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ISCHEMIC PRECONDITIONING PREVENTS PROTEIN AGGREGATION AFTER TRANSIENT CEREBRAL ISCHEMIA

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Abstract

Transient cerebral ischemia leads to protein aggregation mainly in neurons destined to undergo delayed neuronal death after ischemia. This study utilized a rat transient cerebral ischemia model to investigate whether ischemic preconditioning is able to alleviate neuronal protein aggregation, thereby protecting neurons from ischemic neuronal damage. Ischemic preconditioning was introduced by a sublethal 3 min period of ischemia followed by 48 h of recovery. Brains from rats with either ischemic preconditioning or sham-surgery were then subjected to a subsequent 7 min period of ischemia followed by 30 min, 4, 24, 48 and 72 h of reperfusion. Protein aggregation and neuronal death were studied by electron and confocal microscopy, as well as by biochemical analyses. Seven minutes of cerebral ischemia alone induced severe protein aggregation after 4 h of reperfusion mainly in CA1 neurons destined to undergo delayed neuronal death (which took place after 72 h of reperfusion). Ischemic preconditioning reduced significantly protein aggregation and virtually eliminated neuronal death in CA1 neurons. Biochemical analyses revealed that ischemic preconditioning decreased accumulation of ubiquitin-conjugated proteins (ubi-proteins) and reduced free ubiquitin depletion after brain ischemia. Furthermore, ischemic preconditioning also reduced redistribution of heat shock cognate protein 70 and Hdj1 from cytosolic fraction to protein aggregate-containing fraction after brain ischemia. These results suggest that ischemic preconditioning decreases protein aggregation after brain ischemia.

Keywords

brain ischemia; preconditioning; protein aggregation; proteotoxicity; electron microscopy

A brief episode of cerebral ischemia followed by reperfusion leads to delayed neuronal death in CA1 neurons at 48–72 h of reperfusion, while leaving dentate gyrus (DG), CA3 and most cortical neurons largely intact (Ito et al., 1975; Kirino, 1982; Smith et al., 1984). During this delayed period, neurons destined to undergo delayed neuronal death look normal under light microscopy. Under electron microscopy (EM), however, these CA1 neurons contain large quantities of intracellular protein aggregates (Hu et al., 2000).

Cellular proteins in non-native states, i.e. unfolded (newly synthesized), misfolded, denatured, or damaged, expose the sticky hydrophobic segments, and are highly toxic to cells (Taylor et al., 2002; Dobson, 2003). There are several cellular defense systems to process proteins in non-native states and to reduce their proteotoxicity (Dobson, 2003). Molecular chaperones can shield hydrophobic surfaces of proteins in non-native states, thereby blocking their aggregation. Major cellular chaperones are ATPases and assist

protein folding through numerous cycles of binding and release of unfolded protein substrates by hydrolysis of ATP. To avoid protein aggregation, irreparably damaged proteins must be quickly eliminated from cells, mostly by protein ubiquitination followed by proteasomal degradation. Protein ubiquitination is a series of ATP-dependent reactions to form isopeptidyl bonds ligating ubiquitin to hydrophobic segments of unfolded proteins. Proteasomal degradation of ubiquitin-conjugated proteins (ubi-proteins) is also strictly ATP-dependent. Brain ischemia depletes ATP and changes intracellular homeostasis, thereby disabling ATP-dependent molecular chaperones and ubiquitin-proteasomal degradation, resulting in toxic aggregation of non-native proteins during the postischemic phase (Hu et al., 2000, 2001).

A short period of ischemia (ischemic preconditioning) that does not lead to neuronal death (sublethal), is able to induce neuronal tolerance to a subsequent longer or lethal period of ischemia. This phenomenon is known as “ischemic tolerance.” Although definitive mechanisms underlying ischemic tolerance remain incompletely understood, induction of molecular chaperones and folding enzymes has long been proposed to contribute to the acquisition of ischemic tolerance (Kato et al., 1994; Chen and Simon, 1997; Welsh, 1998; Kirino, 2002). Recently, gene microarray and proteomics analyses further suggest that induction of endogenous neuroprotective genes plays an important role in the acquisition of ischemic tolerance (Schaller et al., 2003; Simkhovich et al., 2003; Dirnagl et al., 2003; Stenzel-Poore et al., 2003; Barone, 2004; Dhodda et al., 2004; Kawahara et al., 2004). Molecular chaperones and the ubiquitin-proteasomal system defend virtually all cell types from diverse stressful pathological conditions by reducing proteotoxicity from newly synthesized polypeptides and denatured proteins, and by eliminating irreparably damaged proteins (Yenari et al., 1998; Rokutan et al., 1998; Giffard et al., 2004). Despite such close ties between preconditioning and protein aggregation, it has not been studied whether ischemic preconditioning is able to reduce protein aggregation after brain ischemia. Earlier studies from the authors’ laboratory have demonstrated that accumulation of protein aggregates is a significant pathological event that takes place mainly in neurons destined to undergo delayed neuronal death after both global and focal brain ischemia (Hu et al., 2000, 2001). The results from this study clearly demonstrate that ischemic preconditioning markedly reduces protein aggregation after brain ischemia.

EXPERIMENTAL PROCEDURES

Ischemia model

Brain ischemia was produced using the two-vessel occlusion (2VO) model in rats (Smith et al., 1984). All experimental procedures were approved by the Animal Care and Use Committee at the University of Miami and were performed in compliance with the National Institutes of Health guidelines on the ethical use of animals. All measures were taken to reduce animal suffering and numbers of animals in this study. Male Wistar rats (250–300 g) were fasted overnight. Anesthesia was induced with 4% halothane followed by maintenance with 1–2% halothane in an oxygen/nitrous oxide (30/70%) gas mixture. Catheters were inserted into the external jugular vein, tail artery and tail vein to allow blood sampling, arterial blood pressure recording and drug infusion. Both common carotid arteries were encircled by loose ligatures. Fifteen minutes prior to ischemia induction and 15 min postischemia, blood gases were measured and adjusted to PaO₂ > 90 mm Hg, PaCO₂ 35–45 mm Hg, and pH 7.35–7.45 by adjusting the tidal volume of the respirator. Bipolar EEG was recorded before, during and after ischemia until recovery from anesthesia. Brain ischemia was induced by withdrawing blood via the jugular catheter to produce a MABP of 50 mm Hg, followed by clamping both common carotid arteries either for 3 min for ischemic preconditioning or 7 min for the induction of lethal ischemia. Blood pressure was maintained at 50 mm Hg during the ischemic period by withdrawing or infusing blood

through the jugular catheter. At the end of ischemia, the clamps were removed. Halothane was discontinued and all wounds were sutured. In all experiments, brain temperature was maintained at 37 °C before, during and after ischemia (15 min of reperfusion).

Ischemic preconditioning paradigm

Ischemic preconditioning was induced by subjecting rats to 3 min of ischemia using the 2VO model followed by 2 days of recovery. Sham-preconditioning was produced by subjecting rats to the same surgery but without 3 min of ischemia. Preconditioned rats or their sham-controls were then subjected to 7 min of lethal ischemia followed by 30 min, 4, 24, 48 and 72 h of reperfusion. This preconditioning paradigm has been proven to be very effective at protecting neurons against ischemia in this 2VO ischemic model (Shamloo et al., 1999; Burda et al., 2003). Rats subjected to 3 min of ischemia followed by 30 min, 4 and 24 h of recovery were also utilized to investigate ischemic preconditioning-induced molecular events. Each experimental group consisted of at least four rats. For biochemical studies, brain tissues were obtained by freezing the brains *in situ* with liquid nitrogen (Pontén et al., 1973). For confocal microscopy, rats were perfused with 4% paraformaldehyde in PBS. For EM, rats were perfused with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer.

Histology

Brain slices were cut coronally, dehydrated in ethanol, cleared in xylol and embedded in paraffin. Subsequently, serial 8 µm sections at the dorsal hippocampus were prepared and stained with Celestine Blue and Acid Fuchsin, essentially according to the method of Smith et al. (1984).

EM

Tissue sections from sham-operated control rats and rats subjected to ischemia followed by various periods of reperfusion were stained either by 1% ethanolic phosphotungstic acid (EPTA, purchased from Fisher Scientific, Fairlawn, NJ) or by the conventional osmium–uranyl–lead (Hu et al., 2000). Briefly, coronal brain sections were cut at a thickness of 120 µm with a vibratome through the level of the dorsal hippocampus and postfixed for 1 h with 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). For osmium–uranium–lead staining, sections were postfixed for 2 h with 1% osmium tetroxide in 0.1 M cacodylate buffer, rinsed in distilled water and stained with 1% aqueous uranyl acetate overnight. Tissue sections were then dehydrated in an ascending series of ethanol to 100% followed by dry acetone, and embedded in Durcupan ACM resin. Ultrathin sections were stained with 3% lead citrate prior to examination with an electron microscope. For EPTA staining, sections were dehydrated in an ascending series of ethanol to 100% and stained for 30 min with 1% phosphotungstic acid (PTA) prepared by dissolving 0.1 g of PTA in 10 ml of 100% ethanol and adding 150 µl of 95% ethanol. The EPTA solution was changed once after a 15 min interval during staining. The sections were then further dehydrated in dry acetone and embedded in Durcupan ACM resin.

Confocal microscopy

Confocal microscopy was performed on coronal brain sections (50 µm) from sham-operated control rats and rats subjected to 7 min of ischemia with or without ischemic preconditioning followed by 48 and 72 h of reperfusion. Brain sections were transferred into a 24-well microtiter plate filled halfway with 0.01 M citric acid/sodium citrate buffer (pH 6.0), heated five times for 5 s each in microwave oven set to 30% power, and then washed twice with 0.2% Triton X-100 (TX100)/PBS for 10 min. Non-specific binding sites were blocked with 3% BSA in PBS/0.2% TX100 for 30 min. The brain sections were incubated

overnight at 4 °C with either monoclonal anti-ubiquitin (Chemicon, Temecula, CA, USA) or polyclonal anti-ubiquitin (Sigma-Aldrich, St. Louis, MO, USA) antibody, both at dilutions of 1:200 in PBS containing 0.1% TX100. The sections were washed three times for 10 min each in PBS containing 0.1% TX100 at room temperature and incubated in a mixture of fluorescein-labeled anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) each at a dilution of 1:200, and 4 µg/ml propidium iodide (PI) for 1 h at room temperature, respectively. The sections were washed three times in PBS/0.1% TX100, mounted on glass slides and coverslipped using Gelvatol. The slides were analyzed with a Zeiss 50 confocal microscope.

Subcellular fractionation

Each hippocampus from a given rat was dissected into CA1 and DG regions. The CA1 or DG tissues were homogenized with a Dounce homogenizer (25 strokes) in 10 vol. of ice-cold homogenization buffer containing 15 mM Tris base/HCl pH 7.6, 1 mM DTT, 0.25 M sucrose, 1 mM MgCl₂, 1 µg/ml pepstatin A, 5 µg/ml leupeptin, 2.5 µg/ml aprotinin, 0.5 mM PMSF, 2.5 mM EDTA, 1 mM EGTA, 0.25 M Na₃VO₄, 25 mM NaF and 2 mM sodium pyrophosphate. The homogenates were centrifuged at 10,000×g at 4 °C for 10 min to obtain the pellets and supernatants. The supernatants were centrifuged at 165,000×g at 4 °C for 1 h to get cytosolic fractions (S3) and a microsomal pellet (P3) that contained intracellular light membranes. The 10,000×g pellet was suspended with ice-cold homogenization buffer containing 1% TX100 detergent and 400 mM KCl salt, sonicated three times for 10 s each, washed on a shaker for 1 h at 4 °C, and then centrifuged at 10,000×g for 10 min to obtain a detergent/salt-insoluble-protein aggregate-containing fraction. Protein concentration in subcellular fractions was determined by the micro-bicinchonic acid (BCA) method of Pierce (Rockford, IL, USA).

Western blot analysis

Western blot analysis was carried out with 17% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) for intracellular free ubiquitin in S3 fraction and on 8% SDS-PAGE for remaining subcellular fractions. Samples for Western blotting contained 25 µg of protein in the detergent/salt-insoluble fraction, 50 µg of protein in cytosol (S3) and 40 µg of protein in P3. Four different samples from four rats were analyzed in each experimental group. Following electrophoresis, proteins were transferred to an Immobilon-P membrane. The membranes were incubated overnight at 4 °C with a monoclonal antibody against ubiquitin (1:2000 dilution, Chemicon), HSC70 and HSP40 (Hdj1) (both at 1:2000 dilution, Stressgen, Victoria). The membranes were then incubated with horseradish-peroxidase-conjugated anti-mouse secondary antibody for 1 h at room temperature. The blots were developed with an ECL detection method (Pierce). The films were scanned and the optical densities of protein bands were quantified using Kodak 1D gel analysis software. One-way ANOVA followed by Fisher's PLSD post hoc test was employed to assess statistical significance.

RESULTS

Histopathology

Brain sections were obtained from sham-operated control rats and rats subjected either to 3 min ischemic preconditioning, 7 min lethal ischemia or 3 min ischemic preconditioning plus 7 min of ischemia, followed by 7 days of reperfusion, respectively. They were stained with Acid Fuchsin and Celestine Blue and examined by light microscopy (Fig. 1). Normal neurons were relatively large, pyramid-like or round in shape (Fig. 1, sham, arrowheads), whereas ischemic dead neurons were shrunken and contained dark and polygonal nuclei (N) (Fig. 1, arrows). No neuronal death occurred in CA1 neurons from sham-operated control rats (Fig. 1, sham, upper panel). A few dead neurons were occasionally seen in CA1 region

from rats subjected to 3 min of cerebral ischemia followed by 7 days of reperfusion (Fig. 1, 3 min, upper panel, arrow). More than 80% of CA1 neurons from rats subjected to 7 min of ischemia followed by 7 days of reperfusion were dead (Fig. 1, 7 min, upper panel, arrows). Ischemic preconditioning virtually reduced CA1 neuronal death after 7 min of ischemia (Fig. 1, 3 min + 7 min, upper panel). DG (Fig. 1, lower panel), CA3, and most neocortical neurons (data not shown) remained largely intact after either 3 min or 7 min of ischemia, or 3 min of ischemic preconditioning plus 7 min of ischemia. Occasionally, one or two dead neurons were also seen among several hundred DG granule cell layers after 7 min of ischemia (Fig. 1, 7 min, lower panel, arrows). All these results are consistent with previous reports (Kato et al., 1994; Smith et al., 1984; Shamloo et al., 1999).

Preconditioning reduced protein aggregation after ischemia

EPTA can be utilized to stain protein aggregates in neurons by EM (Hu et al., 2000, 2001). In this EPTA EM protocol, tissue sections are dehydrated through absolute ethanol without prior osmium fixation, leading to extraction of the lipid membranes from tissue sections. Therefore, the endoplasmic reticulum (ER) and mitochondria (M) were visible in negative contrast (Fig. 2, sham). EPTA normally stained N and weakly labeled ribosomes (as small dots, arrowheads) in sham-operated control CA1 neurons (Fig. 2, sham). No obvious ultrastructural alterations were found in CA1 neurons from rats subjected to 3 min of ischemia followed by 24 (data not shown) and 48 h of reperfusion (Fig. 2, 3' + 48h), whereas 7 min of brain ischemia led to severe accumulation of EPTA-stained protein aggregates at both 24 and 48 h of reperfusion (Fig. 2, 7' + 24hR and 7' + 48hR, arrows). Ischemic preconditioning (3 min of ischemia followed by 48 h of recovery) reduced 7 min ischemia-induced intracellular accumulation of protein aggregates at both 24 and 48 h of reperfusion (Fig. 2, 3' + 7' + 24hR and 3' + 7' + 48hR). In comparison with CA1 neurons, EPTA-stained aggregates were rarely found in CA3, DG and most neocortical neurons in all experimental groups after 7 min of ischemia (data not shown, but see Fig. 3). CA3, DG and neocortical neurons survived 7 min of cerebral ischemia (Smith et al., 1984).

Preconditioning reduced aggregation of ubi-proteins and depletion of free ubiquitin in CA1 neurons after ischemia

Neuronal protein aggregates observed after ischemia are composed of ubi-proteins (Hu et al., 2000, 2004). When proteins are ubiquitinated or aggregated after brain ischemia, their ubiquitin immunoreactivity pattern alters, which can be studied by confocal microscopy (Hu et al., 2000, 2001). To follow up the EM observations, we investigated by confocal microscopy changes in ubiquitin immunoreactivity pattern in brain sections. Two sources of anti-ubiquitin antibodies, one from Chemicon (MAB1510, CA) and one from Sigma (U5379), were used in this study. The Chemicon monoclonal anti-ubiquitin antibody labeled both free ubiquitin and ubi-protein aggregates while the Sigma polyclonal anti-ubiquitin antibody preferentially recognized only free ubiquitin in brain sections (Morimoto et al., 1996; Hu et al., 2000). Brain sections were double-labeled with the Chemicon anti-ubi-protein antibody and PI, and were examined by confocal microscopy (Fig. 3). Ubiquitin immunolabeling (green) was relatively evenly distributed in sham-operated control CA1 (Fig. 3, sham, upper panel) and DG (Fig. 3, sham, lower panel) neurons, as well as in neurons from other brain regions (data not shown). At 24 (data not shown) and 48 h of reperfusion after 7 min of ischemia, the immunolabeling pattern was clearly changed from an even distribution to a heterogeneous distribution, with anti-ubiquitin positive aggregates scattered around the N and associated with the dendritic plasmalemma (Fig. 3, 7' + 48h, green, upper panel, arrows). In comparison, changes in the ubiquitin immunoreactivity pattern after ischemia were only very occasionally found in DG neurons after 7 min of cerebral ischemia (Fig. 3, 7' + 48h, lower panel, arrowheads), consistent with the fact that delayed neuronal death occasionally took place in one or two DG neurons after ischemia

(see Fig. 1). By 72 h of reperfusion, ubiquitin immunolabeling (green) virtually disappeared from CA1 neurons (Fig. 3, 7' + 72h, upper panel), but was upregulated in DG (Fig. 3, lower panel, 7' + 72h), CA3 and cortical neurons (data not shown). The PI-stained N were virtually unchanged in CA1 and DG neurons before 48 h of reperfusion, but become shrunken and polygonal in shape in CA1 neurons at 72 h of reperfusion because the CA1 neurons were dead at this time point (Fig. 3, upper panel, 7' + 72h) (Hu et al., 2000). Three minutes of ischemic preconditioning virtually reduced ubi-protein aggregation at 48 h of reperfusion (Fig. 3, 3' + 7' + 48h, upper panel), and abolished delayed neuronal death in CA1 neurons at 72 h of reperfusion after 7 min of ischemia (Fig. 3, 3' + 7' + 72h, upper panel). In addition, preconditioning also led to upregulation of ubiquitin in CA1 and DG neurons after 7 min of ischemia (Fig. 3, 3' + 7' + 48h and 3' + 7' + 72h).

Depletion of free ubiquitin in brain sections was also studied by confocal microscopy with the Sigma polyclonal antibody that recognizes only free ubiquitin in brain sections (Morimoto et al., 1996). Brain sections from rats subjected to 7 min of ischemia either with or without ischemic preconditioning followed by 48 and 72 h of reperfusion were labeled with the anti-free ubiquitin antibody (Fig. 4). Free ubiquitin immunostaining was evenly distributed in sham-operated control CA1 neurons (Fig. 4, sham, upper panel), but was severely depleted at 48 and 72 h of reperfusion in CA1 neurons after 7 min of ischemia without preconditioning (Fig. 4, 7' + 24h and 7' + 48h, upper panel). On the contrary, 3 min ischemic preconditioning upregulated free ubiquitin in CA1 neurons at 48 and 72 h of reperfusion after 7 min of ischemia (Fig. 4, 3' + 7' + 48h and 3' + 7' + 72h, upper panel). Relative to CA1 neurons, free ubiquitin was also upregulated at 48 and 72 h of reperfusion in DG neurons after 7 min of ischemia either with or without ischemic preconditioning (Fig. 4, lower panel). These results were consistent with the quantitative analysis of ubiquitin expression on Western blots illustrated below (Figs. 5 and 6).

Upregulation of free ubiquitin and reduction of ubi-proteins by ischemic preconditioning

The hippocampus was dissected into CA1 region and DG area, and cytosolic S3, microsomal P3 and the detergent/salt-insoluble protein aggregate-containing fractions were prepared for Western blot analyses of ubi-protein aggregates and free ubiquitin after ischemia either with or without preconditioning. As demonstrated in Fig. 5A, high molecular weight ubi-proteins were significantly increased in the detergent/salt-insoluble fractions from CA1 regions at 30min and 4 h of reperfusion after either 3 or 7 min of brain ischemia (Fig. 5A, 3 min and 7 min). However, the increases were much more pronounced and persistent after 7 min of ischemia than after 3 min of ischemia (Fig. 5A and 5C, 3 min and 7 min). By 24 and 48 h of reperfusion, the increases in ubi-proteins virtually returned to control level in CA1 region after 3 min of ischemia, but were continuously increased after 7 min of ischemia (Fig. 5A and 5C, 3 min and 7 min). Ischemic preconditioning (3 min of ischemia plus 48 h of recovery) altered insignificantly the early increases, but reduced the late increases in ubi-proteins in CA1 region after 7 min of ischemia (Fig. 5A and 5C, 3 min + 7 min). CA1 neurons after 7 min of ischemia were destined to die at 72 h of reperfusion (see Figs. 3–4). Relative to the changes in CA1 region, increases in ubi-proteins were less pronounced in DG area after either 3 min, or 7 min of ischemia (Fig. 5B and 5D, 3 min and 7 min). Ischemic preconditioning also reduced accumulation of ubi-proteins in DG region after 7 min of ischemia (Fig. 5B and 5D, 3 min + 7 min). Ubi-proteins were not significantly altered in P3 fraction in CA1 and DG regions after either 3 or 7 min of ischemia (data not shown). Concomitantly, cytosolic free ubiquitin in CA1 region was significantly decreased at 30 min and 4 h of reperfusion after either 3 min, 7 min of ischemia, or 3 min preconditioning plus 7 min of ischemia (Fig. 6A and 6C, 3 min, 7 min, 3 min + 7 min). By 24 and 48 h of reperfusion, however, the CA1 cytosolic free ubiquitin was significantly upregulated after 3 min of ischemia (Fig. 6A and 6C, 3 min), remained significantly lower

than the sham-operated control level after 7 min of ischemia (Fig. 6A and 6C, 7 min), but was upregulated after 3 min preconditioning plus 7 min of ischemia (Fig. 6A and 6C, 3 min + 7 min). Relative to the changes in CA1 neurons, free ubiquitin in the DG area was more modestly but significantly decreased at 30 min and 4 h of reperfusion, and was then upregulated over the control level at 24 and 48 h of reperfusion after either 3 min, 7 min of ischemia, or 3 min preconditioning plus 7 min of ischemia (Fig. 6B and 6D, 3 min, 7 min, 3 min + 7 min).

Redistribution of HSC70 and Hdj1 following ischemia or ischemic preconditioning

As demonstrated in Fig. 7A and 7B, HSC70 protein content in the cytosolic fraction from the CA1 region tended to decrease but the change was insignificant either after 3 min or 3 min ischemic preconditioning plus 7 min of brain ischemia. However, HSC70 protein content in the cytosolic fraction was significantly decreased at 30 min and 4 h of reperfusion after 7 min of brain ischemia (Fig. 7A and 7B). In comparison with HSC70, Hdj1 level in the CA1 region was significantly decreased in the cytosolic fraction at 30 min and 4 h of reperfusion either after 3 or 7 min, or 3 min ischemic preconditioning plus 7 min of brain ischemia (Fig. 7A and 7B). Hdj1 level recovered to the control level in the CA1 cytosolic fraction at 48 h of reperfusion either after 3 min, or 3 min ischemic preconditioning plus 7 min of brain ischemia, but the decrease continued after 7 min of ischemia (Figs. 7A and 5B). The decrease was much more pronounced in Hdj1 than in HSC70 in the cytosolic fraction after ischemia (Fig. 7A and 7B). Concomitantly, HSC70 and Hdj1 were significantly increased in the detergent/salt-insoluble protein aggregate-containing fraction either after 3 or 7 min of ischemia, or 3 min preconditioning plus 7 min of ischemia (Fig. 7C and 7D). By 48 h of reperfusion, both HSC70 and Hdj1 returned to the control level after 3 min of ischemia, but were still significantly higher than the control level either after 7 min of ischemia or 3 min preconditioning plus 7 min of ischemia (Fig. 7C and 7D). The increase in Hdj1 in the detergent/salt-insoluble fraction was more dramatic than that in HSC70, especially after 7 min of ischemia (Fig. 7C and 7D). Similar to the CA1 region, the redistribution of HSC70 and Hdj1 between cytosolic and detergent-insoluble fractions was also seen in the DG area, but the changes were more modest and returned to the control levels at 48 h of reperfusion either after 3 or 7 min of ischemia, or 3 min ischemic preconditioning plus 7 min of ischemia (data not shown).

DISCUSSION

Ischemic tolerance remains one of the most effective measures that protect neurons from delayed neuronal death in CA1 neurons after transient cerebral ischemia (Kato et al., 1994; Shamloo et al., 1999; Kirino, 2002). Therefore, much research effort has been devoted to elucidating the underlying mechanisms (Sommerschild and Kirkeboen, 2002). Because of close relationship between ischemic tolerance, induction of molecular chaperones and protein aggregation, we studied whether ischemic preconditioning is able to block protein aggregation after ischemia in a rat cerebral ischemia model. This study demonstrates that ischemic preconditioning reduces significantly protein aggregation in CA1 neurons destined to undergo delayed neuronal death after ischemia. As evidenced by EM, ischemic preconditioning (3 min ischemia followed by 2 days of recovery) virtually eliminated formation of EPTA-stained protein aggregates in CA1 neurons after 7 min of ischemia. Protein aggregates are composed of detergent/salt-insoluble ubi-proteins (Hu et al., 2000, 2001). As viewed by confocal microscopy, ischemic preconditioning markedly reduces formation of ubi-protein aggregates and depletion of free ubiquitin in CA1 neurons after 7 min of ischemia. Western blot analysis confirms that ischemic preconditioning induces expression of free ubiquitin, and reduces deposition of ubi-proteins into the detergent-insoluble protein aggregate-containing fraction after 7 min of lethal ischemia. Furthermore,

ischemic preconditioning also reduces redistribution of HSC70 and HSP40 from cytosolic fraction to protein aggregate-containing fraction after ischemia. These results suggest that ischemic preconditioning-facilitated neuroprotection may be mediated in part by reduction of a lethal aggregation of ubi-proteins after ischemia.

The presence of dark substances under EM in rat CA1 neurons after transient cerebral ischemia was observed by Kirino et al. (1984). The accumulation of dark materials in ischemic CA1 vulnerable neurons was also reported by Deshpande et al., in 1992. However, the identities of these dark materials were unknown. Hu et al. (2000)) found that EPTA selectively labels neuronal aggregates accumulated after brain ischemia. Because EPTA preferentially stains proteins under EM (Burry and Lasher, 1978), EPTA-stained aggregates are likely protein aggregates. This conclusion is confirmed by the fact that neuronal aggregates accumulated after ischemia contain strong ubiquitin immunoreactivity under electron and confocal microscopy (Hu et al., 2000, 2001). Because protein aggregates are found as early as 2–4 h of reperfusion, and are continuously increased in CA1 neurons until the onset of delayed neuronal death that takes place at 72 h of reperfusion, it was proposed that protein aggregation over an extended period of time will eventually kill neurons in a delayed fashion after transient cerebral ischemia (Hu et al., 2000, 2001). The findings from previous studies support this hypothesis. (1) The acquisition of ischemic tolerance depends on de novo protein synthesis, and induction of a host of neuroprotective stress proteins (Kato et al., 1994; Dewar et al., 1994; Noga et al., 1997; Poe and O'Neill, 1997; Akagawa et al., 1998; Shamloo et al., 1999; Ide et al., 1999; Samali et al., 1999; DeGracia et al., 2002; Budagova et al., 2003; Burda et al., 2003). Stress proteins such as molecular chaperones, folding enzymes and components of ubiquitin-proteasomal system reduce toxic aggregation of unfolded proteins (Nowak, 1991; Chen and Simon, 1997; Sharp et al., 1999; Hu et al., 2000, 2001; Yenari et al., 1998, 2002; Kirino, 2002; Giffard et al., 2004; Hampton et al., 2003). (2) The recovery interval between sublethal ischemic preconditioning and the subsequent lethal period of ischemia is absolutely necessary for the acquisition of ischemic tolerance (Shamloo et al., 1999; Abe and Nowak, 2000; Burda et al., 2003). This time-lag correlates very well with the temporal profile of the cellular stress response after ischemic preconditioning, suggesting that ischemic preconditioning-induced stress proteins (chaperones) may shield hydrophobic surfaces of unfolded proteins, thus reducing their toxic aggregation (Liu et al., 1993; Kato et al., 1994; Shamloo et al., 1999; Burda et al., 2003; Giffard et al., 2004; Dobson, 2003).

The details about what proteins are aggregated and how protein aggregation exerts proteotoxicity after brain ischemia are currently incompletely understood. Newly synthesized polypeptides are the major sources of unfolded proteins in normal cells (Frydman, 2001; Hartl and Hayer-Hartl, 2002). Ribosomes are highly abundant in cells and numerous nascent polypeptides normally emerge from their parent ribosomes at any given moment (Kinjo and Takada, 2003; Chebotareva et al., 2004; Zemel et al., 2004). Partially or newly synthesized polypeptide chains expose their hydrophobic segments and are highly prone to intra-molecular misfolding and inter-molecular aggregation driven by the exposed hydrophobic force (Hartl and Hayer-Hartl, 2002). Therefore, co-translational folding generally requires cooperation among chaperone HSC70, its co-chaperone Hdj1 and a cellular ATP supply. During co-translational folding, HSC70 carries out numerous cycles of binding and release of newly made polypeptide substrate by hydrolysis of ATP. Although cellular HSC70 is highly abundant, the kinetics of the HSC70-coupled substrate binding and release cycles is insufficient to compete with nascent chain aggregation during protein biosynthesis in normal cells, and thus must be accelerated by its cochaperone HDJ1 which promotes HSC70 hydrolysis of ATP (Hartl and Hayer-Hartl, 2002). An ischemia-induced cascade of energy failure and changes in intracellular homeostasis may cumulatively disable ATP-dependent protein quality control machinery for co-translational folding after brain

ischemia (Siesjö et al., 1999; Hu et al., 2004). As a result, newly made polypeptides on ribosomes are unable to fold. Consequently, unprocessed nascent polypeptides may be irreversibly aggregated, resulting in irreversible damage of protein synthesis machinery after ischemia, as evidenced by the irreversible inhibition of protein biosynthesis after brain ischemia (Hossmann, 1993). Ischemic preconditioning induces molecular chaperones and components of the ubiquitin-proteasomal system, thus strengthening protein quality control defense systems to reduce protein aggregation after ischemia. This is consistent with previous reports that ischemic preconditioning resumes protein synthesis after ischemia (Kato et al., 1995; Burda et al., 2003; Schneeloch et al., 2004).

A significant portion of protein aggregates accumulated after brain ischemia is associated with cellular lipid membranes (see Fig. 1, 7' + 48h and Fig. 3, 7 + 38h, arrows). A structural consequence of amphipathic peptide adsorption onto a lipid membrane is the formation of transmembrane peptide pores (Zemel et al., 2004). As a result, membrane leakage after ischemia may change cell homeostasis and contribute to delayed neuronal death after brain ischemia. This study demonstrates that ischemic preconditioning may reduce protein aggregate association with lipid membranes after ischemia.

In general, preconditioning activates a host of functionally distinct families of stress proteins to cooperatively reduce protein aggregation after stress (Qiao et al., 2003; Giffard et al., 2004). The cellular HSC70 (DnaK) concentration is an order of magnitude higher than the Hdj1 (DnaJ) concentration (Laufen et al., 1999). DnaJ stimulates DnaK ATPase activity several hundred-fold in the presence of denature protein substrate (Laufen et al., 1999). When proteins become aggregated, their detergent solubility decreases markedly (Kazantsev et al., 1999; Hu et al., 2001). As demonstrated in this study, HSC70 is highly abundant in the cytoplasm. Only a small proportion of cytosolic free HSC70 pool is redistributed into the detergent-insoluble protein aggregate-containing fraction, whereas a large proportion, if not all, of cytosolic free Hdj1 pool are deposited into the protein aggregate-containing fraction in CA1 neurons after lethal brain ischemia. HSC70 and Hdj1 are partners for folding of nascent polypeptide chains (Hartl and Hayer-Hartl, 2002). The cytosolic free HSC70 and Hdj1 are the active forms (Angelidis et al., 1999). Therefore, deposition or inactivation of Hdj1 may play a major role in the disabilities of protein chaperoning and neuronal damage after brain ischemia. Therefore, induction of HSP70 itself, an inducible form of HSC70, might not be absolutely required for the acquisition of ischemic tolerance owing to the high content of constitutive HSC70 (Abe and Nowak, 2000, 2004; Wada et al., 1999; Nishino and Nowak, 2004). In addition, inhibition of toll-like receptor and cytokine signaling, and induction of ubiquitin, ER stress genes and neurotrophins may also play an important role in ischemic tolerance after ischemia (Welsh, 1998; Ide et al., 1999; Miyazaki et al., 2002; Hayashi et al., 2003; Kariko et al., 2004; Garcia et al., 2004).

Ischemic preconditioning is a very effective way to prevent delayed neuronal death after ischemia (Kato et al., 1994; Shamloo et al., 1999; Kirino, 2002). Yet, there is still much debate concerning whether ischemic preconditioning could be used as a therapeutic measure to treat ischemic neuronal damage. Apparently, ischemic preconditioning is a source of harmful stress. Three minutes of ischemic preconditioning by itself leads to increase in toxic ubi-proteins as demonstrated in this study. On the other hand, toxic ubi-proteins serve as signals to activate heat shock transcription factors to induce expression of molecular chaperones (Voellmy, 2004). Therefore, although it is questionable whether a harmful stress should be used to induce ischemic tolerance against a second lethal stress, understanding molecular pathways underlying ischemic preconditioning may facilitate developing less harmful therapeutic agents and procedures for treatment of brain ischemia.

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Abbreviations

DG	dentate gyrus
EM	electron microscopy
EPTA	ethanolic phosphotungstic acid
ER	endoplasmic reticulum
HSP40	heat shock cognate protein 40
HSP70	heat shock protein 70
M	mitochondria
N	nuclei
PI	propidium iodide
PTA	phosphotungstic acid
SDS-PAGE	SDS–polyacrylamide gel electrophoresis
TX100	Triton X-100
ubi-proteins	ubiquitin-conjugated proteins
2VO	two-vessel occlusion

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Fig. 1.

Histopathology of CA1 (upper panel) and DG (lower panel) neurons after ischemia. Ischemic preconditioning was induced by 3 min of ischemia followed by 3 days of recovery. Rats were subjected to sham-surgery, 3 min or 7 min of ischemia, or 3 min ischemic preconditioning plus 7 min of ischemia (3 min + 7 min). Paraffin brain sections were stained with Celestine Blue and Acid Fuchsin and examined by light microscopy. Delayed neuronal death takes place in CA1 neurons at 3 days of reperfusion after 7 min of ischemia (7 min, upper panel). Arrows indicate damaged neurons after ischemia.

Fig. 2.

Electron micrographs of EPTA-stained protein aggregates in CA1 pyramidal neurons after brain ischemia. Rats were subjected to either sham-surgery without ischemia, or 3 min of ischemia, or 7 min of ischemia with or without preconditioning, followed by 24 or 48 h of reperfusion, respectively. Brain sections were stained with EPTA and examined by EM. The ER and M were often visible in negative contrast due to lipid extraction. EPTA stained N and ribosomes (as small dots, arrowheads) in sham-operated brain sections (sham) or in rats subjected to 3 min of ischemia followed by 48 h of recovery ($3' + 48hR$). EPTA additionally labels intracellular protein aggregates at 24 and 48 h of reperfusion after 7 min of ischemia ($7' + 24hR$ and $7' + 48hR$, arrows). Three minutes of ischemic preconditioning

reduced formation of EPTA-stained protein aggregates at 24 and 48 h of reperfusion after 7 min of ischemia ($3' + 7' + 24\text{hR}$ and $3' + 7' + 48\text{hR}$).

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Fig. 3.

Double-stained confocal microscopic images of CA1 (upper panels) and DG (lower panels) neurons after ischemia. Brain sections were double-labeled with the anti-ubiquitin antibody (green) and PI (red). Sections were from sham-operated control rat and rats either with or without 3 min ischemic preconditioning subjected to 7 min of ischemia followed by 48 and 72 h of reperfusion. Ubiquitin immunoreactivity (green) was evenly distributed in sham-control CA1 neurons (sham, upper panel, arrows). Ubi-protein aggregates (green) were seen at 48 h of reperfusion in CA1 neurons (7' + 48h, upper panel, arrows), and cell death occurred at 72 h of reperfusion (7' + 48h, upper panel) after 7 min of ischemia. Ischemic precondition reduced ubi-protein aggregation at 48 h of reperfusion (3' + 7' + 48h, upper panel, arrows), as well as neuronal death at 72 h of reperfusion (3' + 7' + 72h, upper panel, arrows) after 7 min of ischemia. Ubiquitin (green) was upregulated in DG neurons (lower panel) after ischemia either with or without preconditioning. A few DG neurons contained ubi-protein aggregates (green) after 7 min of ischemia (7' + 48h, lower panel, arrowheads). PI-stained neurons (red) became shrunken and polygonal in shape in CA1 neurons at 72 h of reperfusion because the CA1 neurons were dead (7min + 72h, upper panel).

Fig. 4.

Confocal microscopic images of CA1 (upper panels) and DG (lower panels) neurons after ischemia. Sections were from sham-operated control rat and rats either with or without 3 min ischemic preconditioning subjected to 7 min of ischemia followed by 48 and 72 h of reperfusion. Free ubiquitin immunostaining was depleted in most CA1 neurons at 48 h of reperfusion (7' + 48h, upper panel) and lost at 72 h of reperfusion (7' + 72h, upper panel) after 7 min of ischemia. Three minutes of ischemic preconditioning upregulated free ubiquitin in CA1 neurons at 48 and 72 h of reperfusion after 7 min of ischemia (3' + 7' + 48h, and 3' + 7' + 72h, upper panel). Free ubiquitin was also upregulated in DG neurons (lower panel) at 48 and 72 h of reperfusion after 7 min of ischemia either with preconditioning or without preconditioning (3' + 7' + 48h, and 3' + 7' + 72h, lower panel).

Fig. 5.

(A, B) Immunoblots of ubi-proteins in detergent/salt-insoluble protein aggregate-containing fractions after ischemia. Samples of CA1 (A) and DG (B) tissues were from sham-operated control rats (Ctr) and rats subjected to either 3 min or 7 min of ischemia, or 3 min of ischemic preconditioning plus 7 min of ischemia, followed by 30 min, 4, 24 and 48 h of reperfusion, respectively. Ctr were subjected to sham-surgery without ischemia followed by either 30 min or 48 h of recovery, respectively. The blots were labeled with the anti-ubi-protein antibody and visualized with the ECL system. Molecular sizes are indicated on the left. (C, D) Changes in ubi-proteins in the detergent/salt-insoluble fraction were evaluated with Kodak 1D image software. Mean optical densities of immunoblot bands are expressed as mean $\pm$ S.D. ($n=4$) of a percentage of Ctr. * Denotes $P<0.05$ between sham-operated Ctr and experimental conditions. One-way ANOVA followed by Fisher's PLSD post hoc test was utilized for statistical analysis.

Fig. 6.

(A, B) Immunoblots of cytosolic free ubiquitin. Samples of CA1 (A) and DG (B) tissues were from sham-operated control rats (Ctr) and rats subjected to either 3 min or 7 min of ischemia, or 3 min of ischemic preconditioning plus 7 min of ischemia, followed by 30 min, 4, 24 and 48 h of reperfusion, respectively. The blots were labeled with the anti-ubiquitin antibody and visualized with the ECL system. (C, D) Changes in cytosolic ubiquitin were evaluated with Kodak 1D image software. Mean optical densities of free ubiquitin bands are expressed as mean $\pm$ S.D. ($n=4$) of a percentage of Ctr. * Denotes $P < 0.05$ between Ctr and experimental conditions. One-way ANOVA followed by Fischer's PLSD post hoc test was utilized for statistical analysis.

Fig. 7.

(A, B) Immunoblots of HSC70 and Hdj1 after ischemia. Cytosolic (A) and detergent/salt-insoluble (B) fractions were from CA1 tissues of sham-operated control rats (Ctr) and rats subjected to either 3 min or 7 min of ischemia, or 3 min of ischemic preconditioning plus 7 min of ischemia, followed by 30 min, 4, 24 and 48 h of reperfusion, respectively. The blots were labeled with the anti-HSC70 and Hdj1 antibodies and visualized with the ECL system. (C, D) Changes in HSC70 and Hdj1 immunoblots of the cytosolic (C) and detergent/salt-insoluble (B) fractions were evaluated with Kodak 1D image software. Mean optical densities of the protein bands are expressed as mean $\pm$ S.D. ($n=4$), a percentage of Ctr. * Denotes $P < 0.05$ between Ctr and experimental conditions. One-way ANOVA followed by Fischer's PLSD post hoc test was utilized for statistical analysis.