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ALTERATIONS OF *N*-ETHYLMALEIMIDE-SENSITIVE ATPASE FOLLOWING TRANSIENT CEREBRAL ISCHEMIA

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Abstract

Neuronal repair following injury requires recruitment of large amounts of membranous proteins into synaptic and other cell membranes, which is carried out by the fusion of transport vesicles to their target membranes. A critical molecule responsible for assemblage of membranous proteins is *N*-ethylmaleimide-sensitive factor (NSF) which is an ATPase. To study whether NSF is involved in ischemic neurological deficits and delayed neuronal death, we investigated alterations of NSF after transient cerebral ischemia by means of biochemical methods, as well as confocal and electron microscopy. We found that transient cerebral ischemia induced depletion of free NSF and concomitantly relocalization of NSF into the Triton X-100-insoluble fraction including postsynaptic densities in CA1 neurons during the postischemic period. The NSF alterations are accompanied by accumulation of large quantities of intracellular vesicles in CA1 neurons that are undergoing delayed neuronal death after transient cerebral ischemia. Therefore, permanent depletion of free NSF and relocalization of NSF into the Triton X-100-insoluble fraction may disable the vesicle fusion machinery necessary for repair of synaptic injury, and ultimately leads to synaptic dysfunction and delayed neuronal death in CA1 neurons after transient cerebral ischemia.

Keywords

brain ischemia; molecular chaperone; *N*-ethylmaleimide-sensitive factor; postsynaptic density; electron microscopy

The processes of repair, regeneration and recovery of cells from injuries require recruitment of large amounts of membranous proteins, which is undertaken by the fusion of transport vesicles to their target membranes (Rothman, 1996). A critical molecule required for assemblage of membranous proteins is *N*-ethylmaleimide-sensitive factor (NSF) ATPase. NSF was first discovered by its property of restoring *N*-ethylmaleimide-inactivated vesicles fusion (Block et al., 1988). It was primarily thought that NSF would act as a fusion protein to bridge membranes destined to fuse at the expense of ATP. It is now known that NSF is not a fusion protein in the sense of directly merging two membranes, but acts as a molecular chaperone to change the conformation of fusion protein SNAREs (for soluble NSF attachment protein receptors). During this process, cytosolic free NSF ATPase interacts with SNARE through the adaptor protein soluble NSF attachment protein to form the so-called 20 S particle, which leads to dissociation of the SNARE complex for another cycle of membrane fusion (Morgan and Burgoyne, 1995; Neuwald, 1999; May et al., 2001). Therefore, only cytoplasmic free NSF is the active form for dissociation of the SNARE complex, whereas relocalization of NSF into a Triton-insoluble fraction inactivates NSF

(Block et al., 1988; Rothman, 1996; Mohtashami et al., 2001; Stewart et al., 2002). NSF also plays an important role in the assembly of receptor into postsynaptic membranes (Song et al., 1988; Nishimune et al., 1998; Osten et al., 1998; Noel et al., 1999). To date, only one isoform of NSF is found in most organisms except in *Drosophila* which expresses dNSF-1 and dNSF-2 (Mohtashami et al., 2001). Therefore, inactivation of NSF cannot be compensated in most organisms (May et al., 2001). The *hyh* mouse, which carries a mutant NSF attachment protein gene, has a markedly small cerebral cortex at birth and dies postnatally (Hong et al., 2004).

It is well established that transient global cerebral ischemia causes selective delayed neuronal death in the hippocampal CA1 region, whereas dentate gyrus (DG) neurons and neurons in other regions are relatively resistant (Ito et al., 1975; Kirino, 1982). The search for the mechanisms underlying the vulnerability of CA1 neurons to ischemia has been extensive, but they are still un-clear. In an earlier study, we performed two- and three-dimensional electron microscopic analyses of synapses selectively stained with ethanolic phosphotungstic acid (EPTA) in the hippocampus of rats subjected to 15 min of ischemia followed by various periods of reperfusion (Martone et al., 1999). Hippocampal postsynaptic densities (PSDs) after ischemia became thicker than those in sham-operated controls. A quantitative study indicated that the increase in thickness after ischemia was both greater and more long-lived in area CA1 than in DG. In this study, we found that the cytoplasmic free NSF was immediately and persistently depleted, mainly in CA1 neurons that would die at 2–3 days of reperfusion after a brief period of ischemia, whereas NSF was less affected in surviving neurons such as DG granule cells and cortical neurons after the same ischemic insult. The depletion of cytosolic NSF in CA1 pyramidal neurons was due to, at least in part, relocation of NSF into PSDs, rather than the net loss of NSF protein after brain ischemia.

EXPERIMENTAL PROCEDURES

Materials

Leupeptin, pepstatin, aprotinin, phosphotungstic acid (PTA), and propidium iodide (PI) were purchased from Sigma (St. Louis, MO, USA). A polyclonal antibody to synaptophysin was purchased from StressGen (Victoria, Canada). A monoclonal antibody to NSF was a generous gift from Dr. Mitsuo Tagaya (Tokyo University of Pharmacy and Life Sci, Hachioji, Tokyo, Japan). Fluorescein-anti-mouse and lissamine rhodamine-anti-rabbit IgG were purchased from Jackson ImmunoResearch Laboratory (PA, USA). Peroxidase-linked secondary antibodies were purchased from Amersham (USA).

Ischemia model

A rat two-vessel occlusion ischemia model described previously was used in this study (Smith et al., 1984). All procedures were approved by the Animal Use and Care Committee in the University of Miami. All experiments conformed to the National Institute of Health international guidelines on the ethical use of animals. All measures were taken to minimize the number of animals used and their suffering. Briefly, 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 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 PaO₂ >90 mm Hg, PaCO₂ 35–45 mm Hg, pH 7.35–7.45 during the intubation period. Bipolar electroencephalogram was recorded and brain temperature was maintained with a feedback heating lamp setting at 37 °C during the surgical period until the rat recovered from anesthesia. Heparin (150 IU/kg) was administered i.v. and blood was withdrawn via

the jugular catheter to produce a mean arterial blood pressure (MABP) of 50 mm Hg, and both carotid arteries were clamped. 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 the ischemic period, the clamps were removed and the blood 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 brains were collected at 30 min after ischemia (see below). For groups with reperfusion periods longer than 30 min, halothane was discontinued at the end of ischemia, 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.

Experimental groups

Three separate series, each consisting of sham-operated control rats and rats of 15 min of ischemia followed by 30 min, 4, 24, 48 and 72 h of reperfusion, were prepared for biochemical analysis, electron microscopy (EM) and confocal microscopy, respectively. Each experimental group consisted of at least four rats. For biochemical studies, brains were frozen *in situ* with liquid nitrogen. Briefly, rats were anesthetized with halothane, tracheotomized and artificially ventilated, and MABP was monitored. An incision was made on the top of the head to place a 50 ml plastic centrifuge tube with open bottom on the top of the skull. The plastic tube was filled with liquid nitrogen to freeze the brain until MABP decreased below 10 mm Hg. Then, the brains were removed from the liquid nitrogen-frozen skull with a saw and chisel. This *in situ* brain freezing method preserves brain ATP level and prevents brain biochemical change after decapitation (Pontén et al., 1973). The dorsal hippocampal tissues weighing about 50–80 mg from both hemispheres were dissected out from the 1 mm coronal sections in a –12 °C glove box, and were used for Western blot analyses and for preparing PSDs (see below). For EM, animals were ventilated with halothane. The brains were perfused through the ascending aorta with ice-cold phosphate-buffered saline (PBS) and then 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer. For laser scanning confocal microscopy, the brains were perfused with ice-cold 4% paraformaldehyde in PBS.

EM

Electron microscopic studies were carried out on brain tissue sections. Tissue sections were stained by 1% EPTA method, as described previously (Martone et al., 1999). Briefly, coronal brain sections at the dorsal hippocampal level were cut with a vibratome at a thickness of 150 µm and postfixed for 1 h with 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). The tissue sections were dehydrated in an ascending series of ethanol solution to 100% and stained for 30 min with 1% PTA, prepared by dissolving 0.1 g of PTA in 10 ml of 100 ethanol to which 400 µl of 95% ethanol was added. Sections were then embedded in Durcupan ACM. Ultrathin sections (0.1 µm) were prepared and examined with an electron microscope without additional staining.

Laser-scanning confocal microscopy

Single- or double-label fluorescence confocal microscopy was performed on coronal brain sections (50 µm) at the dorsal hippocampal level. Monoclonal antibody against NSF and polyclonal anti-synaptophysin were used, respectively. The sections were transferred into a 24 well microtiter plate filled with 1 ml of 0.01 M citric acid/sodium citrate buffer (pH 6.0) and heated for 10 s in microwave set to 30% power. The sections were then washed twice with 0.2% Triton X-100 (TX100)/PBS for 10 min. Non-specific binding sites were blocked in 3% BSA in PBS/0.1% TX100 for 1 h. NSF and synaptophysin primary antibodies were diluted at 1:500 in PBS/0.1% TX100 and 3% BSA. After incubation overnight at 4 °C, the sections were washed three times for 10 min at RT in PBS containing 0.1% TX100. The sections were then incubated in a mixture of fluorescein-labeled anti-mouse IgG and

lissamine rhodamine-labeled anti-rabbit diluted 1:200, or PI (15 $\mu\text{g}/\text{ml}$) in PBS containing 0.1% TX-100 and 1% BSA for 1 h at room temperature. The sections were washed several times in PBS, mounted on glass slides and coverslipped using Gelvatol. The slides were analyzed on a Zeiss laser-scanning confocal microscope.

Preparation of PSD

Isolation of PSDs was performed according to the procedure of Carlin et al. (1980) except that sodium orthovanadate (0.1 mM) and the protease inhibitors (10 $\mu\text{g}/\text{ml}$ leupeptin, 5 $\mu\text{g}/\text{ml}$ pepstatin, 5 $\mu\text{g}/\text{ml}$ aprotinin, and 0.2 mM phenylmethylsulfonyl fluoride) were included in all buffers. Thirty-two sham-operated control rats and 32 rats subjected to 15 min of ischemia followed by 4 h of reperfusion were used to prepare four separate control samples and four separate postischemia samples, respectively. Briefly, hippocampal tissue samples were pooled from four rats (about 1 g) and homogenized with a Dounce homogenizer. The homogenate was subjected to centrifugation to obtain the crude synaptosomal fraction (P2) as described previously (Hu et al., 1998). This P2 fraction was loaded onto a sucrose density gradient of 0.85 M/1.0 M/1.2 M and centrifuged at $82,500\times g$ for 2 h at 4 °C. The light membrane (LM) fraction was obtained from the 0.85/1.0 sucrose interface and the synaptosomal fraction was collected from the 1.0M/1.2 M sucrose interface. After washing with 2% TX100/300 KCl, the synaptosomal pellets were collected by centrifugation and then subjected to a second 1.0 M/1.5 M/2.0 M sucrose density gradient centrifugation at $201,000\times g$, at 4 °C for 2 h. The isolated PSD fraction was obtained from the 1.5 M/2.0 M interface of the sucrose gradients. The PSD fraction was diluted with an equal volume of 1% TX100/300 mM KCl solution, mixed for 5 min and centrifuged at $275,000\times g$ for 1 h. The PSDs were suspended in a buffer containing 50 mM Tris-HCl, pH 7.4, 0.5 mM DTT, 100 mM KCl, 10 $\mu\text{g}/\text{ml}$ leupeptin, 5 $\mu\text{g}/\text{ml}$ pepstatin, 5 $\mu\text{g}/\text{ml}$ aprotinin, 0.2 mM phenylmethylsulfonyl fluoride, and 0.2 mM sodium orthovanadate. A fraction of the PSDs was used for electron microscopic examination (Hu et al., 1998). The rest of the PSD portion was dissolved in 0.3% SDS for Western blot analysis. The nuclear fraction (N) was isolated by the method of Thompson (1973). Protein concentration was determined by the micro-bicinchoninic acid method of Pierer (Rockford, USA).

Subcellular fractionation

In the study of time course of NSF relocalization after brain ischemia, we prepared several subcellular fractions. Rats were subjected to either sham-operation or to 15 min of ischemia followed by 30 min, 4, 24, 48 and 73 h of reperfusion. Each experimental group consisted of at least four rats. Each hippocampal tissue sample obtained from a given rat was 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 MgCl_2 , 1 $\mu\text{g}/\text{ml}$ pepstatin A, 5 $\mu\text{g}/\text{ml}$ leupeptin, 2.5 $\mu\text{g}/\text{ml}$ 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. Part of the homogenate (H) was directly used for Western blot analysis, and the rest was further centrifuged at $10,000\times g$ at 4 °C for 10 min to obtain a supernatant (S) and pellet. The supernatant was further centrifuged at $165,000\times g$ at 4 °C for 1 h to get a cytosolic fraction (S3) and a microsomal fraction (P3) that contained intracellular membranes. The $10,000\times g$ pellet was suspended with ice-cold homogenization buffer containing 2% TX100 and 300 mM KCl, sonicated three times for 10 s, washed on a shaker for 1 h at 4 °C, and then centrifuged at $10,000\times g$ for 10 min to obtain the TX100-insoluble pellet.

Western blot analysis

Western blot analysis was carried out on 8% SDS-PAGE as described previously (Hu and Wieloch, 1994). Samples for Western blotting contained 100 μg of protein in the

homogenates (H), 40 μ g in the LM, 4 μ g in the PSDs and 25 μ g in nuclear fractions. In a separate set of experiments, samples for Western blotting contained 25 μ g of protein in the TX100-washed pellets, 40 μ g of protein in cytosol (S3) and 40 μ g of protein in P3. We regularly loaded two separate sham-operated controls and two separate samples from every postischemic condition onto one gel. Four separate samples were analyzed in every experimental group. Following electrophoresis, proteins were transferred to an Immobilon-P membrane. The membranes were incubated overnight at 4 °C with primary monoclonal antibody against NSF (1:5000). 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 (Amersham). Quantification of NSF protein in the subcellular fractions was done with Kodak 1D image software. Mean optical densities of immunoblot bands are expressed as percentage of control (mean $\pm$ S.D., $n=4$). * Denotes significant difference ($P<0.01$) between control and 4 h of reperfusion (Student's t -test or ANOVA followed by Fischer's PLSD post hoc test).

RESULTS

As we reported previously, 15 min of transient global cerebral ischemia followed by 24 h of reperfusion did not lead to apparent morphological changes in neurons relative to sham-operated controls when examined by light microscopy. However, frank neuronal necrosis occurred in CA1 pyramidal neurons at approximately 72 h of reperfusion, while DG and most neocortical neurons remained largely intact after ischemia (Martone et al., 1999). At the electron microscopic level, most neurons possessed relatively normal cell membranes and nuclei, but protein aggregates were found in the CA1 neurons after 4 h of reperfusion, consistent with our previous reports (Hu et al., 2000).

To study whether NSF is possibly involved in ischemic neuronal damage, we either labeled the brain sections with a monoclonal anti-NSF antibody alone (Fig. 1A), or double-labeled with the anti-NSF antibody (Fig. 1B, green) together with either anti-synaptophysin (Fig. 1B, red) or PI (Fig. 1C, red). The sections were examined by confocal microscopy. The punctate NSF immunostaining in the cytoplasm and apical dendrites of CA1 neurons, probably representing cytoplasmic free NSF, was observed in sham-operated controls (Fig. 1A, Ctr, arrows). The dense punctate NSF immunostaining was also found in the control CA1 neuropil (Fig. 1A, Ctr), which probably reflected presynaptic terminals because the staining was colocalized with synaptophysin immunolabeling. The colocalization of green color NSF and red color synaptophysin creates an orange or yellow color (Fig. 1B, Ctr, arrowheads). Fifteen minutes of cerebral ischemia followed by 24 h of reperfusion virtually depleted cytoplasmic NSF in CA1 neuronal somata (Fig. 1A, 24 h, stars and Fig. 1B, 24 h, arrows). In the CA1 neuropils, NSF appeared increased at 24 h of reperfusion (Fig. 1A, 24 h, green color, arrowheads). There was no apparent change in synaptophysin staining at 24 h of reperfusion after brain ischemia (Fig. 1B, 24 h, red color). The time course study showed that depletion of neuronal cytoplasmic NSF took place only in CA1 neurons starting as early as 30 min of reperfusion and lasting until 72 h of reperfusion (Fig. 1C, upper panel, green color). At 72 h of reperfusion, NSF immunoreactivity declined in the CA1 neuropil region (Fig. 1C, upper panel, 72h, green color), most probably because neuronal death occurred in the CA1 region as revealed by the PI staining (see below). The NSF immunostaining was less affected in the ischemic surviving neurons such as DG granule cells (Fig. 1C, middle panel, green color), and most neocortical neurons (Fig. 1C, lower panel, green color). PI is a fluorescent dye that stains nucleic acids and is often utilized to stain necrotic cells in culture because it is unable to pass lipid membranes. In fixed brain sections, however, PI is able to penetrate into both normal and damaged cells to stain nucleic acids (Fig. 1C, red color). PI staining can distinguish normal from damaged neurons by their nuclear size and shape under confocal microscopy. PI-stained normal CA1 and DG nuclei were spherically shaped before

24 h of reperfusion (Fig. 1C, red color). At 72 h of reperfusion, however, the CA1 nuclei were shrunken and assumed to a polygonal shape, indicating that neuronal death took place (Fig. 1C, CA1, 72 h, red color, arrows). The morphologies of PI-stained surviving or dead neurons in postischemic brain sections were confirmed by pathological staining with acid fuchsin and Celestin Blue and by EM as we reported previously (Hu et al., 2000).

To investigate NSF relocation further, we prepared hippocampal homogenate (H), nuclear, LM and PSD fractions from sham-operated and postischemic subjects. Western blot analyses demonstrated that NSF was unchanged in the homogenates, LMs and isolated nuclear fractions, but was increased in the purified PSD fraction after ischemia (Fig. 2A). The protein concentrations in these fractions were determined using Lowry method. Equal amounts of proteins among different samples were loaded onto SDS-PAGE for Western blot analyses. In addition, we probed these fractions on Western blots with a tubulin antibody as an internal control to assure further the even loading of the protein samples. The tubulin protein levels were unchanged in these fractions after ischemia (Fig. 2A). The PSD fraction was not contaminated with other subcellular components as examined by EM (Fig. 2B). Quantification of NSF bands in Western blots showed that NSF protein level was very low in sham-operated control PSDs but was significantly increased by about six- to seven-fold in PSDs isolated from rats subjected to 15 min of ischemia followed by 4 h of reperfusion (Fig. 2C).

To study the time course of NSF relocation, we subjected rats to sham-operation or 15 min of ischemia followed by 30 min, 4, 24, 48 and 72 h of reperfusion, and prepared subcellular fractions. As demonstrated in Fig. 3, a decrease in cytosolic (S3) NSF occurred as early as 30 min of reperfusion and lasted until 72 h of reperfusion. Concomitantly, NSF was markedly increased in the TX100-insoluble pellets during the early period of reperfusion but declined to a certain degree at 72 h of reperfusion after brain ischemia (Fig. 3A). NSF was unchanged in the P3 fraction during reperfusion (Fig. 3A). Quantification of NSF bands in Western blots showed that the NSF protein level was high in the cytosol (S3) in sham-operated control but decreased by about 70% at 30 min and 4 h of reperfusion after ischemia. After 24 h of reperfusion, NSF in the S3 fraction tended to recover and the changes were insignificant (Fig. 3B). In comparison with the S3 fraction, the NSF protein level was also low in the TX100-insoluble fraction prepared from sham-operated controls but gradually and significantly increased during the postischemic phase in this fraction after brain ischemia (Fig. 3B). NSF was not significantly altered in the P3 fraction after brain ischemia (Fig. 3B).

Cytoplasmic free NSF is the active form (Rothman, 1996). Depletion of the cytoplasmic NSF indicates its inactivation, which brings membrane fusion machinery to a standstill, resulting in accumulation of intracellular vesicles as observed in numerous previous studies (Block et al., 1988; Brunger, 2001; May et al., 2001; Mohtashami et al., 2001). To test whether depletion of NSF in CA1 neurons was also accompanied by accumulation of intracellular vesicles in these neurons, we examined the brain sections by EM. We utilized the EPTA EM method to stain brain sections because our previous studies have shown that EPTA can selectively stain intracellular vesicles in brain sections after ischemia (Hu et al., 2000). As demonstrated in Fig. 4, EPTA normally stained nuclear structure (Fig. 4, Ctr, N), but additionally stained intracellular vesicles that accumulated in CA1 neurons at 4 and 24 h of reperfusion (Fig. 4, CA1, 4 h and 24 h, arrows). Intracellular vesicles were individually scattered in the cytoplasm at 4 h of reperfusion (Fig. 4, CA1, 4 h, arrows), but they were mostly aggregated with each other at 24 h of reperfusion after ischemia (Fig. 4, CA1, 24 h, arrows). There was no intracellular vesicle accumulation in surviving neurons such as DG granule cells after the same period of ischemia (Fig. 4, DG).

DISCUSSION

This study has provided strong evidence that following transient global cerebral ischemia in rats, the levels of NSF are depleted in the cytoplasm and translocated to the TX100-insoluble fraction in the ischemia-vulnerable hippocampal CA1 neurons undergoing delayed cell death after transient cerebral ischemia. The confocal microscopic study further showed that NSF changes take place mainly in CA1 neurons but not in DG and neocortical neurons after ischemia. The relocalization of NSF is accompanied by accumulation of intracellular vesicles in the CA1 neurons. Relocalization of NSF into the TX100-insoluble fraction reflects NSF inactivation as demonstrated in numerous previous studies (Block et al., 1988; Mohtashami et al., 2001; Stewart et al., 2002), and causes synaptic dysfunction and cell death in various organisms including rodents (Block et al., 1988; Mohtashami et al., 2001; Stewart et al., 2002; Hong et al., 2004). Therefore, inactivation or relocalization of NSF may contribute to synaptic dysfunction and delayed neuronal death in CA1 neurons after transient cerebral ischemia.

The underlying mechanism for depletion of neuronal cytoplasmic free NSF and relocalization of NSF into the TX100-insoluble fraction remains elusive. We propose that ischemia-induced alterations of intracellular homeostasis, such as ATP depletion, intracellular calcium overload, acidosis and overproduction of reactive oxygen species, change NSF conformation and lead to NSF inactivation after ischemia. This is because ATP and normal cellular homeostasis are required for NSF-mediated dissociation of fusion protein complex (Whiteheart et al., 1994). Inactivation of NSF may cause accumulation of intracellular vesicles as demonstrated in Fig. 4. These vesicles are associated with protein aggregates stained with EPTA (Hu et al., 2000). Why is NSF permanently trapped into the TX100-insoluble fraction including PSDs in CA1 dying neurons after transient cerebral ischemia? NSF is a chaperone protein and has high affinity for hydrophobic segments of unfolded proteins (Hu et al., 2000; Hu and Martone, 2001). It is therefore highly likely that NSF is irreversibly aggregated with sticky hydrophobic segments of unfolded proteins that are overproduced during and after brain ischemia (Hu et al., 2000).

Many factors, including energy failure, calcium overload, acidosis, inhibition of protein synthesis, reactive oxygen species, protein aggregation, ER stress, heat-shock proteins and many others, have been postulated to play roles in delayed neuronal death or survival after brain ischemia (Magnusson and Wieloch, 1989; Siesjö, 1989; Nowak, 1991; Sharp et al., 1999; Hossmann, 1993; Chan, 1996; Hu et al., 2000; Paschen, 2003). Although the present study has clearly demonstrated that inactivation and relocalization of NSF in the TX100-insoluble fraction and accumulation of vesicles occur in CA1 dying neurons after ischemia, the causal effects between these NSF changes and delayed CA1 neuronal death remain elusive. It should be important to demonstrate that, for instance, by overexpression or mutation of NSF or NSF-related biological processes, the CA1 neurons will survive after transient cerebral ischemia. However, NSF transgenic animals are not available and the hyh mouse, which carries a mutant NSF attachment protein gene, has a markedly small cerebral cortex at birth and dies postnatally (Hong et al., 2004). Based on the well-established roles of NSF in cell survival, ischemia-induced NSF depletion should have severe consequences. Numerous studies have consistently demonstrated that depletion of free NSF brings membrane fusion machinery to a standstill, resulting in accumulation of intracellular vesicles (Malhotra et al., 1988; Block et al., 1988; Tagaya et al., 1993; Rothman, 1996; Mohtashami et al., 2001). An NSF mutant loses its function and leads to cell death under stressful conditions (May et al., 2001; Mohtashami et al., 2001). Evidence suggests that persistent NSF depletion may play an important role in protein trafficking and delayed neuronal death after transient cerebral ischemia.

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Abbreviations

DG	dentate gyrus
EM	electron microscopy
EPTA	ethanolic phosphotungstic acid
LM	light microscope
MABP	mean arterial blood pressure
NSF	<i>N</i> -ethylmaleimide-sensitive fusion protein
PBS	phosphate-buffered saline
PI	propidium iodide
PSD	postsynaptic densities
PTA	phosphotungstic acid
SNAREs	soluble NSF attachment protein receptors
TX100	Triton X-100

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Fig. 1.

(A) High-resolution confocal microscopic images of hippocampal CA1 neurons labeled with anti-NSF antibody. Brain sections were obtained from rats subjected to either sham-operated control (Ctr) or 15 min of ischemia followed by 24 h of reperfusion. The intra-neuronal NSF immunostaining at 24 h of reperfusion disappeared in CA1 neurons (stars) while it correspondingly increased in the neuropil (arrowheads). (B) Confocal microscopic images of hippocampal CA1 neurons double-labeled with anti-NSF (green color) and anti-synaptophysin antibodies (red color). Brain sections were also from control (Ctr) and 24 h reperused rats. NSF labeling was colocalized or associated with synaptophysin immunostaining in the neuropil region (arrowheads). (C) Confocal microscopic images of hippocampal CA1 (upper panel), DG (middle panel) and neocortical (lower panel) regions double-labeled with anti-NSF antibody (green color) and PI (red color). Brain sections were from sham-operated control rats and rats subjected to 30 min, 4, 24 and 72 h of reperfusion after 15 min of ischemia. The intra-neuronal NSF immunostaining persistently disappeared in CA1 region while unchanged in the DG and cortical area after ischemia. At 72 h of reperfusion, CA1 neuropil NSF staining declined (green color), and CA1 neurons stained with PI became polygonal shape, indicating neuronal death in the CA1 region (red color, arrows).

Fig. 2.

(A) Immunoblots of NSF in homogenate, LM, PSD and nuclear (N) fractions. Hippocampal tissue samples were prepared from sham-control rats (Ctr) and rats subjected to 15 min of ischemia followed by 24 h of reperfusion. Two separate samples in each experimental group were run in the SDS-PAGE and shown. The blots were labeled with an anti-NSF antibody and visualized with an ECL system. (B) An electron micrograph of purified PSD fraction. The PSDs were prepared from rats subjected to 15 min of ischemia followed by 4 h of reperfusion. PSD fraction was examined by conventional EM. (C) Quantification of NSF protein in the subcellular fractions with Kodak 1D image software. Mean optical densities of immunoblot bands are expressed as percentage of control (mean $\pm$ S.D., $n=4$). * Denotes significant difference ($P<0.01$) between control and 4 h of reperfusion (Student's t -test).

Fig. 3.

(A) Immunoblots of NSF in S3, P3 and TX100-insoluble pellets (P2). Hippocampal tissue samples were obtained from sham-control rats (Ctr) and rats subjected to 15 min of ischemia followed by 30 min, 4, 24, 48 and 72 h of reperfusion. The blots were labeled with an anti-NSF antibody and visualized with an ECL system. Two separate samples in each experimental group were run on SDS-PAGE and are shown. (B) Quantification of NSF protein bands on Western blots of subcellular fractions with Kodak 1D image software. Mean optical densities of immunoblot bands from four separate samples are expressed as percentage of control (mean ± S.D., $n=4$). * Denotes significant difference ($P < 0.01$) between control and experimental conditions (ANOVA followed by Fischer's PLSD post hoc test).

Fig. 4.

Electron micrographs of EPTA-stained CA1 and DG neurons from sham-operated control (Ctr, upper panel) rat and rat subjected to 15 min of ischemia followed by 4 h (middle panel) and 24 h (lower panel) of reperfusion. EPTA-stained intracellular vesicles (arrows) were accumulated in CA1 neurons but they were not found in DG neurons after the same period of ischemia. Scale bar=0.5 μ m.