PROLONGED TRANSLATION ARREST IN REPERFUSED HIPPOCAMPAL CORNU AMMONIS 1 IS MEDITATED BY STRESS GRANULES

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Inhibition of protein synthesis is a part of a programmed response of cells to stress (Anderson and Kedersha, 2002). Cerebral ischemia and reperfusion (I/IR) are clinically relevant forms of profound neuronal stress that lead to a transient brain-wide inhibition of neuronal protein synthesis. This inhibition is mediated, at least in part, by phosphorylation of the alpha subunit of eukaryotic initiation factor 2 [eIF2α; eukaryotic initiation factor 2 phospho-form, eIF2α(P)] (reviewed in DeGracia et al., 2002). However, while levels of eIF2α(P) return to control by 4–8 h reperfusion (Althausen et al., 2001; Martin de la Vega et al., 2001; Kumar et al., 2003), translation remains irreversibly inhibited in the selectively vulnerable cornu Ammonis (CA) 1 region (Dienel et al., 1980; Bodsch et al., 1985; Thilmann et al., 1986; Araki et al., 1990) of the hippocampal formation [HF, taken to include CA1, CA2, CA3, hilus and the dentate gyrus (DG), after Amaral and Witter (1989)]. Although post-translational modifications of other regulators of protein synthesis have been evaluated in the reperfused brain, no definitive mechanism of irreversible translation inhibition in CA1 has been identified to date (reviewed in DeGracia, 2004).

One consequence of eIF2α(P)-mediated translation inhibition is the formation of stress granules (SGs) (Kedersha et al., 1999; Kedersha and Anderson, 2002; Kimball et al., 2002). SGs are phase dense particles that appear in the cytoplasm of cells subjected to environmental stresses (Nover et al., 1983). Phosphorylation of eIF2α leads to accumulation of stalled 48S preinitiation complexes (Kedersha and Anderson, 2002), and enhanced cytoplasmic translocation of the nuclear RNA binding proteins T cell internal antigen (TIA-1) and T cell internal antigen-1 related protein (TIAR) (Taupin et al., 1995). TIA-1 and TIAR are multifunctional, prion-like proteins that both self-aggregate and bind poly-adenylated (poly-A) mRNAs to form SGs (reviewed in Anderson and Kedersha, 2002). SGs form transiently, sequestering small ribosomal subunits and dynamically sorting poly-A mRNAs during periods of stress-induced translational arrest. Removal of the stress results in the dissolution of SGs and a return of the cell to a translationally active state. However, SGs persist in cells in which the induced stress leads to cell death (Kedersha et al., 1999).
We have used double-labeling immunofluorescence to visualize small ribosomal subunit (40S) S6 (small ribosomal subunit protein 6) and TIA-1 in neurons of the rat HF following cardiac arrest-induced transient global ischemia. Cytoplasmic localization of TIA-1 in the form of punctate somal subunit protein 6) and TIA-1 in neurons of the rat HF visualize small ribosomal subunit (40S) S6 (small ribosomal subunits. Further, we provide electron microscopic evidence that SGs in control neurons are electron dense cytoplasmic bodies of 100–200 nm, interspersed throughout endoplasmic reticulum and adjacent to the cytoplasmic face of the outer nuclear membrane. At 10 min reperfusion, neuronal SGs quantitatively increased in CA3, DG, and hilus, but not in CA1, and returned to control values by 90 min reperfusion. However, by 4 h reperfusion, both TIA-1 and S6 decreased in HF, and S6 was exclusively sequestered within SGs only in CA1 pyramidal neurons. As SGs are sites of inhibited protein synthesis, our observations provide a structural mechanism for the continued inhibition of protein synthesis in reperfused CA1 pyramidal neurons at a time when eIF2α(P) is almost completely cleared. Additionally, we describe changes in the nuclear morphology of TIA-1 in resistant CA3, DG and hilus, that did not occur in CA1, suggesting that the nuclear behavior of TIA-1 may also play a role in the selective vulnerability of CA1 pyramidal neurons.

EXPERIMENTAL PROCEDURES

TIA-1 goat IgG primary antisera was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). S6 ribosomal protein and phospho-S6 ribosomal protein (Ser240/244) antisera were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Antibody specific for serine-51 phosphorylated eIF2α was purchased from Biosource International (Camarillo, CA, USA). Ribosomal P antigen antisera was purchased from Immunovi-sion (Springdale, AR, USA). Anti-eIF4E was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Monoclonal anti-eIF4G directed against a fragment corresponding to nucleotides 288–1811 of the rat sequence was a gift from Dr. Scot Kimball (Pennsylvania State University, Hershey, PA, USA). Alexa FluorR 488 donkey anti-goat IgG and Alexa FluorR 555 donkey anti-rabbit IgG were purchased from Molecular Probes (Eugene, OR, USA). Cy3 donkey anti-human IgG (secondary antisera for ribosome P antigen) was from Biomedica (Foster City, CA, USA). All other chemicals were reagent grade.

I/R 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 1996). All steps were taken to minimize the number of animals and to prevent their suffering. The thoracic compression method of cardiac arrest was used to induce global brain ischemia in male Long Evans rats (250–300 g) (Indianapolis, IN, USA) by methods we have previously described (DeGracia et al., 1997; Kumar et al., 2003). This model results in profound inhibition of protein synthesis, inhibited translation initiation, and rapid accumulation of eIF2α(P) (DeGracia et al., 1996, 1997; Kumar et al., 2003). All animals were maintained normothermic during both the ischemic and reperfusion periods by means of a homeostatic blanket control unit (Harvard Apparatus, Holliston, MA, USA). Experimental groups (n = 3 per group) were: nonischemic controls (NIC), 10 min ischemia and 10 min reperfusion (10R), 10 min ischemia and 90 min reperfusion (90R), and 10 min ischemia and 4 h reperfusion (4hrR).

Immunocytochemistry

At appropriate times, animals were transcardially perfused with 40 ml of 0.9% NaCl solution followed by 300 ml of 4% paraformaldehyde (PFA) in 0.1 M PBS solution at a flow rate of 20 ml/min. Brains were post-fixed overnight by immersion in 4% PFA/0.1 M PBS. Thirty-five micron slices through the dorsal hippocampus were obtained via vibratome and stored at −20 °C in cryostat solution until used.

Double-labeling immunohistochemistry was performed as follows. Brain slices were washed 10 min × 4 in 0.1 M PBS then blocked in 10% normal donkey serum (NDS) in 0.1 M PBS containing 0.3% Triton X-100 (PBS-Tx) solution for 45 min, followed by washing × 3 in 0.1 M PBS for 10 min. Slices were then immersed in primary antibodies in 1% NDS in PBS-Tx at room temperature with rocking overnight. Following × 3 washes in 0.1 M PBS, slices were incubated in the dark in secondary antibody solution for 2 h and again washed × 3 in 0.1 M PBS for 10 min. Slices were mounted, dried, sealed under coverslips and stored at −20 °C. Primary antisera dilutions were as follows: 1:150, anti-TIA-1; 1:25, anti-S6; 1:100, anti-eIF4G; 1:150; anti-eIF4E; and 1:5000, anti-ribosomal P antigen. Secondary antisera dilutions in 1% NDS in PBS-Tx were: 1:300, Alexa 488; 1:300, Alexa 555; 1:300, Cy3.

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 (or Cy3) (red), respectively. Digital images were acquired with the AxioCam MRm high resolution digital camera and processed with Axiovision version 4.2 (Carl Zeiss). ApoTome optical sectioning was performed under ×40 and ×63 oil immersion objectives. With the ×40 objective, 15 μm z-stacks were acquired generating 20 sequential optical sections of 0.775 μm thicknesses (“z-stack” refers to the set of sequential optical sections in the xy plane that are acquired along the depth of the z axis of the tissue slice). For the ×63 objective, 10 μm z-stacks were acquired generating 24 optical sections of 0.425 μm thicknesses.

For ×40 data, to enhance visualization of punctate cytoplasmic staining, orthogonal projections of the 15 μm z-stack were constructed in Axiovision version 4.2 and then exported to Photoshop version 5 (Adobe Systems, Inc., San Jose, CA, USA). Green and red channels were “added” (merged) to generate standard composite images (Fig. 2A). Using set theory terminology, addition of the green and red channels is equivalent to calculating the union of the two channels, producing the standard merged images of double-labeling immunofluorescence. Green and red channels were also “multiplied” to generate yellow channels for SG analysis (Fig. 2B). Multiplication of green and red channels is equivalent to calculating the intersection of the two channels, thus isolating only those parts of the image that overlap and form yellow. Yellow channels were exported as gray-scale TIFF files and imported into Intelligent Quantifier version 3.1 (BioImage, Inc., Jackson, MI, USA) and analyzed to generate SG counts and intensities. Yellow channels were also subtracted from red channels to generate images of S6 immunostaining devoid of S6 and TIA-1 co-localization (Fig. 7). All quantitative data from the experimental groups were compared by...
ANOVA followed by LSD post hoc with statistical significance set at $P<0.05$.

**Fig. 1.** (A) Immunoblots and densitometric analysis for A. eIF2α(P), B. ribosomal protein S6, and phsopho-S6, and C. TIA-1 in 125 μg of unfractionated whole HF of NIC, or after 10R, 90R or 4hrR following a 10 min cardiac arrest. Positive control for eIF2α(P) was derived from tg treated (tg+) NB104 cells and negative control from untreated (tg−) NB104 cells (Kumar et al., 2003). Densitometric analysis shows mean±standard deviation of the respective immunoblots. For eIF2α(P), data are normalized to the NIC group. Bottom gels in B and C are Sypro Ruby stains of the respective nitrocellulose membranes, shown as protein loading controls, with less than 5% variation among the experimental groups. (* $P<0.05$; ** $P<0.01$; LSD post hoc compared with NIC).

Z-stacks acquired under the ×63 objective were analyzed in Axiovision version 4.2 with Inside4D module in order to perform
cut-view analysis (Fig. 10) and to generate 3D reconstructions (Fig. 11). A cut-view is a software generated reconstruction of the xz and yz planes of the z-stack, allowing visualization through the depth of the acquired z-stack. Three dimensional reconstruction was by the Transparency rendering method as described in the Axiovision software documentation. This method produces 3D reconstructions by pixel interpolation between the z-stack optical sections. Depending on the angle of rotation of the 3D construct, the individual optical sections can be seen to be stacked on top of each other, producing a striated appearance, such as that seen in the 3D reconstruction shown in Fig. 11C and 11D.

Western blotting

The same experimental groups as above were repeated, and, at appropriate times, the HF was dissected bilaterally. Whole HFs were immediately sonicated on ice in homogenization buffer containing 20 mM HEPES pH 7.5, 10% glycerol, 1 mM EDTA, 10 mM tetrasodium pyrophosphate, 100 mM NaF, 17.5 mM β-glycerophosphate, 1 mM PMSF, 4 μg/ml aprotinin, 2 μg/ml pepstatin A, and 1 μM okadaic acid. Aliquots of unfractonated homogenates were used to determine protein concentrations by the Folin phenol reagent method, and the remainder frozen on dry ice/ethanol then stored at −80 °C until used. For immunoblotting of unfractonated HF, 125 μg protein were loaded per lane. Western blotting for ribosomal S6 (1:250), phospho-S6 (1:500), TIA-1 (1:250) and eIF2α(P) (1:750) were by methods previously described (DeGrazia et al., 1996, 1999; Kumar et al., 2003).

Positive and negative controls for eIF2α(P) were obtained by exposing NB104 cells (derived from rat sympathetic ganglia, Prasad, 1975) to 1 μM thapsigargin (tg) or no tg, respectively, for 2 h at 37 °C. Phosphorylated S6 and dephosphorylated S6 were prepared by culturing NB104 cells in DMEM containing 1% horse serum, or serum-free DMEM, respectively, for 2 h. Whole cell lysates were prepared by sonication in the same buffer as above, and 125 μg of lysate protein was loaded as controls on SDS-PAGE gels.

Relative band densities were determined utilizing BioImage Intelligent Quantifier v3.1 and groups were compared by ANOVA followed by LSD post hoc with statistical significance set at \( P < 0.05 \). As a loading control, nitrocellulose membranes were stripped and then stained with Sypro Ruby (Amersham Biosciences, Piscataway, NJ, USA) to visualize total protein loading.

Electron microscopy (EM)

For transmission electron microscopy (TEM), whole brains were sectioned coronally at a thickness of approximately 1 μm. Single sections through the dorsal hippocampus were placed in 0.1 M PBS in a Petri dish and the CA1 and CA3 regions were trimmed

Fig. 2. Illustrations of channel manipulations: A. Addition and B. multiplication of red (Alexa 555-conjugated secondary IgG) and green (Alexa 488-conjugated) color channels are depicted both graphically (colored ovals in boxes) and histologically for the CA3 region (bottom images in the figure). Using set theory terms, addition of the red and green channels is the same as taking the union of two sets, while multiplication of the channels gives an intersection of the two sets. This intersection was used to generate the yellow channel (i.e. SGs) in digitally acquired, orthographic projections of sequential images which were dissected optically (ApoTome microscope) through 15 μm z-stacks in the various regions (CA1, CA3, hilus, DG) of HF. (C) After omission of the primary antibodies against S6 and TIA-1, the hilus of the HF shows only faint granular fluorescence. (D) While a few fluorescent neurons are seen in the retrosplenial cortex (D, arrow), the glia cells in the corpus callosum show no detectable labeling. (E) Immunoreexpression of S6 and TIA-1 in the pyramidal cell layer of the CA2 region of HF (E, between arrows) is consistently less intense than that in CA1 (to the right of right pair of arrows) or CA3 (to the left of left pair of arrows).
from the other hippocampal regions under a dissecting microscope. Tissue blocks (1–2 mm³) of CA1 and CA3 were washed 3×10 min in 0.1 M PBS, fixed in 1% osmium tetroxide (EM Science, Gibbstown, NJ, USA) at room temperature for 2 h, washed in 0.1 M PBS 3×10 min, dehydrated in graded ethanol, cleared in propylene oxide and embedded in araldite. Toluidine Blue–Pyronin B-stained, semithin (1 μm) sections were obtained from the blocks for orientation of the pyramidal cell layer. Ultrathin (approximately 90 nm) sections were cut from the trimmed blocks, placed on 200 mesh copper grids, stained with uranyl acetate and lead citrate and visualized with a JEOL JEM-1010 electron microscope.

RESULTS

Detection of eIF2α(P), S6, phospho-S6, and TIA-1 by Western blot

In whole HF, levels of eIF2α(P) peaked at 12-fold controls at 10 min reperfusion, and then decreased to eight-fold at 90R, and 3.3-fold at 4hrR (Fig. 1A), consistent with our previous findings (Kumar et al., 2003). As was reported previously, (DeGracia et al., 1997), there was no change in total eIF2α levels (D. J. DeGracia, unpublished observation). S6 (Fig. 1B) and TIA-1 (Fig. 1C) were detected by Western blot, at Mr of 32 kDa and 42 kDa, respectively. The lack of nonspecific background staining on Western blot confirmed the suitability of the TIA-1 and S6 antisera for immunohistochemical staining. S6 protein showed a progressive reduction with reperfusion duration, decreasing to 50% control levels by 4hrR (ANOVA, P < 0.006). The S6 antisera detected phosphorylated S6 from serum-treated NB104 cells, which migrated slightly slower than dephosphorylated S6 from DMEM-treated NB104 cells (Fig. 1B, top blot), and this was confirmed using phosphospecific S6 antisera (Fig. 1B, middle blot). The S6 in un-fractionated HF homogenates displayed only dephosphorylated S6 that comigrated with S6 from DMEM-treated NB104 cells (Fig. 1B, top blot), and this was confirmed using phosphospecific S6 antisera (Fig. 1B, middle blot). The S6 in un-fractionated HF homogenates was performed with TIA-1 and either eIF4E, eIF4G or ribosomal P antigen. The ribosomal P antigen consists of three proteins of the 60S ribosomal subunit, PO, P1, and P2 (Bonfa et al., 1988), and thus ribosome P antigen antisera detects the 60S subunit. The proteins eIF4E and eIF4G are components of the mRNA cap binding complex eIF4F (Gingras et al., 1999). eIF4F binds the 40S ribosomal subunit as part of the 48S preinitiation complex (Gingras et al., 1999). The distribution of eIF4E in the cytoplasm of CA1 neurons was distinctly granular (Fig. 3B), whereas eIF4G (Fig. 3F) showed both granular and diffuse staining in the cell body and proximal dendrites. Ribosomal P antigen (Fig. 3J) showed a relatively homogenous staining throughout the cell body and proximal dendrites, as well as a prominent circular structure inside the nucleus (Fig. 3J and 3K, asterisks). Following standard merging of each respective channel with the green TIA-1 channel, eIF4E (Fig. 3C, arrow) and eIF4G (Fig. 3G, arrow) colocalized with cytoplasmic TIA-1 in control CA1 neurons in the form of yellow punctate cytoplasmic particles. TIA-1 and ribosomal P antigen did not colocalize, and green punctate TIA-1 particles were observed in the neurons (Fig. 3K, arrow). When yellow channels were calculated, distinct and intense particles were observed for both eIF4E (Fig. 3D, arrow) and eIF4G (Fig. 3H, arrow) colocalization with TIA-1, but only a few very faint particles were observed for ribosomal P antigen (Fig. 3L, arrow). Similar results were obtained for hippocampal CA3, DG, hilus, and cerebral cortical neurons (data not shown). Hence, as it has been established that SGs contain 40S ribosomal subunits and eIF4F components, but not 60S ribosomal subunits (Kedersha and Anderson, 2002; Kimball et al., 2003), our data strongly support that SGs are present constitutively in brain neurons.

SGs in CA1

Fig. 4 illustrates the reperfusion time course of S6 and TIA-1 double-labeling in the CA1 region of HF. In NIC animals, S6 [red (r) channel fluorescence] was localized throughout the cell body cytoplasm and the proximal apical dendrite trunk of neurons in both granular and diffuse staining patterns. The granular S6 staining in the cytoplasm is consistent with the distribution of the Nissl substance, and the diffuse S6 staining likely represents dispersed polyribosomes and free ribosomes normally found throughout the cell body and dendrites. In contrast, TIA-1 in NIC CA1 [green fluorescence (g)] was observed primarily in the nucleus. In merged S6/TIA-1 images (left colored panel), some TIA-1 co-localized with cytoplasmic S6 in the
Double labeling immunofluorescence in NIC CA1 for: A–D, TIA-1 and eIF4E; E–H, TIA-1 and eIF4G; and I–L TIA-1 and ribosomal P antigen. Panels A, E and I show TIA-1 staining. Panels B, F and J are staining for eIF4E, eIF4G and ribosomal P protein, respectively. Panels C, G and K are the respective merges (additions) for TIA-1 and eIF4E, eIF4G and ribosomal P protein. Panels D, H and L are the yellow channels resulting from multiplying the TIA-1 and eIF4E, eIF4G and ribosomal protein P channels, respectively. Scale bar = 10 μm for all panels.
Fig. 4. Formation of SGs in the CA1 region of HF in the four experimental groups (NIC, 10R, 90R, and 4hrR). The left, colored panel for each experimental group illustrates the orthographic projections through 15 μm z-stacks, after merging (adding) the red (S6) and green (TIA-1) color channels. The rectangular area outlined by white dashed lines in each of the colored panels is further illustrated to the right, first representing the red S6 channel alone (r), then the green TIA-1 channel (g), and finally the multiplied red and green channels resulting in the yellow (y, SGs) channel. Magnification of all panels is indicated by the 20 μm bar in the 4hrR colored panel. Putative basket cells of the pyramidal cell layer are indicated by arrows in the 90R and 4hrR panels.
form of yellow [yellow fluorescence (y)], perinuclear granules, which indicated a basal level of SGs in control CA1 (Fig. 4, NIC arrows). The size and number of SGs in the cytoplasm of 10R CA1 pyramidal neurons (colored panel and y channel) were similar to those of the NIC group. However, an orange (rather than red) dispersed fluorescence was visible in the cell bodies of 10R CA1 pyramidal neurons. This was due to translocation of TIA-1 from the nucleus to the cytoplasm as seen by increased TIA-1 staining of the cell bodies in the 10R g channel (Fig. 4, 10R, arrows). At 90R, the cytoplasmic SG fluorescence in S6/TIA-1 merged images decreased (although not statistically significant, see below and Fig. 9). The 90R CA1 pyramidal neurons could be clearly distinguished from pyramidal layer interneurons, the latter of which possessed a distinctly red cytoplasm and larger cell bodies (Fig. 4, 90R, arrow in all four 90R panels pointing to a putative basket cell interneuron). By 4hrR reperfusion, the diffuse S6 staining in the cytoplasm of CA1 pyramidal neurons was wholly absent, and, in merged images, all that was visible were roughly spherical, yellow SGs surrounding the nucleus and extending into the proximal dendrites (Fig. 4, 4hrR). In contrast, 4hrR CA1 interneurons showed diffuse red cytoplasmic staining as well as perinuclear SGs (Fig. 4, 4hrR, arrow). Thus, in 4hrR CA1 pyramidal neurons, but not CA1 interneurons, all S6 staining was confined to SGs, indicating an essentially complete sequestration of the 40S ribosomal subunits in SGs.

SGs in CA3 and hilus (Figs. 5 and 6, respectively)
CA3 pyramidal neurons and large hilar neurons, putatively identified as mossy cells, showed similar qualitative staining patterns. In NIC HF these cells had distinct yellow granules (SGs) dispersed throughout the cell body and proximal dendrites. At 10R, the number and intensity of the SGs were significantly increased from controls, which resulted in the most intense fluorescence obtained among the experimental groups of the study. By 90R there was a qualitative decrease in SGs coincident with a diffuse orange fluorescence in the cell body of CA3 pyramidal neurons and hilar cells compared with those at 10R; the only exception to this pattern being found in putative CA3 interneurons (Fig. 5, arrows in the 90R panels). Unlike CA1, by 4hrR both CA3 and hilar neurons resembled controls, with the cytoplasm of the cell body and proximal dendrites returning to a predominantly red fluorescence and containing dispersed SGs. Significant cytoplasmic staining of S6 devoid of TIA-1 indicates the potential for CA3 and hilar neurons to assume a translationally active state.

SGs in DG (Fig. 7)
Because SGs were hard to visualize in the sparse cell body cytoplasm of the DG granule cells, analysis of these neurons was carried out under the ×63 oil immersion objective rather than the ×40 objective used for the other HF regions (compare the 10 μm bar in Fig. 7 with the 20 μm bars used for the other regions). In general, the pattern of S6 and TIA-1 fluorescence in DG granule cells was similar to those found in CA3 and hilus. Very fine SGs were found in the NIC granule cell cytoplasm (Fig. 7, NIC, y). At 10R, larger and more prominent SGs (arrows) appeared in granule cells. A diffuse orange cytoplasm was visible in merged images of 10R DG (Fig. 710R, left panel), indicating translocation of TIA-1 into the cytoplasm. Also at 10R, S6 staining became more diffuse (r channel) suggesting disaggregation of polysomes. However at 90R and 4hrR, all of the prominent SGs had disappeared and the fine, residual yellow channel was no different from that of controls. In addition, S6 staining at 90R and 4hrR returned to a predominantly granular form, similar to that of controls, suggesting reformation of polysomes and return to a translationally active state, consistent with the recovery of protein synthesis in reperfused DG within hours of reperfusion (Hossmann, 1993). Finally as in the CA region, the r channel fluorescence in the cell body of DG interneurons was very intense in all experimental groups (not shown), indicating a different pattern of response to ischemic stress.

Comparison of SG-associated S6 protein in CA1 and CA3 pyramidal neurons at 4hrR
To evaluate the extent to which S6 was colocализed with TIA-1 in CA1 and CA3 pyramidal neurons in NIC and 4hrR groups, subtraction of the yellow channel (TIA-1 × S6) from the r channel (S6) allowed visualization of S6 that was not co-localized with TIA-1 (Fig. 8). In pyramidal neurons of NIC CA1 and CA3, and 4hrR CA3, diffuse TIA-1-free S6 staining revealed the cytoplasm and proximal dendrites. Punctate S6 staining showed many granules free of TIA-1, suggesting S6 localization in Nissl bodies rather than being associated with SGs. However, in 4hrR CA1, the cell bodies of pyramidal neurons were no longer discernible and only a few punctate granules defined the proximal dendrites (arrowheads). In contrast, 4hrR CA1 interneurons showed cyttoplasmic S6 staining (arrow, note this is the same field as shown in Fig. 4, 4hrR panel). Thus, at 4hrR reperfusion in CA1 pyramidal neurons, the vast majority of S6 staining co-localized with TIA-1. The observation that S6, and by extension 40S ribosomal subunits, are mostly sequestered in SGs provides a mechanism underlying the well-established prolonged translation inhibition in reperfused CA1 pyramidal neurons, at a time when eIF2α(P) levels of whole HF have decreased 80% (Fig. 1A).

Quantification of SGs in HF
Isolating the yellow channel (described in Experimental Procedures) allowed quantification of the amount and intensities of SGs. When the total number of SGs was normalized to the number of cells, all four HF regions showed a similar baseline of about four SGs per cell. This value did not change for CA1 across all experimental groups (Fig. 9A). However, CA3, hilus and DG increased to ~15 SGs per cell at 10R, returning to control levels at 90R and 4hrR (Fig. 9A). A similar result was obtained when the total intensity of all SGs was normalized per cell (Fig. 9B). There was no statistically significant change in CA1, although there was a trend toward an ~four-fold increase at 4hrR. CA3 and hilus showed 11-fold, and DG showed five-fold increases in the intensity of SGs at 10R, and all
Fig. 5. Formation of SGs in the CA3 region of NIC, 10R, 90R and 4hrR animals. Scale bar = 20 μm for all panels, indicated in the 4hrR colored panel. Arrows in the 90R panels point at an interneuron (i.e. basket cell) with a red channel of greater intensity than that in the surrounding pyramidal cells. Abbreviations and panel layouts are as in Fig. 4.
Fig. 6. Formation of SGs in the hilus of all experimental groups. Scale bar=20 μm for all panels, indicated in the 4hrR colored panel. Abbreviations and panel layouts are as in Fig. 4.
Fig. 7. Formation of SGs in the DG. In order to visualize the extremely fine SGs in the tightly packed cell bodies of DG granule cells, analysis had to be carried out under a ×63 oil immersion objective. Scale bar = 10 μm for all the panels, indicated in the 4hrR colored panel. Abbreviations and panel layouts are as in Fig. 4.
returned to control values at 90R and 4hrR. When the intensity of SGs was divided by the total number of SGs, this ratio tripled in CA3 and hilus and doubled in DG at 10R indicating an increased intensity of the SGs, and CA1 did not change. Thus, CA3, hilus and DG neurons, but not those in CA1, undergo a quantitative increase in the number and intensity of SGs at 10R, the same time when eIF2α(P) is at its maximum (Fig. 1A).

Cut-view analysis of SGs

High resolution (×63 oil immersion objective) cut-view analysis revealed distinct morphologies of SGs in different HF regions at different reperfusion times (Fig. 10). SGs in neurons of all NIC and 10R groups had a "trichromatic" columnar organization, that was discerned in the software reconstructed xz plane (Fig. 10, lower images between light green lines in each panel). We term these “trichromatic” due to the orderly columnar arrangement of the three fluorescence colors in which green TIA-1 was at the base, yellow composite TIA-1/S6 in the middle, and red S6 at the apex of the column. Trichromatic SGs are shown for NIC CA1 (Fig. 10A), 10R DG (Fig. 10C) and 10R CA3 (Fig. 10D). The long axis of the columns was typically 5–8 μm and the short axis 2–4 μm, with the long axis perpendicular to the microscope field of view (the microscope field is shown in the top images of each panel in Fig. 10). By 4hrR, SGs in CA1 pyramidal neurons were entirely yellow (Fig. 10B). SGs in 4hrR CA3 and hilar neurons (Fig. 10E and 10F, respectively) were composed of a yellow "core" surrounded by a red “shell,” with an orange region of variable thickness between the core and shell. The complete overlap of the S6 and TIA-1 proteins in single SGs of CA1 may

Fig. 8. S6 that is not co-localized with TIA-1 in CA1 and CA3 pyramidal neurons. To illustrate S6 staining that is free of co-localized TIA-1, the yellow channel representing co-localized S6 and TIA-1 (see Experimental Procedures) was subtracted from the red S6 channel for CA1 (top panels) and CA3 (bottom panels) in the NIC (left panels) and 4hrR (right panels) groups. Note the white areas in each of the four images, where TIA-1-co-localized S6 has been subtracted from total S6 staining. Scale bar≈20 μm for all panels.
Fig. 9. Quantification of SGs in HF. Orthographic projections (as shown in Figs. 4–7) of each of the HF regions were converted to yellow channels, and particles were counted and quantified for total intensity (as described in Experimental Procedures). Counts were obtained from three images per group, and each image contained approximately 50 cells, except DG which contained ~200 cells. (A) The total number of SGs was divided by the total number of cells for each HF region. (* P<0.05 as compared with respective NICs for each region). (B) Total SG intensity divided by total number of cells (** P<0.01, * P<0.05, compared with respective NICs for each region). (C) Total density of SGs divided by the total number of SGs (* P<0.05 compared with NICs for each region).
reflect a tighter sequestration of the S6 into the granule, while those in CA3 and hilus may indicate a more loose interaction between the proteins, and possibly, a dispersal of the S6 protein toward the granule periphery. This last assumption is born by the fact that by 4hrR, S6 in CA3 and hilus is present in the cytoplasm and is similar to controls (e.g. Figs. 5 and 6, compare NIC to 4hrR).

3D reconstructions of SG distribution and continuity of nuclear and SG TIA-1

Fig. 11 contrasts different patterns of SG and S6 distribution in the cytoplasm of CA3 and CA1 pyramidal neurons at 4hrR as revealed by 3D reconstructions of the respective optical sections. SGs in CA3 were few, but S6 (red) staining was abundant and dispersed throughout the cell bodies and proximal dendrites (Fig. 11A). In contrast, 4hrR CA1 pyramidal neurons displayed a stark absence of cytoplasmic S6 (red) staining, and an abundance of roughly spherical SGs clustered in close vicinity to the nucleus (Fig. 11C). This data suggests that the loss of S6 in unfractionated HF observed by Western blot (Fig. 1B) is occurring predominantly in CA1 pyramidal neurons.

Because of the close relationship of SGs to the nucleus, we aimed at demonstrating a continuity between the green (TIA-1) fluorescence in the cell nucleus with that in a trichromatic column (SG) adjacent to the nucleus. Such morphological relationship is shown in 3D reconstruction of a 10R CA3 pyramidal neuron using merged TIA-1 and S6 channels (Fig. 11C), and in the TIA-1 3D reconstruction (Fig. 11D). Narrow streams of green fluorescence appeared to radiate from the nuclear TIA-1 and then become continuous with the green fluorescence at the base of a trichromatic columnar SG next to the nucleus (arrows in Fig. 11C and 11D insets). This kind of visual image suggests a continuity of nuclear-localized and SG-localized TIA-1.

Patterns of nuclear TIA-1 after ischemia/reperfusion

Variations in nuclear TIA-1 distribution in HF neurons after I/R correlated with ischemic resistant and vulnerable cell types. In NICs, the pattern of nuclear TIA-1 fluorescence of CA3, hilus and DG neurons was aggregated into distinct "patches" or clumps surrounding areas devoid of staining, a pattern we shall refer to as "condensed." In contrast, CA1 pyramidal neurons showed a more homogeneously dispersed, fibrous pattern containing one or two areas absent of TIA-1 staining, which we shall term "decondensed" (compare the four NIC panels in Fig. 12, and also see Fig. 11B). TIA-1 nuclear staining remained decondensed in CA1 neuronal nuclei in all experimental groups. At 10R and 90R, the nuclear TIA-1 staining pattern was decondensed in all HF regions. At 4hrR, nuclear TIA-1 in CA3, hilus and DG neurons tended to condense at the core of the nucleus and around the inner aspect of the nuclear envelope, in roughly radial patches, thus giving the nucleus a characteristic "cart-wheeled" appearance. While the significance of this TIA-1 reorganization is not understood, we note these changes occur in the ischemia-resistant HF regions. Altered TIA-1 nuclear morphology could underlie a transcriptional mechanism related to the cells' ability to disassemble SGs, or some other mechanism to resist further reperfusion injury.

SG ultrastructure

We performed TEM to identify the ultrastructural correlate of the cytoplasmic granules identified by immunofluorescence. Guided by the immunofluorescence results, we evaluated CA1 and CA3 pyramidal neurons from control brains or brains subjected to 10 min ischemia and 20 min reperfusion (10I/20R, equivalent to the 10I/10R group with respect to translation arrest and eIF2α(P) levels). In agreement with previous EM descriptions (Gilks et al., 2004), SGs consisted of irregular or roughly circular electron dense bodies, with diameters of 0.1–0.2 μm, often surrounded by ribosomes or ribosomal subunits.

Fig. 13 shows progressive magnifications of a control (A–C) and a 10I/20R (D–F) CA1 pyramidal neuron. We observed several well-established features of reperfused neurons (Fig. 13D–F), including disaggregation of poly-somes, dilation and vesicularization of the Golgi apparatus (Fig. 13E, arrow) and clumping of nuclear chromatin. In both the control and reperfused neurons, several electron dense bodies (marked by white circles) were observed interspersed among endoplasmic reticulum and free ribosomes, or adjacent to the cytoplasmic face of the outer nuclear membrane. Fig. 13C and 13F are high magnifications of such bodies. Their diameters were approximately 100 nm, and they were surrounded by free ribosomes, some of which were in direct contact with the SG (Fig. 13C and F, arrows). In the control CA1 neuron, the SG is seen adjacent to an ER membranous sac (Fig. 13C). In the reperfused CA1 neuron, the SG is located immediately adjacent to the cytoplasmic face of the outer nuclear membrane (Fig. 13F). The bodies are not membrane bound, in contrast to an adjacent, membrane-bound lysosome (Fig. 13E, arrow head). Notably, consistent with a lack of increase in SGs in reperfused CA1 (Fig. 9A), there was no obvious increase in the number of the SGs between the control and the reperfused neuron (compare the number of white circles between Fig. 13A and 13D).

Fig. 14 contrasts a control (A–C) and a reperfused (D–F) CA3 pyramidal neuron. The control CA3 pyramidal neuron possessed abundant polysome rosettes and ribosome-studded rough ER (Fig. 14A). In contrast, the reperfused CA3 neuron showed almost complete disaggregation of poly-somes (Fig. 14D). Scattered throughout the region of the cytoplasm abundant in ER, careful inspection of both images again revealed many circular or irregularly shaped electron dense bodies of 100–200 nm, often surrounded by free ribosomes. In fact, the number of such bodies increased following reperfusion (compare the number of white circles in Fig. 14A to those in Fig. 14D), corresponding to the increase in SGs in CA3 observed by immunofluorescence (Fig. 5, 10R). As with the CA1 neurons, the high resolution images revealed these bodies to be surrounded by or in contact with free ribosomes (Fig. 14C and 14F, arrows), lacking a membrane, and granular or particulate in form. For both control and reperfused
neurons, the high magnification images show the SGs adjacent to ER membranous sacs (Fig. 14B and 14E, respectively). The granular nature of these bodies is consistent with SGs being composed of 48S preinitiation complexes enmeshed in a matrix of TIA-1 molecules.

**DISCUSSION**

The present study has produced several findings: (1) SGs containing TIA-1, S6, eIF4E and eIF4G, but not 60S ribosomal subunits, were observed in control HF neurons, (2) at 10 min reperfusion following 10 min ischemia, SGs quantitatively increased in resistant CA3, hilar and DG neurons, but not vulnerable CA1 pyramidal cells, (3) both ribosomal protein S6 and TIA-1 decreased in unfractionated HF homogenates at 4hrR, (4) in 4hrR CA1 pyramidal neurons, but not other HF cell types, S6 became exclusively localized in SGs, (5) SGs underwent distinct morphological changes in different HF regions, (6) TIA-1 underwent a change in its nuclear distribution in resis-
tant HF regions, but not in the vulnerable CA1 region, and (7) the ultrastructural correlate of the neuronal SG is a 0.1–0.2 μm, irregularly shaped particle, localized in the cytoplasm in the vicinity of ER or the outer nuclear membrane, and surrounded by free ribosomes or ribosomal subunits.

Fig. 11. Three dimensional reconstructions from optically sectioned 15 μm thick tissue blocks illustrating the 3D spatial distribution of S6 and TIA-1 in reperfused CA1 and CA3 neurons. (A) 3D reconstruction of merged TIA-1 (green) and S6 (red) channels of 4hrR CA3 showing abundant free 40S subunits (red color). (B) 3D reconstruction of merged TIA-1 and S6 4hrR CA1 shows all S6 is colocalized in TIA-1 containing SGs. (C) 3D reconstruction of merged TIA-1 and S6 channels of a 10R CA3 pyramidal neurons clearly shows columnar structure of SGs (inset). (D) 3D reconstruction of the same field as in C, with only the TIA-1 (green) channel. C and D illustrate the possible migration of TIA-1 from the nucleus to a perinuclearly located SG (inset). Because of the 3D reconstruction method, depending on the angle of rotation of the 3D construct, the individual optical sections can be seen to be stacked on top of each other, producing a striated appearance, as seen in the right side of Fig. 10C and 10D and in the respective inset images. Scale bars shown for each image; scale in D same as C.
SGs and prolonged translation arrest in reperfused CA1

Persistent translation arrest following brain reperfusion has been proposed to be causally linked to delayed neuronal death (Hossmann, 1993). Therefore, a key issue addressed by the present study was to investigate whether SGs played a role in the irreversible translation inhibition that occurs in CA1 pyramidal neurons during reperfusion. We note here that we did not feel compelled to simultaneously measure in vivo protein synthesis because it is well established that translation recovers in CA3, DG and hilus, but not CA1, following the duration of transient brain ischemia used in the present study (Dienel et al., 1980; Bodsch et al., 1985; Thilmann et al., 1986; Araki et al., 1990; also reviewed in Hossmann, 1993). Further, we previously showed in the present model that in vivo protein synthesis in whole HF was 10% and 25% of controls at 90R and 4hrR, respectively (Kumar et al., 2003), indicating a net recovery of translational competence in 4hrR HF.

Using eIF2\(\alpha\)(P) immunohistochemistry, we also previously demonstrated that all four HF regions underwent maximal eIF2\(\alpha\) phosphorylation at 10 min reperfusion, which cleared almost completely from the cytoplasm of all HF neurons by 4 h reperfusion (DeGracia et al., 1997). In fact, the modest residual eIF2\(\alpha\)(P) at 4hrR was detected in the nuclei of CA1 pyramidal neurons (DeGracia et al., 1997), precluding eIF2\(\alpha\)(P)-mediated inhibition of cytoplasmic protein synthesis. In the present study, at 4 hr reperfusion, eIF2\(\alpha\)(P) decreased 80% from its peak at 10 min reperfusion to 3.3-fold controls (Fig. 1), translation has recovered 2.5-fold from 90R (Kumar et al., 2003), but CA1 pyramidal neurons are well known to display complete translation arrest (Dienel et al., 1980; Bodsch et al., 1985; Thilmann et al., 1986; Araki et al., 1990). Hence, eIF2\(\alpha\) phosphorylation by itself cannot account for translation arrest at reperfusion durations greater than several hours.

Our present results indicate that it is the continued presence of SGs in reperfused neurons which prolongs...
Fig. 13. Transmission electron micrographs illustrating the distribution of SGs in a control (A–C) or a 10 min ischemic and 20 min reperfused (D–F) CA1 pyramidal neuron. White circles in A and D mark electron dense areas of putative SGs. Black dashed lines in A and C delimit the plasma membrane of the CA1 cell of interest. B and E are blowups of the white dashed boxes in A and D, respectively. C and F are blowups of the white dashed boxes in B and E, respectively. Arrows in C and F point to ribosomes associated with the electron dense particle that is approximately 100 nm in diameter. Arrowhead in E points to a membrane bound lysosome. ER, endoplasmic reticulum; NUC, nucleus. Scale bars = 500 nm in A and C, in B and D are 250 nm and in C and E are 125 nm. Magnifications are: A, 28,000×; B, 56,000×; C, 168,000×; D, 28,000×; E, 54,000×; F, 180,000×.
Transmission electron micrographs illustrating the distribution of SGs in a control (A–C) or a 10 min ischemic and 20 min reperfused (D–F) CA3 pyramidal neuron. White circles in A and D mark electron dense areas of putative SGs. B and E are blowups of the white dashed boxes in A and D, respectively. C and F are blowups of the white dashed boxes in B and E, respectively. Arrows in C and F point to ribosomes associated with the electron dense particle that is approximately 100 nm in diameter. ER, endoplasmic reticulum; NUC, nucleus. Scale bars in A and C, in B and D are 500 nm and in C and E are 125 nm. Magnifications are: A, 27,000×; B, 56,000×; C, 188,000×; D, 28,000×; E, 68,000×; F, 176,000×.
translation arrest beyond the duration of eIF2α phosphorylation. Further, CA1 pyramidal neurons were distinguished from all other HF neurons by the simultaneous loss of cytoplasmic matrix S6 and the complete sequestration of S6 in SGs. We therefore suggest that these events are early markers of irreversible inhibition of protein synthesis in CA1 pyramidal neurons following I/R.

There is precedence for this suggestion. Kedersha et al. (1999) discussed the imperfect correlation between eIF2α phosphorylation and translation arrest, citing the example from heat shock in which eIF2α phosphorylation is transient, but translation arrest persists (Duncan and Hershey, 1989). Kedersha et al. (1999) also suggested that eIF2α(P) is required for the initiation, but not the maintenance of translational arrest, and further stated that if dephosphorylation of eIF2α(P) occurs in the continued presence of stress, mRNA and 40S subunit sequestration in SGs may become the “major determinant” of continued translational arrest. We propose that our present observations in reperfused CA1 pyramidal neurons support this suggestion.

Ultrastructural correlates and SG subcellular localization

Our TEM observations of SGs bear directly on previous studies of neuronal ultrastructure following I/R. A number of ultrastructural modifications have been observed in CA1 pyramidal neurons by EM following I/R. These include membranous “whorls” located in sites normally occupied by the ER and Golgi apparatus (Petito and Pulsinelli, 1984), ribosomal aggregates enclosed within some of the larger whorls (Rafols et al., 1995), proliferation of ER (Kirino and Sano, 1984), protein aggregation (Hu et al., 1992) or deposition of dark material (Kirino et al., 1984; Deshpande et al., 2000, 2001), and the appearance of what was variously described as electron dense material (Deshpande et al., 1992) or deposition of dark material (Kirino et al., 1984).

The electron dense material identified by Kirino et al. (1984) and Deshpande et al. (1992) was not membrane enclosed but was associated with membranous sacs of the ER in the soma and dendrites of reperfused CA1 neurons. Although both authors presented various suggestions as to the nature of these depositions, to our knowledge no further studies have investigated these structures. Significantly, the electron dense bodies persisted until cell death at 3 days’ reperfusion, leading both authors to suggest they play a role in delayed neuronal death. We suggest the electron dense material identified in the Kirino et al. (1984) and Deshpande et al. (1992) studies in CA1 neurons is in fact SGs, albeit of a modified nature.

Variations in SG physiology following I/R

Two of our findings indicate that SG physiology is different between CA1 and the other hippocampal regions following I/R. First, SGs increased along with eIF2α(P) in resistant regions, but not CA1 pyramidal neurons. Second, by 4hR, SGs in CA1 pyramidal neurons displayed a different morphology than those in CA3 or hilus.

Anderson and Kedersha (2002) presented the model that polysomes and SGs are mutually exclusive structures, the former a structural correlate of active protein synthesis, the latter a structural correlate of inhibited protein synthesis. SGs and polysomes are in equilibrium with each other, and the extent to which the equilibrium proceeds toward SG formation is dependent upon any factor that will dissociate polysomes. Under physiological conditions, cells respond to stress by phosphorylation of eIF2α and thereby shift the equilibrium from polysomes to SGs. Therefore, when levels of eIF2α(P) rise, polysomes should dissociate and SGs form. This is exactly what we observed in CA3, hilus and DG. However, a quantitative increase in SGs did not occur in CA1 pyramidal neurons, despite the fact that polysomes disaggregated (Fig. 13D) and eIF2α(P) increases massively in these cells (DeGracia et al., 1997). This indicates that there is a modification in SG physiology in CA1 pyramidal neurons following I/R.

Second, SGs in CA1 pyramidal neurons displayed a different structure from those in the resistant HF regions at 4hR. In controls and at 10 min reperfusion, the SGs in all HF regions were morphologically similar (e.g. the trichromatic columns). At 4 h reperfusion, the SGs in 4hR CA3 and hilar neurons, containing a yellow core surrounded by a red shell, suggest a looser association of S6 and TIA-1 in which, perhaps, these are in the process of returning to the basal “trichromatic” state. In contrast, the solid yellow SGs of 4hR CA1 intimate a tight association of S6 and TIA-1 in a more permanent structure. Given that SGs in all HF regions were initially of similar form (e.g. trichromatic columns), their divergent morphologies at 4hR reperfusion are an additional indication that SG physiology is modified in CA1 pyramidal neurons following I/R.

Hence, taking our current finding that SG physiology is modified in reperfused CA1 pyramidal neurons, in conjunction with the ultrastructural observations of the persistence of electron dense bodies in CA1 (Kirino et al., 1984; Deshpande et al., 1992), leads to the conclusion that altered SG physiology in CA1 pyramidal cells is the cause of persistent translation arrest, and therefore causally linked to delayed neuronal death.

Variations in SG morphology following I/R

The present study has not directly investigated the link between the three SG morphologies we identified by immunofluorescence (e.g. trichromatic columns, yellow core/red shell, and solid yellow particle) and the SG structures we observed by TEM. By TEM we observed particles on the order of 100–200 nm. By light microscopy, we observed 3D columnar structures with a cross-sectional area of 2–5 μm that extended 5–8 μm in the z direction. It is reasonable to presume that the particles observed by light microscopy are composed of many of the 100 nm particles, particularly as these particles form clusters over areas of several microns. One possibility is that the primary and secondary antibodies used to detect the SGs by immunofluorescence accumulated on the individual SGs, linking them artificially in the 3D structures we observed. However, this possibility does not explain the very ordered and regular structures we observed as a function of reperfusion duration (Fig. 10). An alternative possibility is that the
100–200 nm particles are cross sections of a more extensive 3D structure, analogous to the ER membrane, the latter of which forms a complex 3D labyrinth in the cytoplasm.

This latter interpretation is plausible insofar as our current understanding of the structure and composition of SGs is incomplete. In some instances, different stresses will lead to different SG compositions. For example, heat shock stress leads to recruitment of HSP27 to SGs, but arsenic and UV-induced stresses do not (Kedersha et al., 1999). The minimum composition of SGs appears to involve eIF5-deficient 48S preinitiation complexes, TIA-1 and TIAR (Kedersha and Anderson, 2002), with some controversy as to if eIF2 is an SG component (Kedersha and Anderson, 2002; Kimball et al., 2003). TIA-1’s association with SGs is highly dynamic, with TIA-1 entering and leaving SGs rapidly (Kedersha et al., 2000) in a prion-like aggregation (Gilks et al., 2004). Formation of SGs is also dependent upon intact microtubules (Ivanov et al., 2003), and the “survival motor neuron” (SMN) protein (Hua and Zhou, 2004). Our present results support the dynamic view of SGs. Further investigation is necessary to fully determine the significance of the three SG structures we have identified here, how they relate to the TEM structures, and how SGs are modified to contribute to delayed neuronal death following reperfusion.

Ribosomal protein S6 in HF after I/R

To our knowledge, ours is the first study to evaluate ribosomal protein S6 directly in reperfused HF. Ribosomal protein S6 is a 32 kDa protein component of the 40S ribosomal subunit. Serine/threonine phosphorylation of S6 by S6 kinase shifts the mRNA binding specificity of ribosomes such that they preferentially translate mRNAs coding for ribosomal proteins, initiation and elongation factors (Meyuhas, 2000). Regulation of ribosome activity via S6 phosphorylation has long been recognized (Wettenhall et al., 1983), and S6 kinase is controlled by the mTOR pathway (Kimball and Jefferson, 2004).

To date, three studies have evaluated S6 kinase activity following brain I/R and all showed a decrease in the phosphorylation of S6 kinase in the first several hours of reperfusion (Althausen et al., 2001; Janelidze et al., 2001; Martin de la Vega et al., 2001). To our knowledge, there have been no studies of the phosphorylation of S6 following brain I/R. In our unfractionated HF samples, all S6 was dephosphorylated and did not react with phospho-S6 specific antisera (Fig. 1B). This indicates that S6 is not constitutively phosphorylated in HF. Alternatively, although our homogenization buffer is designed to prevent elf2α(P) dephosphorylation by inhibition of endogenous elf2α(P) phosphatases, it is possible that endogenous phospho-S6 may have been dephosphorylated during homogenization by a phosphatase not inhibited by our isolation buffer.

Our Western data show a 50% decrease in S6 in unfractionated HF by 4 h reperfusion (Fig. 1B). In CA3, hilus and DG, SGs quantitatively increased at 10R (Fig. 9), indicating that S6 shifted from polysomes to SGs at this time. However, in these regions, SG counts returned to control levels and TIA-1-free cytoplasmic S6 staining resembled controls by 4hrR (Figs. 5–8), suggesting that there was not a gross loss of S6 in CA3, hilus or DG. On the other hand, in 4hrR CA1 pyramidal neurons, essentially all S6 cytoplasmic staining was colocalized with TIA-1 in the form of SGs (Fig. 8). Since SGs did not quantitatively change in CA1 (Fig. 9), our quantitative data argue that the S6 decrease occurred in TIA-1-free cytoplasmic S6 in CA1 pyramidal cells. This finding implies a loss of 40S ribosomal subunits in CA1 pyramidal neurons by 4hrR. The cause of S6 decrease, whether by proteolysis or some other mechanism, has not been investigated in the present study and provides an important avenue for further investigation.

Cytoplasmic TIA-1 in HF neurons

TIA-1 is a cytoplasmic protein containing three RNA recognition motifs (Tian et al., 1991). RNA recognition motifs are 90 amino acid modules present in proteins involved in various aspects of RNA metabolism (Varani and Nagai, 1998). TIA-1 has been shown to suppress translation of the mRNA coding tumor necrosis factor alpha (Gueydan et al., 1999), and in promoting alternative splicing of “weak” 5’ splice sites (Del Gatto-Konczak et al., 2000; Forch et al., 2000).

We have shown here that control CA1, CA3 and hilus showed prominent TIA-1 staining in some, but not all, of the cytoplasmic S6 granules (e.g. Fig. 8) indicating that there are basal levels of SGs in these neurons. TIA-1 has been independently observed in the cytoplasm of control rodent neurons (N. L. Kedersha, Division of Rheumatology, Immunology, and Allergy Brigham and Women’s Hospital, personal communication). These observations suggest that TIA-1 plays a physiologic role in neurons. It has been suggested that cytoplasmic TIA-1, and TIAR, may play a general role in physiologic translation control, for example in promoting cytoplasmic deadenylonation or readenylonation (Antic and Keene, 1998). Our observations of the constitutive presence of SGs in neurons give rise to the suggestion that they may play a role in the normal cycle of protein synthesis: upon translation termination, at least some mRNA and modified 48S preinitiation complexes are recruited to SGs, which serve to protect mRNA from degradation and provide a pool to recruit mRNA and 48S preinitiation complexes back into active protein synthesis. Given potential physiologic functions for cytoplasmic TIA-1, Kedersha et al. (1999) have suggested that assembly of SGs under stress conditions may represent an extreme case of SG formation. Our observation of SGs in control neurons supports this suggestion.

Nuclear TIA-1 after I/R

We observed both a decondensed and a condensed distribution of nuclear TIA-1 (Fig. 12). The ischemia-resistant HF regions showed a dynamic change between these patterns, suggesting that lack of this change may play a role in the vulnerability of CA1 pyramidal neurons. Other investigations have shown TIA-1 to be predominantly located in the nucleus and to shift to cytoplasmic SGs fol-
lowing cell stress (e.g. Kedersha et al., 1999, 2000). To our knowledge there has been no focus on the relationship between TIA-1’s subcellular morphology and its biochemical function. In the decondensed TIA-1 pattern, we observed one or two roughly spherical volumes absent of TIA-1 staining. Interestingly, these “holes” showed positive staining for ribosomal P antigen (asterisks, in Fig. 3K and 3J), suggesting they are the nucleolus. In studies from other laboratories, nuclear TIA-1 staining also shows one or up to several of these “holes” devoid of TIA-1 staining (Kedersha et al., 1999; Kedersha and Anderson, 2002; Kimball et al., 2003). We observed the nuclear TIA-1-deficient areas to be expanded greatly in resistant HF at 4 h reperfusion (Fig. 12). If these “holes” are indeed the nucleolus, then perhaps nucleolar expansion in resistant HF regions indicates increased synthesis of ribosomes, a potentially protective response to ischemic stress. Alternatively, the condensed pattern of TIA-1 staining may represent a stress-induced genetic response, perhaps one involved in dissolution of cytoplasmic SGs.

Finally, we observed a 40% decrease in TIA-1 in un-fractionated HF at 4 h reperfusion. As the immunofluorescence data suggest that S6 is being lost predominantly from the cytoplasm of CA1 pyramidal neurons, the morphological changes we have observed with TIA-1 are also consistent with its loss. The condensed distribution of TIA-1 in the nuclei of CA3, DG and hilar neurons at 4 h reperfusion suggests loss of TIA-1 from these cells. Again, we have made no attempt here to investigate the cause of TIA-1 loss. We speculate that this may be part of the mechanism of abating the SG response. Perhaps in the dissolution of cytoplasmic SGs, cytoplasmic TIA-1 undergoes proteolysis as one means of dissipating SGs and returning the cell to a translationally active state.

CONCLUSION

Here we have provided the first in vivo description of SGs in hippocampal neurons and the behavior of SGs out to 4 h reperfusion after 10 min of global ischemia. We have provided morphological evidence that prolonged translation arrest in reperfused CA1 pyramidal neurons is due to loss of 40S ribosomal subunits and the complete sequestration of the remaining 40S ribosomal subunits in SGs at a time when eIF2α(P) has dephosphorylated 80% from its peak. This mechanism relates the persistent translation arrest in CA1 to the acute phase of translational arrest mediated by eIF2α phosphorylation (DeGracia, 2004), supporting Hossmann’s (1993) suggestion that persistent translation inhibition in CA1 represents a lack of recovery from acute translation inhibition. The present study opens the door to investigate why CA1 pyramidal neurons display differential behavior from the other HF regions with respect not only to SGs and S6, but also nuclear localized TIA-1.

The connection between persistent translation arrest and cell survival is that cells need to synthesize proteins to express genetic repair programs elicited by ischemic stress (Paschen, 2003; DeGracia and Montie, 2004), and to simply continue synthesis of constitutive proteins required for neurons to operate. The present data support the hope that pharmacologic interventions that can restore protein synthesis will facilitate neuronal survival following brain I/R, as occurs following stroke and resuscitation from cardiac arrest.

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