the mitochondria marker COX IV (Figure 1D), and two cytoskeleton components, α -tubulin (Figure 1E) and NF-H/M (Figure 1F). Thus, the mRNA granules did not colocalize with any of the organelle markers tested.

Figure 2 shows antigens representing mRNA binding systems. In NICs, the large (60S) ribosomal subunit, as marked by RPA, showed a diffuse cytoplasmic colocalization with poly(A) mRNAs (Figure 2A), as would be expected for cells active in protein synthesis. However, the mRNA granules did not colocalize with RPA in reperfused samples. We previously showed a lack of colocalization of mRNA granules with the 40S marker S6 following brain I/R (Jamison et al., 2008). As it is well-known that polysomes are fully dissociated at the 1hR time point (Hossmann, 1993; Martin de la Vega et al., 2001), our results indicate that the dissociated ribosomal subunits are not associated with the mRNA granules.

We also previously showed that the mRNA binding protein HuR colocalized with mRNA granules in CA3 but not CA1 pyramidal neurons at 1hR (Jamison et al., 2008). Steitz and colleagues (Gallouzi et al., 2001) have shown that HuR interacts with two protein ligands, APRIL and pp32, during CRM1-dependent nucleocytoplasmic transport of HuR-bound mRNAs. We observed no colocalization of APRIL (Figure 2B) or pp32 (Figure 2C) with the mRNA granules in the reperfused samples. Unexpectedly, the well-known neuronal nuclear marker NeuN strongly colocalized with the mRNA granules in both CA1 and CA3 during reperfusion (Figure 2D).

For all of the antisera used above, Western blots on brain homogenate proteins produced single bands at the correct molecular weights (Figure 3E). The only exception was RPA which produced several bands, but this result is expected because RPA cross reacts with many of the large ribosomal subunit proteins on Western blots (Lin et al., 1982).

RIP Results

RIP of HuR or PABP was performed on cytoplasmic fractions from forebrain homogenates of NIC and 8hR samples (Figure 3A–D). When mRNA was extracted and the cDNA amplified via probes for either *gapdh* or *hsp70*, HuR RIPs selectively brought down *hsp70* mRNA but not *gapdh*. PABP RIPs brought down both *hsp70* and *gapdh* mRNA. Thus HuR demonstrated selective mRNA binding compared to PABP.

DISCUSSION

Because transient mRNA structures tend to be labile upon cell disruption (Mili & Steitz, 2004; Kedersha and Anderson, 2007), here we primarily used histochemical methods to further assess the colocalization properties of the mRNA granules formed in reperfused neurons. The mRNA granules present in the reperfused CA1 and CA3 neurons did not colocalize with markers of major intracellular organelles or cytoskeleton. Nor did the mRNA granules colocalize with a marker of the 60S subunit. In combination with our previous result showing mRNA granules did not colocalize with 40S subunits (Jamison et al., 2008), we conclude that poly(A) mRNAs are sequestered away from both ribosomal subunits following polysome dissociation during the initial hours of brain reperfusion. This stands in contrast to stress granules, which contain a modified form of the 40S subunit (Anderson and Kedersha, 2006). In spite of mRNA granules in CA3 neurons colocalizing with HuR at 1hR (Jamison et al. 2008), there was no colocalization with the HuR accessory proteins APRIL and pp32, suggesting that the function of HuR in the mRNA granules may be distinct from its role in nuclear to cytoplasmic transport of mRNAs mediated in conjunction with APRIL and pp32 (Gallouzi et al., 2001).

Unexpectedly, the mRNA granules strongly colocalized with NeuN outside of the nucleus following brain I/R (Figure 2D). This result is consistent with a recent study that identified NeuN as an mRNA splicing factor, FOX-3 (Kim et al., 2009). The colocalization of NeuN and the mRNA granules suggests NeuN may function similarly to other mRNA binding proteins such as HuR (Gorospe, 2003) or TIA-1 (Anderson and Kedersha, 2006) that reside in the nucleus under normal conditions, but export into the cytoplasm under conditions of cell stress to contribute to the genetic reprogramming to a stress response phenotype (DeGracia et al. 2008).

The RIP experiments show HuR selectively bound *hsp70* mRNA compared to PABP. It has been reported that *hsp70* mRNA contains an adenine and uridine rich element (ARE) in its 3'-untranslated region (Zhao et al., 2002). The ARE sequence is the binding target for HuR (Chen et al., 2002). HuR binding to ARE-containing mRNAs, such as *c-fos*, contribute to their stabilization and selective translation (Fan and Steitz, 1998). On the other hand, PABP binds any mRNA with a poly(A) tail of sufficient length (Mangus et al., 2003), and is expected to pull down a variety of mRNAs. For the purpose of the present report, the isolation of *hsp70* mRNA following HuR RIP from the soluble fraction is consistent with the lack of colocalization of the mRNA granules with the organelle systems we tested.

In conclusion, the present study confirms our previous observation of mRNA granules (Jamison et al., 2008), and extended these to show that the mRNA granules: (1) do not colocalize with several major intracellular organelle systems, and (2) are sequestered from both ribosomal subunits. Further, we reported the unexpected finding that NeuN strongly colocalized with the mRNA granules. The present observations support the that mRNA regulation plays an important role in the post-ischemic response of all neurons to ischemic stress (DeGracia et al., 2008). Understanding the intrinsic response of the neurons will be crucial to developing effective therapies to combat ischemic brain damage as occurs

following cardiac arrest and resuscitation and stroke, therapies which to this point have remained frustratingly elusive.

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Abbreviations used

2VO/HT	bilateral carotid artery occlusion and hypovolemic hypotension
40S	small ribosomal subunit
APRIL	acidic protein rich in leucine
ARE	adenine and uridine rich element
CA	Cornu Ammonis (Ammon's horn)
COX IV	cytochrome c oxidase subunit IV
FISH	fluorescence in situ hybridization
FOX3	homologue 3 of feminizing locus on X protein 1
gapdh	mRNA for glyceraldehyde-3-phosphate dehydrogenase
GM130	Golgi matrix protein 130
hsp70	mRNA for 70 kDa inducible heat shock protein
HuR	also called HuA or ELAV
I/R	ischemia and reperfusion
IF	immunofluorescence histochemistry
NeuN	neuronal nuclei
NF H/M	neurofilament heavy and medium chain
NIC	sham-operated, non-ischemic control
PABP	poly(A) binding protein
PDI	protein disulfide isomerase
poly(A)	poly-adenylated mRNAs
pp32	polypeptide 32 kDa
RIP	RNA immunoprecipitation
S6	small ribosomal subunit protein 6
TA	translation arrest
TGN38	trans Golgi network 38
TIA-1	T cell internal antigen

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Highlights

1. mRNA granules formed after brain ischemia do not colocalize with organelles.
2. mRNA granules formed after brain ischemia do not colocalize with ribosomes.
3. RNA immunoprecipitation of HuR co-precipitates *hsp70* mRNA.

Figure 1.

Representative photomicrographs of poly(A) mRNAs and organelle markers as indicated in the figure for nonischemic control (NIC) and following one hour of reperfusion (1hR) in hippocampal layers CA1 and CA3. All images were acquired under $\times 63$ oil immersion and are orthographic projections of ten sequential 0.35 micron optical slices. Scale bar in lower right most panel is 10 microns and applies to each image. Each image is a 1/3rd crop from the original photomicrograph.

Figure 2.

Representative photomicrographs of poly(A) mRNAs and mRNA binding systems as indicated in the figure for nonischemic control (NIC) and following one hour of reperfusion (1hR) in hippocampal layers CA1 and CA3. All images were acquired under $\times 63$ oil immersion and are orthographic projections of ten sequential 0.35 micron optical slices. Scale bar in lower right most panel is 10 microns and applies to each image. Each image is a 1/3rd crop from the original photomicrograph.

Figure 3.

RNA immunoprecipitation of HuR and PABP. **(A)** and **(D)** Western blots of immunoprecipitations of HuR and PABP, respectively. “Input” is 75 mg total protein of cytoplasmic fraction; “sham” and “8hR” are IP results from respective experimental groups; “-input” is IP reaction run in absence of cytoplasmic fraction for HuR. Molecular weights are as indicated, and HuR and PABP bands indicated as labeled. **(B)** and **(C)** Agarose gels (1%) of PCR amplification products (30 cycles) after extraction of nucleic acids from HuR and PABP IPs, respectively. “g” and “h” indicate amplification with primers for *gapdh* and *hsp70* mRNAs, respectively. “bp”, base pair weight standards. **(E)** Western blots validating the specificity of the antisera used for microscope analysis. Positions of molecular weight standards are indicated to the left. Abbreviations for anti-sera are given in Materials and Methods.

Table 1

antiser	dilution	1° buffer	time	temperature	membrane
APRIL	1:500	5% milk	1 hr	25° C	PVDF
α-tubulin	1:1000	10% milk	1 hr	25° C	PVDF
COX IV	1:150	5% milk	overnight	4° C	PVDF
GM130	1:500	5% milk	1 hr	25° C	PVDF
NeuN	1:1000	5% milk	overnight	4° C	NC
NFH/M	1:1000	5% milk	overnight	4° C	NC
PDI	1:1000	2% milk	overnight	4° C	NC
pp32	1:333	-	overnight	4° C	NC
ribo P	1:300	5% milk	overnight	4° C	NC
TGN38	1:250	5% milk	overnight	4° C	PVDF