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Subcellular stress response and induction of molecular chaperones and folding proteins after transient global ischemia in rats

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Abstract

Brain ischemia induces the toxic accumulation of unfolded proteins in vulnerable neurons. This cellular event can trigger the unfolded protein response (UPR) and activate the expression of a number of genes involved in pro-survival pathways. One of the pro-survival pathways involves the sequestration and elimination of misfolded and aggregated proteins. Recent evidence suggests that the endoplasmic reticulum (ER), mitochondria, and cytoplasm respond individually to the accumulation of unfolded proteins by induction of organelle specific molecular chaperones and folding enzymes. This study utilized a rat model of transient (15 min) global ischemia (2-vessel occlusion) to investigate the regional and temporal induction of some of these key stress proteins after ischemia. Electron microscopy demonstrated that visible protein aggregates accumulated predominately in the cytoplasm. We used *in situ* hybridization (forebrain structures) and western blot (hippocampus) analysis to measure changes in expression of heat shock protein 70 (HSP70 cytoplasmic), HSP60 (mitochondrial), ER luminal proteins glucose response proteins GRP78 and GRP94, protein disulphide isomerase (PDI), homocysteine-inducible, endoplasmic reticulum stress-inducible protein (HERP), and calnexin. Induction of mRNA for HSP70 occurred earlier (beginning at 30 min) and at a higher level relative to the delayed (4–24 h) and more moderate induction of mRNAs for mitochondrial matrix HSP60 and the ER lumen HERP, GRP78, GRP94, calnexin and PDI. Increases in hippocampal proteins were observed at 4 h (HSP70) and 24 h (HSP60, GRP78, GRP94) after reperfusion. These results demonstrate that after a transient ischemic insult, the subcellular responses to the accumulation of unfolded proteins varies between cellular compartments and are most prevalent in the cytoplasm and, to a lesser degree, in the mitochondrial matrix and ER lumen.

Keywords

Brain ischemia; Protein aggregation; Chaperone and folding protein; CA1 hippocampus; Herp-GRP78-GRP94-calnexin-PDI-HSP70-HSP60

1. Introduction

In rat forebrain ischemia models, transient cerebral ischemia followed by reperfusion leads to delayed selective neuronal death in hippocampal CA1 pyramidal neurons after 48–72 h of reperfusion while leaving dentate gyrus (DG) granule cells largely intact (Pulsinelli et al.,

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1982; Ito et al., 1975; Kirino, 2000; Smith et al., 1984). This model has been commonly utilized to study molecular events occurring uniquely either in CA1 vulnerable neurons or in dentate gyrus surviving neurons after ischemia. During the early post-ischemic period 0–48 h, CA1 neurons destined to die appear normal, as viewed by routine light microscopy. Under electron microscopy (EM), however, visible protein aggregates progressively accumulate in CA1 neurons from 2–4 h of reperfusion and continue until delayed cell death takes place at 48–72 h of reperfusion after ischemia (Hu et al., 2000; Liu et al., 2004, 2005b; Zhang et al., 2006). Although delayed neuronal death after transient cerebral ischemia has been intensively studied, the molecular mechanisms underlying selective neuronal vulnerability remain incompletely understood (Kirino, 2000; Yamashima et al., 2007).

Accumulating evidence suggests that the endoplasmic reticulum (ER), mitochondria and cytoplasm each respond to the accumulation of unfolded proteins by compartment-specific signaling pathways (Paschen, 2003; Yoneda et al., 2004; DeGracia and Hu, 2007). The maturation of newly synthesized membranous and secreted proteins begins within the endoplasmic reticulum (Hebert and Molinari, 2007). As many as 1/3 of all newly synthesized proteins are targeted to the ER and as a consequence, chaperone and folding enzymes outnumber newly synthesized proteins and function to prevent protein aggregation and assist in protein folding (Ghaemmaghami et al., 2003). However, various pathological insults induce a state of stress in which the number of unfolded proteins exceeds the processing capacity of molecular chaperones and folding enzymes. To increase the capacity of processing unfolded proteins produced after stress, the ER initiates signaling pathways that increase gene transcription of molecular chaperones and shut off protein translation. These ER-mediated regulatory processes are referred to as the “unfolded protein response” (UPR) (Mori et al., 1996). The ER stress response kinase (PERK) and glucose-regulated protein 78 (GRP78) are key sensors of the UPR (Lee, 2005; DeGracia and Hu, 2007). Accumulation of unfolded proteins in the ER lumen: (i) induces activation of PERK to shut off protein synthesis via phosphorylation of eukaryotic initiation factor 2 (eIF-2), and (ii) binds GRP78, thus promoting uncoupling of GRP78 from GRP78-bound transcription factors. The uncoupled transcription factors are then translocated into the nucleus to induce expression of genes encoding molecular chaperones and folding enzymes such as GRP78 itself, HERP, GRP94, calnexin and PDI (Kokame et al., 2000, 2001). UPR components such as GRP78 and GRP94 have been observed after focal brain ischemia (Aoki et al., 2001; Hayashi et al., 2005; Paschen, 2004; Wang et al., 1993).

In contrast to the UPR, accumulating unfolded proteins in the cytoplasm induce expression of chaperones via a different signaling pathway that involves the activation of HSF (heat shock transcription factor) (Abravaya et al., 1992). HSP70 is the major stress-induced cytoplasmic chaperone (Vass et al., 1988; Nowak, 1991; Sharp et al., 1999) and has been intensively studied after brain ischemia (Giffard et al., 2004). Therefore, induction of HSP70 is indicative of the cytoplasmic buildup of non-native-state proteins (Naylor and Hartl, 2001). HSP60 is a major molecular chaperone predominantly residing in the mitochondrial matrix. Recent studies clearly demonstrate that the *HSP60* gene is selectively induced by mitochondrial accumulation of unfolded proteins, but not affected either by heat-shock (mainly eliciting cytoplasmic stress) or ER stress (Corydon et al., 2005; Lee, 2005; Martin, 1997; Martin and Hartl, 1997; Yoneda et al., 2004).

The purpose of this study was to elucidate some of the cellular compartment specific stress responses involved in delayed neuronal death. We utilized a transient global ischemia model to study induction of cellular compartment-specific molecular chaperones and folding enzymes in the hippocampus after brain ischemia and reperfusion. The results indicate that protein aggregation and the induction of molecular chaperones occur strongest in the cytoplasm, followed by mitochondrial matrix, and to a lesser degree in the ER lumen of vulnerable CA1

neurons. These findings indicate that ischemic stress affects the genetic response of vulnerable and non-vulnerable brain regions by targeting multiple cellular organelles.

2. Results

2.1. Histopathology

To verify the temporal profile and patterns of CA1 neuron selective vulnerability in sham and postischemic rats, 50 μ m sections were stained with acid fuchsin and celestine blue. Under light microscopy, normal neurons are round (Fig. 1, arrows), whereas ischemic dead neurons have shrunken nuclei with polygonal and elongated shapes (Fig. 1, arrowheads). No morphological differences were seen in hippocampal CA1, CA3 and DG neurons between sham-operated control rats and rats subjected to 15 min of ischemia followed by 24 h of reperfusion (Fig. 1, arrows). Delayed neuronal death was observed after 48 h of reperfusion only in dorsal CA1 pyramidal neurons of the hippocampus (Fig. 1, arrowheads), DG neurons were virtually intact after ischemia. These results are consistent with previous reports (Hu et al., 2000; Smith et al., 1984).

2.2. Subcellular localization of protein aggregates in CA1 neurons

To study regional and temporal distribution of protein aggregates after transient cerebral ischemia, we performed EM analysis of hippocampal sections. Neurons from sham-operated control CA1 (Fig. 2A) and DG (Fig. 2B) regions contained normal polyribosomes (arrows), nuclei (N), mitochondria (M), rough endoplasmic reticulum (ER) and Golgi apparatus (G). Abnormal protein aggregates were not seen in CA1 (Fig. 2A) and DG (Fig. 2B) neurons from sham-operated control, but found mainly in the cytoplasm of CA1 neurons at as early as 1 h of reperfusion (Fig. 2C, arrowheads). EM-visible protein aggregates were continuously accumulating in the cytoplasm from 1 h of reperfusion onward and were then associated with mitochondria and the ER in CA1 neurons at 24 h of reperfusion (Fig. 2E, double arrows). In comparison, EM-visible protein aggregates were rarely seen in DG surviving neurons after transient cerebral ischemia (Figs. 2D and F). After 48 h of reperfusion, more than 95% of CA1 neurons were damaged, whereas virtually all DG neurons appeared normal (see Fig. 1).

2.3. Gene expression after transient ischemia

2.3.1. HSP70—In sham-operated animals HSP70 mRNA levels were undetectable in any regions of the brain. HSP70 was rapidly and intensely upregulated beginning at 30 min after reperfusion throughout the forebrain (Fig. 3). Expression was seen throughout the cortex, hippocampus, thalamus, and hypothalamus. By 4 h after reperfusion, the expression had reached maximum levels in these regions, with a slight decrease in the hypothalamus. At 24 h, HSP70 mRNA was more confined to the outer cortical layers, hippocampus, and thalamus, and by 48 h, expression was still elevated above sham but was limited to the CA1 hippocampus and outer cortical layers (Fig. 3A). Region of Interest (ROI) measurements were taken in the outer cortical layer of the cerebral cortex, CA1, and DG. By means of a radioactive standard, values were converted to nCi/g and expressed as mean $\pm$ SEM ($n=5$ per group, $*p<0.05$). Quantitation of HSP70 mRNA intensity in the CA1 hippocampus and dentate gyrus is shown in Fig. 3B.

2.3.2. HSP60—Mitochondrial matrix chaperone HSP60 was also induced after brain ischemia (Fig. 3). Induction of HSP60 mRNA was observed at 4 h after reperfusion in the cortex, hippocampus, and thalamus and persisted at 24 h, decreasing but still remaining above sham levels at 48 h. The levels in the CA1 and dentate gyrus were significantly elevated relative to sham at 4 and 24 h ($p<0.05$) with only CA1 levels remaining elevated at 48 h (Fig. 3C).

2.3.3. Endoplasmic reticulum stress gene expression—GRP78 was upregulated in the CA1 at 4 h persisting to 24 h and in the dentate gyrus beginning at 30 min (Fig. 4). HERP was upregulated beginning 4 h after reperfusion in the CA1, DG, and cortex (Fig. 4B). HERP remained elevated in the CA1 for up to 48 h. GRP94 was increased later starting at 24 h, $p < 0.05$. The GRP94 expression seems to be limited to the medial portion of CA1, and more diffusely distributed in the pyramidal layer. This would be suggestive of expression also in some type of reactive glia in the region.

2.3.4. ER folding enzymes—PDI and calnexin are both constitutive proteins involved in the normal processing of proteins through the ER. PDI showed a modest and transient increase in expression only in the DG at 4 h after reperfusion. Calnexin mRNA was elevated in the CA1 and DG at 24 h (Figs. 5A and B; $*p < 0.05$).

The largest increases in mRNA were observed for HSP70, between 6 and 14 folds higher than sham. HSP60 showed an increase of 3–6 folds. The ER stress proteins GRP78, GRP94, and HERP showed more modest increases of 2–4 folds, and the ER folding enzymes calnexin and PDI showed only slight 1.5–2 fold increases.

2.4. Organelle-specific chaperone proteins after brain ischemia

To study whether ischemia-induced upregulation of mRNAs for molecular chaperones and folding enzymes were translated into proteins after brain ischemia, we measured the protein levels of HSP70, HSP60, GRP78, GRP94, PDI and calnexin by western blot analysis (Fig. 6). Dorsal hippocampal tissue lysates were prepared from sham-operated control animals and animals subjected to 15 min of ischemia followed by 4 and 24 h of reperfusion and analyzed on 8% SDS-PAGE. The level of HSP70 protein was low in sham-operated control, and increased significantly at 4 and 24 h of reperfusion (Figs. 6A and B). This was consistent with the increases seen in mRNA. It should be pointed out that although overall rate of protein synthesis may be irreversibly inhibited, some stress RNAs, such as HSP70 mRNA, may be partially and selectively translated through the internal ribosome-entry site in CA1 neurons after brain ischemia (Holcik and Sonenberg, 2005; Liu et al., 2005a; DeGracia and Hu, 2007). While mRNA levels were increased in the hippocampus as early as 4 h, protein levels of HSP60 and GRP78 were increased over sham-operated at 24 h after ischemia. Both the message and protein levels of GRP94 were slightly, but significantly, increased at 24 h after brain ischemia in the CA1 region (Figs. 6A and B). PDI mRNA was increased at 4 h in the dentate gyrus only, but protein levels were not measurably changed. Calnexin mRNA was significantly increased at 24 h in both CA1 and DG, but the protein levels appeared to be slightly decreased at 24 h in the total hippocampus samples (Fig. 6B).

3. Discussion

In the present study, we investigated the subcellular localization of protein aggregates and the induction of molecular chaperones and folding enzymes after transient cerebral ischemia. It is well known that irreversible translational arrest occurs in neurons destined to die after ischemia/reperfusion (DeGracia and Hu, 2007). EM-visible protein aggregates were mainly localized to the cytoplasm of CA1 neurons. Cytoplasmic HSP70 mRNA was induced earliest and strongest, mRNAs for mitochondrial matrix HSP60 and the ER luminal HERP, GRP78, GRP94, calnexin and PDI were upregulated more moderately. Increases in HSP70 mRNA was detected as early as 30 min of reperfusion and peaked at 4 h. The mRNA levels declined thereafter in surviving neurons but remained elevated in neurons destined to die. In comparison, induction of ER-luminal and mitochondrial matrix chaperone mRNAs was delayed, starting at 4 h of reperfusion mainly in ischemically vulnerable CA1 neurons. Since unfolded proteins are the natural inducers for molecular chaperones and folding enzymes, these results demonstrate a cascade

of events where overproduction of unfolded proteins results in over-expression of molecular chaperones and folding enzymes as an adaptive measure to counter unfolded protein toxicity.

Cytoplasmic denatured proteins have a higher binding affinity to Hsp90-containing multi-chaperone complexes, resulting in heat-shock transcription factor-1 (HSF1) release from the complexes. Subsequently, HSF1 translocates to the nucleus and stimulates production of molecular chaperones like HSP70 (Voellmy, 2004). In this study we observed that the CA1 region shows the most persistent expression of HSP70. This may reflect that accumulation of unfolded/misfolded proteins is persistently present in CA1 neurons. Similarly, accumulated ER-lumen unfolded proteins are able to sequester GRP78 from its binding partners activating transcription factor 6 (ATF6) and IRE-1. ATF6 and XBP1 subsequently translocate to the nucleus to activate the promoters of many genes including GRP78, HERP, GRP94, calnexin and PDI (Kokame et al., 2000, 2001) initiating the UPR. In the mitochondria, HSP60 is selectively induced by perturbations in protein folding. Neither heat-shock stress nor ER stress induce HSP60 expression. This indicates that mitochondria respond to stress via an unidentified signaling pathway different from the heat-shock response and UPR (Corydon et al., 2005; Lee 2005; Martin and Hartl, 1997; Yoneda et al., 2004). The observed upregulation of these genes in the present study would support the hypothesis that the ER and mitochondria are also reacting to the accumulation of unfolded proteins following cerebral ischemia.

The ER luminal unfolded proteins are normally transported retrogradely from the ER lumen to the cytosol for degradation by the ubiquitin–proteasome system. HERP is a novel ER stress-inducible transmembrane protein localized in the ER membrane and is indicative of the ER stress-associated degradation pathway (ERAD) (Kokame et al., 2001; Yamamoto et al., 2004). The early depletion of ubiquitin and proteasome inactivation render the ubiquitin–proteasome system unable to process all of the unfolded proteins overproduced in the ER lumen after ischemic stress (Ge et al., 2007). As a result, unfolded and newly produced proteins, together with their residing ER-associated polyribosomes, are aggregated on the ER outer membranes (Liu et al., 2005a, b). Activation of the ERAD pathway is indicated in this study by the induction of HERP.

Brain ischemia depletes cellular ATP and leads to malfunction of the ATP-dependent protein folding and degradation machinery (Hu, 2007). During reperfusion, blood flow and cellular ATP levels are soon recovered, but unfolded protein aggregates are progressively accumulated from 2–4 h of reperfusion onward until delayed neuronal death occurs at 48–72 h of reperfusion after an episode of ischemia. This strongly suggests that protein aggregation is due to overproduction of unfolded proteins, rather than directly caused by energy failure during the post-ischemic phase.

Fifteen minutes of cerebral ischemia leads to delayed neuronal death in CA1 neurons but leaves DG granule neurons largely intact (Smith et al., 1984). Neurons containing EM-visible protein aggregates have intact cell membranes and relatively normal appearing mitochondria and nuclei suggesting that protein aggregate-containing neurons are viable. However, these post-ischemic vulnerable neurons die in a delayed fashion (Liu et al., 2005b; Hu et al., 2000, 2001; Kirino, 2000; Ito et al., 1975). Although irreversible inhibition of overall rate of protein synthesis in CA1 neurons, some stress mRNAs may be partially and selectively translated after brain ischemia (Liu et al., 2005a; DeGracia and Hu, 2007). The ischemia-induced expression of stress mRNAs may also reflect that stress signal, i.e., accumulation of unfolded/misfolded proteins, is persistently present in CA1 neurons. Therefore, induction of molecular chaperones and folding enzymes by ischemia in the CA1 hippocampus may be too little to protect unfolded proteins from aggregation after ischemia.

In summary, brain ischemia depletes ATP and changes intracellular homeostasis, thus disabling ATP-dependent protein quality control systems including molecular chaperones, folding enzymes, and protein degradation components during and after ischemia (Hu et al., 2001; Hu and Liu, 2004). These cellular alterations can lead to the accumulation of intracellular unfolded proteins. Overloaded unfolded proteins in neurons are endogenous stimulators of molecular chaperones via their specific stress response signaling pathways in various subcellular compartments. Consequently, compartment-specific genes encoding molecular chaperones and folding enzymes are induced in order to cope with unfolded protein toxicity after ischemia. However, because unfolded proteins are too numerous to be protected by molecular chaperones, they aggregate with protein translational and transport machinery during the post-ischemic phase and eventually contribute to delayed neuronal death. Continued investigation into the expression of cell compartment-specific molecular chaperones and methods to target these cellular events should provide critical information on unfolded protein accumulation in vulnerable neurons and subcellular compartments thereby leading to more targeted treatment strategies.

4. Experimental procedures

4.1. Ischemia model

The two-vessel occlusion (2VO) brain ischemia model was used in this study (Smith et al., 1984). All animal experimental procedures were approved by the Animal Care and Use Committee of the University of Miami Miller School of Medicine, and were performed in compliance with the National Institutes of Health guidelines on the ethical use of animals.

Male Wistar rats (250–300 g) were fasted overnight and anesthetized with halothane. Catheters were inserted into the external jugular vein, tail artery and tail vein to allow blood sampling, arterial blood pressure (MABP) recording and drug infusion. A neck incision was made and both common carotid arteries were isolated and encircled by loose ligatures. Blood gases were measured and adjusted to $\text{PaO}_2 > 90$ mmHg, PaCO_2 35–45 mmHg, pH 7.35–7.45 during the intubation period. Bipolar EEG was recorded and core and brain temperature, measured by rectal and temporalis probes, were maintained at 37 °C with a heating lamp. Heparin (150 IU/kg) was administered i.v. and blood was withdrawn via the jugular catheter to produce a MABP of 50 mmHg, and both carotid arteries were clamped. Blood pressure was maintained at 50 mmHg during the ischemic period by withdrawing or infusing blood through the jugular catheter. At the end of 15 min, the clamps were removed and the blood slowly reinfused through the jugular catheter, followed by 0.5 ml of 0.6 M sodium bicarbonate. For the 30 min reperfusion group, halothane was continued and animals were sacrificed at 30 min after ischemia. For groups with reperfusion periods longer than 30 min, halothane was discontinued following the initiation of the reperfusion period, all wounds were sutured and animals returned to their cages. Sham-operated rats were subjected to the same surgical procedures but without induction of brain ischemia ($n=4$).

4.2. Experimental groups

Three separate series, each consisting of sham-operated control rats and rats subjected to 15 min of ischemia followed by 30 min, 4, 24 or 48 h of reperfusion, were prepared for *in situ* hybridization, electron microscopy and histopathology, respectively. Each experimental group consisted of at least four rats. For *in situ* hybridization, the animals were sacrificed by deep anesthesia followed by decapitation, and whole brains were removed and immediately frozen. Coronal sections were cryosectioned at 12 μm at the dorsal hippocampal level at bregma -3.5 (Zilles 1985).

For EM, animals were ventilated with halothane. The brains were perfused through the ascending aorta with ice-cold PBS for 30 s and then 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer. Brains were removed, post-fixed in the same fixative for 2 h, and then processed for embedding. For histopathology, animals were anesthetized with 3% halothane and perfused through the ascending aorta with ice-cold phosphate-buffered saline (PBS) for 30 s and then 4% paraformaldehyde in PBS. Brains were removed, post-fixed in the same fixative for 24 h, sectioned at 50 μ m with a vibratome (Leica Microsystems Inc., Bannockburn, IL) and stained with fuchsin and celestine blue (Smith et al., 1984).

4.3. In situ hybridization

Sections were thawed to room temperature and immediately fixed for 5 min in 4% formaldehyde in PBS. Sections were acetylated for 10 min at room temperature in 0.25% acetic anhydride and 0.1 M triethanolamine HCl (pH 8), then dehydrated through a series of graded ethanol solutions, delipidized in 100% chloroform for 5 min, rinsed in 100% ethanol, and allowed to air dry. Sections were hybridized to ³⁵S-labeled riboprobes generated by *in vitro* transcription of the anti-sense (for positive probe) and sense (for negative probe) strands of cDNA clones subcloned into transcription vectors using the Promega Riboprobe™ System. cDNA clones for HSP60, Herp, GRP94, calnexin and PDI were generously provided by Dr. Koichi Kokame (National Cardiovascular Center Research Institute, Osaka, Japan) and the cDNA clone for GRP78 was provided by Dr. Amy S. Lee (USC/Norris Cancer Center). HSP70 cDNA clone was subcloned in this lab from the rat inducible HSP70 cDNA clone supplied by Dr. Frank Sharp (Longo et al., 1993). Hybridization was conducted as follows: the denatured probe (2×10^7 dpm/ml) was added to a solution containing 100 μ g/ml salmon sperm DNA (ssDNA), 250 μ g/ml each of yeast total RNA and tRNA, 50% formamide, 20 mM Tris HCl (7.4), 1 mM EDTA, 300 mM NaCl, 10% dextran sulfate, and 1 $\times$ Denhardt's. The hybridization solution was added to the sections, covered with coverslips, and hybridized under humid conditions at 55 °C for 20 h. After removal of the coverslips, the sections were washed at room temperature in a series of decreasing amounts of standard saline citrate (SSC) with 1 mM DTT to a final concentration of 0.1 $\times$ SSC. The slides were treated with 20 μ g/ml RNase A for 30 min at 37 °C. The final high-stringency wash was carried out in 0.1 $\times$ SSC, 1 mM DTT for 1 h at 65 °C. Sections were dehydrated through a series of ethanol solutions containing 300 mM ammonium acetate, ending with 100% ethanol. Sections were exposed to Kodak BIOMAX MR film at 4 °C for various lengths of time. Negative control (sense strand) probes showed no hybridization signals (data not shown).

Autoradiographs were digitized by a charge-coupled device camera (Xillix Technologies Corp., Canada) with a Micro Nikon 55 mm lens, which was interfaced to an image-analysis system (MCID Model M2, Imaging Research, Inc., Canada) and captured at 50 μ m resolution. [¹⁴C] Methylmethacrylate standards placed on the films were digitized as well. Image files were transferred to a DEC Alpha Station for analysis. Optical density values of regions of interest were converted to activity values (μ Ci/g of ¹⁴C-equivalents) by means of the standards. Measurements were taken of regions that showed a positive signal for each probe and similar regions in the control animals. Means and standard deviations were calculated ($n=5$ per group). Statistical assessments were performed using one-way ANOVA or Student's *t*-test where appropriate.

4.4. Electron microscopy

EM was carried out on brain tissue sections stained with the conventional osmium–uranyl–lead method as described previously (Hu et al., 2000). Briefly, coronal brain sections were cut at a thickness of 120 μ m with a vibratome at the level of the dorsal hippocampal level. Brain sections were postfixed for 2 h in 1% osmium tetroxide in 0.1 M cacodylate buffer, rinsed in

distilled water, and stained with 1% aqueous uranyl acetate overnight. Tissue sections were dehydrated in an ascending series of ethanol to 100% followed by dry acetone, and embedded in Durcupan ACM. Small squares from the hippocampal CA1 and DG regions (3.8 mm caudally from bregma) were dissected and glued onto resin blocks. Ultrathin sections (0.1 μm) were prepared and stained 1 min with 3% lead citrate prior to examination with a transmission electron microscope (model EM10C, Zeiss, Germany).

4.5. Western blot analysis

Hippocampal tissue sample were homogenized with a Dounce homogenizer in ice-cold homogenization buffer containing 15 mM Tris/HCl (7.6), 1 mM DTT, 0.25 M sucrose, 1 mM MgCl_2 , 1 $\mu\text{g/ml}$ pepstain A, 5 $\mu\text{g/ml}$ leupeptin, 2.5 $\mu\text{g/ml}$ aprotonin, 0.5 mM PMSF, 2.5 mM EDTA, 1 mM EGTA, 0.25 M Na_3VO_4 , 25 mM NaF and 2 mM sodium pyrophosphate. Protein concentration was determined by the micro-bicinchoninic acid (BCA) method of Pierer (Rockford, USA). 30 μg of total protein were analyzed by western blot on 8% SDS-PAGE. Following electrophoresis, proteins were transferred to Immobilon-P. The membranes were incubated overnight at 4 °C with primary antibodies against GRP78 (Stressgen, Ann Arbor, Mi. SPA-826), GRP94 (Stressgen, SPA-851), HSP70 (Stressgen, SPA-812), HSP60 (Stressgen, SPA-828E), PDI (Stressgen, SPA-891) and calnexin (Stressgen, SPA-860). The membranes were then incubated with horseradish-peroxidase conjugated anti-rabbit or anti-mouse secondary antibody for 1 h at room temperature. The blots were developed with an ECL detection method (Amersham). Immunoblots were analyzed with Kodak ID image analysis software. Each immunolabeled protein band was calculated as the mean intensity value minus background, and then presented as mean $\pm$ SEM ($n=4$) of a percentage of mean sham-operated control. Data were analyzed by one-way ANOVA followed by post-hoc Fisher's LSD.

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Fig. 1. Histopathology of hippocampal CA1 and DG neurons stained with acid fuchsin and celestine blue after ischemia. No neuronal death was found in brain sections from the sham-operated control rats and rats subjected to 15 min of ischemia followed by 24 h of reperfusion in the 2VO brain ischemia model. Normal neurons are shown by arrows. However, most CA1 neurons were dead (arrowheads) and almost all DG neurons had survived at 48 h of reperfusion after ischemia.

Fig. 2.

Electron micrographs of CA1 (A, C, E) and DG (B, D, F) neurons. Brain sections were from sham-operated control and animals subjected to 15 min of ischemia followed by 1 and 24 h of reperfusion: (A) sham CA1 neuron; (B) a sham DG neuron; (C) CA1 neuron at 1 h of reperfusion; (D) DG neuron at 1 h of reperfusion; (E) CA1 neuron at 24 h of reperfusion; (F) DG neuron at 24 h of reperfusion. Scale bar=1 μ m. N=nucleus; M=mitochondrion; ER=rough endoplasmic reticulum; G=Golgi apparatus; Arrows indicate ribosomes, arrowheads indicate protein aggregates and double-arrows point to protein aggregates on mitochondria.

Fig. 3.

(A) *In situ* hybridization of HSP70 and HSP60 in sham operated (sh) and 30 min, 4 h, 24 h, and 48 h of reperfusion. Induction of molecular chaperone genes took place in both hemispheres in the 2VO forebrain ischemia model, but only one hemisphere at bregma level -3.3 is shown. (B) Quantitative analysis of HSP70 mRNA expression after brain ischemia. Region of interest measurements for cortex, CA1 and DG regions of the hippocampus are shown. * $p < 0.05$. (C) Quantitative analysis of HSP60 mRNA expression after brain ischemia. Region of interest measurements for cortex, CA1 and DG regions of the hippocampus are shown. mean $\pm$ SEM, * $p < 0.05$.

Fig. 4.

(A) *In situ* hybridization of GRP78, HERP and GRP94 mRNAs in sham (sh), 30 min, 4 h, 24 h, and 48 h of reperfusion. (B) Quantitative analysis of GRP78, HERP and GRP94 mRNA expression after brain ischemia. Sections were obtained from the same brains as in Fig. 3. GRP78 and HERP mRNAs in coronal sections show basal expression in sham-operated and 30 min of reperfusion, and were moderately up-regulated at 4 h in CA1 and DG. At 24 h, GRP78 and HERP mRNAs were further increased in vulnerable CA1 neurons, but had returned to the basal levels in DG. GRP94 mRNA was only moderately increased at 24 h in the CA1 neurons. Data are expressed as mean $\pm$ SEM ($n = 4$) * $p < 0.05$.

Fig. 5. (A) *In situ* hybridization of PDI and calnexin (CNX). (B) Quantitative analysis of PDI and CNX mRNA expression after brain ischemia. Sections are from the same brains as Fig. 3. PDI mRNA was unchanged in most brain regions, but was slightly elevated in DG at 4 h of reperfusion. (C) Calnexin mRNA was increased in CA1 and DG regions at 24 h of reperfusion. Data are expressed as mean $\pm$ SEM ($n=4$) * $p<0.05$.

Fig. 6.

Western blot analysis of HSP70, HSP60, GRP78, GRP94, PDI and calnexin (CNX) protein levels. Hippocampal tissue homogenates were prepared from sham-operated control rats (sh) and rats subjected to 15 min of cerebral ischemia followed by 4 and 24 h of reperfusion. Each sample was derived from one rat. (A) Western blots labeled with antibodies against HSP70, HSP60, GRP78, GRP94, PDI and CNX, and visualized with the ECL system. (B) Quantitative analysis of HSP70, HSP60, GRP78, GRP94, PDI and CNX protein levels. Data are expressed as mean $\pm$ SEM ($n=4$) * $p < 0.05$.