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Late Calcium EDTA Rescues Hippocampal CA1 Neurons from Global Ischemia-Induced Death

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Transient global ischemia induces a delayed rise in intracellular Zn^{2+}, which may be mediated via glutamate receptor 2 (GluR2)-lacking AMPA receptors (AMPARs), and selective, delayed death of hippocampal CA1 neurons. The molecular mechanisms underlying Zn^{2+} toxicity *in vivo* are not well delineated. Here we show the striking finding that intraventricular injection of the high-affinity Zn^{2+} chelator calcium EDTA (CaEDTA) at 30 min before ischemia (early CaEDTA) or at 48–60 hr (late CaEDTA), but not 3–6 hr, after ischemia, afforded robust protection of CA1 neurons in ~50% (late CaEDTA) to 75% (early CaEDTA) of animals. We also show that Zn^{2+} acts via temporally distinct mechanisms to promote neuronal death. Early CaEDTA attenuated ischemia-induced GluR2 mRNA and protein downregulation (and, by inference, formation of Zn^{2+}-permeable AMPARs), the delayed rise in Zn^{2+}, and neuronal death. These findings suggest that Zn^{2+} acts at step(s) upstream from GluR2 gene downregulation and implicate Zn^{2+} in transcriptional regulation and/or GluR2 mRNA stability. Early CaEDTA also blocked mitochondrial release of cytochrome *c* and Smac/DIABLO (second mitochondria-derived activator of caspases/direct inhibitor of apoptosis protein-binding protein with low pI), caspase-3 activity (but not procaspase-3 cleavage), $p75^{NTR}$ induction, and DNA fragmentation. These findings indicate that CaEDTA preserves the functional integrity of the mitochondrial outer membrane and arrests the caspase death cascade. Late injection of CaEDTA at a time when GluR2 is downregulated and caspase is activated inhibited the delayed rise in Zn^{2+}, $p75^{NTR}$ induction, DNA fragmentation, and cell death. The finding of neuroprotection by late CaEDTA administration has striking implications for intervention in the delayed neuronal death associated with global ischemia.

Key words: **zinc; global ischemia; neuronal death; apoptosis; excitotoxicity; AMPA receptors; $p75^{NTR}$**

Introduction

Under physiological conditions, Zn^{2+} serves as a neuronal signaling molecule and is colocalized with glutamate in presynaptic vesicles at a subset of excitatory synapses (Choi and Koh, 1998). Zn^{2+} is uniquely high in mossy fiber tracts of the hippocampus. The zinc transporter 3 (ZnT-3) is localized to synaptic vesicle membranes within mossy fiber boutons and mediates loading of vesicular Zn^{2+} (Palmiter et al., 1996). Zn^{2+} is coreleased with glutamate spontaneously and in an activity-dependent manner and achieves synaptic concentrations of 10–100 μM (Choi and Koh, 1998) (but see Kay, 2003). Synaptically released Zn^{2+} modulates the activity of postsynaptic receptors. Whereas Zn^{2+} inhibits NMDA receptors (NMDARs) and $GABA_A$ receptors (Peters et al., 1987; Westbrook and Mayer, 1987; Christine and Choi, 1990), it potentiates AMPA receptors (AMPARs) (Rassendren et al., 1990; Bresink et al., 1996). Zn^{2+} enters neurons via voltage-sensitive Ca^{2+} channels, NMDARs, glutamate receptor 2 (GluR2)-lacking AMPARs, and/or the Na^+/Zn^{2+} antiporter (Choi and Koh, 1998). Of these, GluR2-lacking AMPARs exhibit highest permeability to Zn^{2+} (Sensi et al., 1999), but under physiological conditions are expressed at low density on distal dendrites of CA1/3 pyramidal neurons (Lerma et al., 1994; Toomim and Millington, 1998; Yin et al., 1999). Within neurons, Zn^{2+} exists as a functionally important component of metalloenzymes and zinc finger-containing transcription factors. Synaptically released Zn^{2+} may be essential for long-term potentiation induction at CA3 synapses (Lu et al., 2000; Vogt et al., 2000; Li et al., 2001). Accordingly, Zn^{2+}-deficient rats, monkeys, and humans exhibit cognitive impairment (Henkin et al., 1975; Golub et al., 1995; Lu et al., 2000).

Zn^{2+} at high concentrations is a critical mediator of the neuronal death associated with global ischemia, seizures, traumatic brain injury, and other brain disorders (Choi and Koh, 1998; Weiss et al., 2000; Dineley et al., 2003; Zukin et al., 2004). Global ischemia elicits a delayed rise in Zn^{2+} in selectively vulnerable CA1 neurons, evident at late times after insult but before onset of

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cell death (Koh et al., 1996; Park et al., 2000). The membrane-impermeant metal chelator calcium EDTA (CaEDTA), administered before ischemia, blocks the rise in Zn^{2+} and protects CA1 neurons. Global ischemia induces suppression of GluR2 mRNA and protein and promotes expression of GluR2-lacking AMPARs on CA1 neurons (Pellegrini-Giampietro et al., 1997). Injurious stimuli promote Zn^{2+} influx into neurons via GluR2-lacking AMPARs (Yin et al., 2002) and/or release of Zn^{2+} from intracellular stores (Aizenman et al., 2000; Lee et al., 2000).

Although the importance of Zn^{2+} to ischemic death is well established, the mechanisms underlying Zn^{2+} toxicity *in vivo* are less clear. The present study was undertaken to address these mechanisms and examine whether CaEDTA administered after ischemia can rescue CA1 neurons. EDTA exhibits higher affinity for Zn^{2+} than Ca^{2+}; CaEDTA effectively chelates Zn^{2+}, replacing it with equimolar Ca^{2+} (Koh et al., 1996). Here we show that CaEDTA rescues CA1 neurons when administered as late as 60 hr after ischemia. Early CaEDTA attenuates mitochondrial release of cytochrome *c* and Smac/DIABLO (second mitochondria-derived activator of caspases/direct inhibitor of apoptosis protein-binding protein with low pI), caspase-3 activity (but not procaspase-3 cleavage), and GluR2 downregulation. Late CaEDTA blocks the rise in Zn^{2+}, $p75^{NTR}$ expression, and DNA fragmentation. Thus, Zn^{2+} acts via temporally distinct mechanisms to promote ischemic cell death.

Materials and Methods

Animals. Age-matched adult male Mongolian gerbils weighing 60–80 gm (Tumblebrook Farms, Wilmington, MA) and male Sprague Dawley rats weighing 100–150 gm (Charles River, Wilmington, DE) were maintained in a temperature- and light-controlled environment with a 14/10 hr light/dark cycle and were treated in accordance with the principles and procedures of the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals*. Protocols were approved by the Institutional Animal Care and Use Committee of the Albert Einstein College of Medicine.

CaEDTA administration. Animals were administered CaEDTA (300 mM in 5 μl of saline; $CaNa_2$ EDTA; Sigma, St. Louis, MO) or saline only (5 μl) by a single unilateral injection into the right lateral ventricle at a flow rate of 1 μl/min at 30 min before or at indicated times after global ischemia or sham surgery. EDTA is a broad-spectrum divalent-cation chelator that binds Zn^{2+} with higher affinity than Ca^{2+} (Bers et al., 1994); CaEDTA chelates Zn^{2+} and replaces it with equimolar Ca^{2+} (Dawson et al., 1984; Koh et al., 1996). Although CaEDTA chelates other metals such as Fe^{2+} and Cu^{2+}, they do not appear causally related to ischemic cell death, nor does ZnEDTA block Fe^{2+} or Cu^{2+} neurotoxicity (Koh et al., 1996). For intracerebroventricular injections, animals were positioned in a stereotaxic frame, and a cannula (28 gauge; stainless steel; inner diameter, 0.18 mm; outer diameter, 0.36 mm) was lowered stereotaxically into the right cerebral ventricle to a position defined by the following coordinates: 0.92 mm posterior to bregma, 1.2 mm lateral to bregma, 3.6 mm below the skull surface (rats) or 0.4 mm posterior to bregma, 1.2 mm lateral to bregma, 2.6 mm below the skull surface (gerbils).

Global ischemia. All experiments except cytochrome *c* and Smac/DIABLO immunolabeling and caspase activity were performed in gerbils. Gerbils offer an advantage compared with rats in that global ischemia can be produced by the relatively simple procedure, bilateral occlusion of the carotid arteries (BCCO). The day before surgery, CaEDTA- and saline-injected animals were fasted overnight and anesthetized with halothane (4% for the first 5 min, followed by 1% for maintenance) delivered by mask in a mixture of N_2–O_2 (70:30) by means of a Vapomatic anesthetic vaporizer (CWE, Ardmore, PA). Gerbils were subjected to global ischemia by temporary BCCO (5 min) or to sham operation followed by reperfusion, as described previously (Oguro et al., 1999; Opitz et al., 2000). Global ischemia in rats was as described previously (Calderone et al., 2003). In all cases, anesthesia was discontinued immediately after initiation of occlusion. Body temperature was monitored and maintained at 37.5 ± 0.5°C with a rectal thermistor and heat lamp until recovery from anesthesia. Animals that failed to show complete loss of the righting reflex and dilation of the pupils from 2 min after occlusion was initiated until the end of occlusion, and the rare animals that exhibited obvious behavioral manifestations (abnormal vocalization when handled, generalized convulsions, hypoactivity) or loss of >20% body weight by 3–7 d were excluded from the study. After reperfusion, arteries were visually inspected to ensure adequate reflow.

Zn^{2+} staining. To measure intracellular free Zn^{2+} concentrations in neurons, brain sections were labeled with the Zn^{2+}-selective fluorescent quinoline derivative *N*-6-(methoxy-8-quinolyl)-*para*-toluenesulfonamide (TSQ) (Frederickson et al., 1987). Fresh-frozen coronal brain sections (18 μm) were thawed, air dried, labeled with TSQ (0.01%), and washed (once in PBS, followed by once in distilled water). Images were viewed under a Nikon (Tokyo, Japan) Eclipse TE300 fluorescence microscope and acquired with a SPOT RT CCD-cooled camera equipped with Diagnostic Software version 3.0. Settings were held constant for imaging of all sections.

Histological analysis. Neuronal cell loss was assessed by histological examination of toluidine blue-stained brain sections at the level of dorsal hippocampus from animals killed at 72 hr, 5, and 7 d after ischemia or sham operation, as described previously (Calderone et al., 2003). Animals were deeply anesthetized with pentobarbital (50 mg/kg, i.p.) and fixed by transcardiac perfusion with ice-cold 4% paraformaldehyde in PBS (0.1 M, pH 7.4). Brains were removed and immersed in fixative (4°C overnight). Coronal sections (15 μm) were cut at the level of the dorsal hippocampus with a cryotome and stained with toluidine blue. For Figure 2, *a–p*, the number of surviving pyramidal neurons per 250 μm length of the medial CA1 region from six to eight gerbils per group (four sections per gerbil) was counted under a light microscope at 40× magnification in sections. For Figure 2*q*, neuronal damage in the left and right hippocampus was scored on a scale of 0–3 (in which 0 is no damage and 3 is maximal damage) according to Pulsinelli and Brierely (1979). Statistical analysis was assessed by ANOVA, followed by Scheffe's *post hoc* tests.

GluR2 in situ *hybridization.* A [^{35}S]UTP-labeled RNA probe directed to the GluR2 mRNA was transcribed *in vitro* using a commercially available kit (Stratagene, La Jolla, CA), as described previously (Calderone et al., 2003). mRNA expression was assessed by *in situ* hybridization on coronal sections of brain at the level of the hippocampus from experimental and control animals at 48, 72 hr, and 5 d after surgery (Calderone et al., 2003). In brief, brains were rapidly removed, frozen by immersion in 2-methylbutane at −42°C, and stored at −70°C until use. Coronal sections (18 μm) were cut by cryotome and thaw mounted onto glass slides. For *in situ* hybridization, brain sections were acetylated, incubated with prehybridization solution (2 hr at 50°C), and hybridized with ^{35}S-labeled GluR1 or GluR2 RNA probes (1 ng/μl or 10^6 cpm/section; overnight at 49°C). Sections were washed, treated with RNase A (20 μg/ml; 30 min at room temperature), dehydrated by immersion in graded ethanol solutions, and apposed to Kodak XAR-5 film (Eastman Kodak, Rochester, NY) for 3–4 d. For quantitation of mRNA expression, autoradiograms were analyzed with a Scan Jet 4-C computing densitometer using NIH Image 1.61 image-analysis software, as described previously (Calderone et al., 2003).

Western blot analysis. To assess GluR2 protein abundance, protein samples were isolated from microdissected hippocampal subfields of experimental and control animals ($n = 3$–5) at 48 and 72 hr after reperfusion and subjected to Western blot analysis, as described previously (Calderone et al., 2003). In brief, animals were deeply anesthetized and killed by decapitation at 24, 48, and 72 hr after ischemia or sham operation. Hippocampi were rapidly dissected out, and transverse slices of dorsal hippocampus (1 mm) were cut with a Mcllwain tissue chopper. The CA1 and dentate gyrus containing the hilus and CA3c (hereafter termed dentate gyrus) were rapidly microdissected, placed in ice-cold PBS supplemented with the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (1 mM; Sigma), and stored at −70°C until use. Tissue samples were homogenized by sonication in 200 μl of 1 mM Na_2C0_3 buffer, pH 6.8, containing PMSF, and lysed in Laemmli sample buffer (0.025 M Tris-HCI, 5% glycerol, 1% SDS, 0.5% PBS, 0.1 M dithiothreitol, 2.5 mM

β-mercaptoethanol, 1 mM PMSF, 0.5 mM $NaHNO_3$ buffer, pH 6.8). Protein concentration of samples was measured using the BCA protein assay kit (Pierce, Rockford, IL). Protein samples (10 μg) were separated by gel electrophoresis (10% polyacrylamide mini-gels; Bio-Rad, Richmond, CA) and transferred to nitrocellulose membranes for immunolabeling. After reaction, membranes were treated with ECL reagents (Amersham Life Science, Arlington Heights, IL) and apposed to XAR-5 x-ray film (Eastman Kodak). Membranes were reprobed for β-actin as a loading control.

To quantitate protein abundance, bands on Western blots were analyzed with a Scan Jet 4-C computing densitometer using NIH Image 1.61 software. Bands of samples from experimental animals were normalized to β-actin (as a loading control) and expressed as a percentage of the corresponding control value. Protein standard curves were constructed to ensure that samples were in the linear range.

Caspase activity assay. Caspase assays were performed on sections of fresh-frozen rat brain using the FAM-DEVD-FMK caspase detection kit according to manufacturer's instructions (APO LOGIXTM) (Tanaka et al., 2004). FAM-DEVD-FMK is a carboxy-fluorescein analog of benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone (zDEVD-FMK), a broad-spectrum cysteine protease inhibitor that enters cells and irreversibly binds activated caspases. FAM-DEVD-FMK exhibits higher affinity for caspase-3 than for caspase-8 or caspase-7 and exhibits much lower affinity for the calpains than for caspases; thus, at 5 μM, FAM-DEVD is a relatively selective inhibitor of caspase-3 (Pozarowski et al., 2003). Moreover, FAM-DEVD-FMK labeling of CA1 neurons correlates well with caspase-3 activation, as assessed by Western blot analysis (Tanaka et al. 2004). In this study, we therefore refer to FAM-DEVD-FMK labeling as indicative of caspase-3 activity. Brain sections were labeled with 5 μM FAM-DEVD-FMK (1 hr; 37°C), washed three times, and viewed under a Nikon Eclipse TE-300 fluorescent microscope equipped with an image analysis system at an excitation wavelength of 488 nm and emission wavelength of 565 nm. Images were acquired with a SPOT RT CCD-cooled camera with Diagnostic Software version 3.0. For quantitation of caspase-3 activity, images were analyzed by NIH Image 1.61 software. Statistical analysis was by ANOVA and Newman–Keuls test.

Detection of DNA cleavage and $p75^{NTR}$. DNA fragmentation and $p75^{NTR}$ were detected in neurons by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)–$p75^{NTR}$–Hoechst 33342 triple labeling of fresh-frozen brain sections from control and experimental gerbils at 72 hr after ischemia using an *in situ* cell death detection kit (Roche Molecular Biochemicals, Mannheim, Germany), as described previously (Jover et al., 2002). In brief, sections were fixed in 4% paraformaldehyde for 20 min at 4°C and processed for TUNEL. Sections were then blocked and labeled with an anti-$p75^{NTR}$ antibody, followed by biotinylated goat anti-rabbit IgG (1:200) and avidin-conjugated Texas Red (1:200; Vector Laboratories, Burlingame, CA). During washes, sections were labeled with the nuclear stain Hoechst 33342 according to manufacturer's instructions (Molecular Probes, Eugene OR). Images were viewed under a Nikon Eclipse TE300 fluorescence microscope and acquired with a SPOT RT CCD-cooled camera equipped with Diagnostic Software version 3.0. Settings were held constant for imaging of sections from control and experimental animals. TUNEL-positive cells were identified directly by the fluorescence signal of incorporated fluorescein-dUTP.

Antibodies. The following antibodies were used in this study: (1) a monoclonal antibody directed to a sequence within the N-terminal domain of the GluR2 subunit (1:1000; PharMingen, San Diego, CA); (2) rabbit anti-$p75^{NTR}$ polyclonal antibody directed to the cytoplasmic domain of human $p75^{NTR}$ (1:4000; gift from Dr. Moses V. Chao, New York University School of Medicine, New York, NY); (3) anti-cytochrome *c* mouse monoclonal antibody raised against peptides 1-80, 81-104, and 66-104 of pigeon cytochrome *c* (1.5 μg/ml; clone 7H8.2C12; PharMingen); (4) anti-Smac/DIABLO antibody, raised against a synthetic peptide corresponding to amino acid residues 222–237 of murine Smac/DIABLO (0.5 μg/ml; Axxora, San Diego, CA); and (5) anti-caspase-3p20, a goat polyclonal antibody that recognizes both procaspase-3 and activated caspase-3 (1:75; Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies were HRP-conjugated rabbit anti-goat IgG (1:2000; Vector Laboratories) for caspase-3, biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories) for $p75^{NTR}$, or HRP-conjugated horse anti-mouse IgG (1:2000; Vector Laboratories) for GluR2, cytochrome *c*, and Smac/DIABLO.

Results

The rise in Zn^{2+} in selectively vulnerable CA1 neurons is highly delayed

Considerable evidence indicates that global ischemia elicits a rise in Zn^{2+} in selectively vulnerable CA1 neurons and that Zn^{2+} is a mediator of neuronal death (Choi and Koh, 1998; Weiss et al., 2000; Koh, 2001; Dineley et al., 2003). However, the time course of Zn^{2+} accumulation in CA1 neurons is not well delineated. To address this issue, we assessed levels of intracellular Zn^{2+} in hippocampal neurons with the Zn^{2+}-specific fluorescence indicator dye TSQ at times after ischemia. In control hippocampus, TSQ labeling revealed intense fluorescence in the mossy fiber axons of dentate granule neurons in the hilus and stratum lucidum of CA3, as well as faint fluorescence in the stratum radiatum and stratum oriens of CA1 (Fig. 1*a*,*b*). Zn^{2+} was not detectable in the pyramidal cell layer of CA1 or CA3. At 0 and 1 hr after ischemia, the pattern and intensity of TSQ staining did not differ detectably from that observed in control hippocampus (data not shown). Global ischemia induced a slight reduction in Zn^{2+} fluorescence in mossy fibers and Schaffer collaterals, evident at 24 hr after reperfusion, and bright fluorescence in the cell bodies of scattered hilar neurons (Fig. 1*e*,*f*). At 48 hr after ischemia, Zn^{2+} was visible in pyramidal neurons of the CA3a, extending into the CA1/CA3 transition zone, but not CA1 (Fig. 1*g*,*h*). At 72 hr after ischemia, Zn^{2+} fluorescence was pronounced in cell bodies of CA1 pyramidal neurons (Fig. 1*i*,*j*). These findings indicate a substantial delay (nearly 72 hr) between ischemic insult and Zn^{2+} accumulation in selectively vulnerable neurons. This late rise in Zn^{2+} is thought to occur via GluR2-lacking AMPARs, which are highly permeable to Zn^{2+} (Sensi et al., 1999; Yin et al., 2002) and exhibit enhanced expression in CA1 neurons at 60–72 hr after ischemia (Gorter et al., 1997).

EDTA is a broad-spectrum divalent-cation chelator that binds Zn^{2+} with higher affinity than Ca^{2+}; CaEDTA chelates Zn^{2+} and replaces it with equimolar Ca^{2+} (Dawson et al., 1984; Bers et al., 1994). Although CaEDTA chelates other metals such as Fe^{2+} and Cu^{2+}, they do not appear causally related to ischemic cell death, nor does ZnEDTA block Fe^{2+} or Cu^{2+} neurotoxicity (Koh et al., 1996). Administration of CaEDTA 30 min before ischemia (early CaEDTA) did not detectably alter the rise in Zn^{2+} in the CA3a or transition zone, evident at 48 hr (data not shown) but markedly attenuated the late rise in Zn^{2+} in CA1 neurons at 72 hr (Fig. 1*k*,*l*), in confirmation of findings in rats (Koh et al., 1996). In contrast, administration of CaEDTA (300 mM) into the lateral ventricles of control animals did not detectably alter the pattern or intensity of Zn^{2+} fluorescence in hippocampus, assessed at 72 hr after sham operation (Fig. 1*c*,*d*).

Early and late injections of CaEDTA protect against ischemia-induced death of CA1 neurons

If Zn^{2+} indeed accumulates in CA1 neurons at 48–72 hr after ischemia, CaEDTA might be protective not only if given before ischemia, but also when administered as late as 48–60 hr after ischemia (late CaEDTA). To examine the time window in which CaEDTA was effective, we subjected gerbils to global ischemia or sham operation and administered CaEDTA (300 mM, i.c.v.) at times before or after surgery; neuronal death was assessed histologically. As expected,

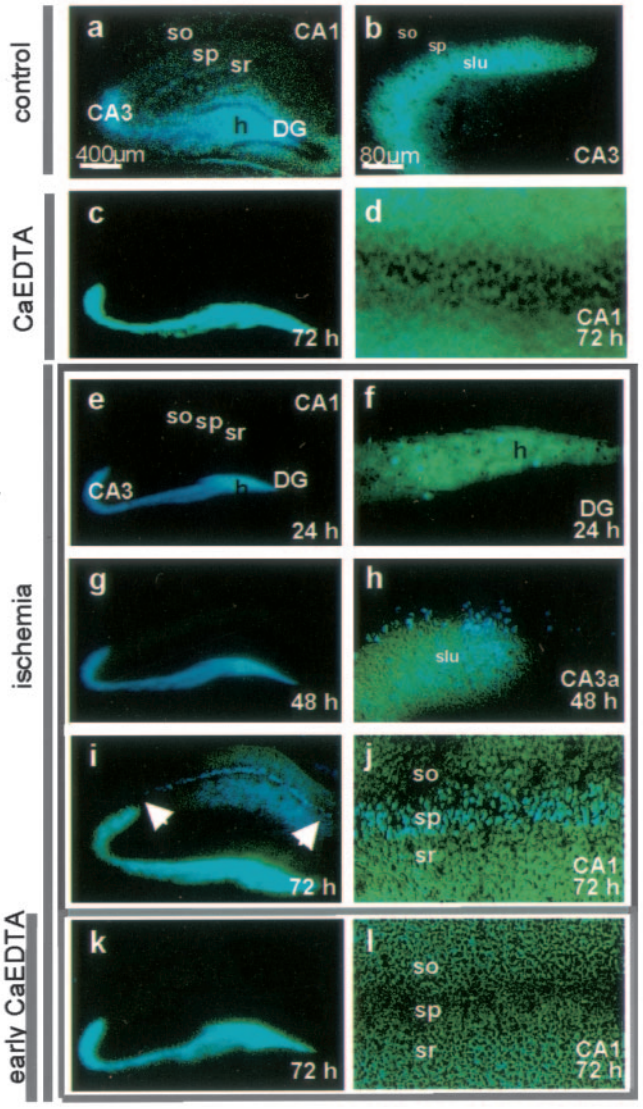


Figure 1. Global ischemia elicits a highly delayed rise in Zn^{2+} in selectively vulnerable CA1 neurons. Zn^{2+} fluorescence in TSQ-stained coronal brain sections from sham (*a–d*) and experimental animals subjected to global ischemia (*e–l*) or to CaEDTA, followed by global ischemia (*k*, *l*). In control hippocampus, TSQ labeling revealed intense fluorescence in the mossy fiber axon terminals of dentate granule neurons in the hilus (h) and stratum lucidum of CA3 (slu) and faint fluorescence in the stratum radiatum and stratum oriens of CA1 (*a*, *b*). CaEDTA injection at 30 min before surgery did not detectably alter the pattern of Zn^{2+} fluorescence in sham-operated control animals, assessed at 72 hr after surgery (*c*, *d*). Global ischemia induced a pronounced increase in Zn^{2+} fluorescence in the cell bodies of scattered hilar neurons, evident at 24 hr after insult (*e*, *f*). At 48 hr, Zn^{2+} fluorescence was visible in CA3a pyramidal neurons, extending into the CA1/CA3 transition zone, but was not visible in CA1 (*g*, *h*). At 72 hr, Zn^{2+} fluorescence was pronounced in cell bodies of CA1 pyramidal neurons [*i* (arrows), *j*]. Injection of CaEDTA 30 min before ischemia did not affect the increase in Zn^{2+} in the transition zone but attenuated the late rise in Zn^{2+} fluorescence in the CA1 (*k*, *l*). Scale bars: (in *a*) *a*, *c*, *e*, *g*, *i*, *k*, 400 μm; (in *b*) *b*, *d*, *f*, *h*, *j*, *l*, 80 μm. so, Stratum oriens; sp, stratum pyramidale; sr, stratum radiatum.

global ischemia induced extensive death of pyramidal cells in the hippocampal CA1, evident at 5 d; the few remaining pyramidal neurons were severely damaged and appeared pyknotic (93.0 ± 1.45 cells/250 μm in control animals; 2.1 ± 1.45 cells/250 μm in ischemic animals; $n = 5$; $p < 0.01$ vs control) (Fig. 2*e*,*f*,*o*). Neuronal death was specific in that little or no cell loss occurred in the nearby CA2 or transition zone, CA3, or dentate gyrus (Fig. 2*e*). With the exception of a few scattered hilar neurons and/or pyramidal neurons in the cortex, no other neurons exhibited cell death. Neuronal death was delayed in that little or no death was manifest at 72 hr (data not shown) (but see Gorter et al., 1997). These data corroborate findings in rats (Calderone et al., 2003) and gerbils (Colbourne et al., 2003). CaEDTA administered 30 min before ischemia greatly reduced the loss of CA1 neurons (CaEDTA pretreatment, 63.44 ± 5.49 cells/250 μm; $n = 5$; $p < 0.01$ vs ischemia only) (Fig. 2*g*,*h*,*s*). In contrast, CaEDTA administered to control animals did not detectably alter neuronal counts (Fig. 2*c*,*d*,*s*). These findings are in confirmation of others (Koh et al., 1996) and are consistent with a model in which Zn^{2+} plays a role in early events leading to delayed death of CA1 neurons.

Administration of CaEDTA at 60 hr after ischemic insult afforded substantial survival of CA1 neurons in ~50% of animals, relative to that of saline-injected animals subjected to ischemia (saline, 14.9 ± 3.4 cells/250 μm, $n = 20$, $p < 0.01$ vs control; CaEDTA at 60 hr, 38.3 ± 4.9 cells/250 μm, $n = 25$, $p < 0.01$ vs ischemia) (Fig. 2*o–r*,*t*). Similar results were observed when CaEDTA was given at 48 hr. In contrast, CaEDTA was ineffective when injected at 72 hr, a time when intracellular Zn^{2+} accumulation in CA1 neurons had already occurred (Fig. 2*u*), although the relatively low number of animals sampled precludes a definitive conclusion. These findings are consistent with the concept that CaEDTA administered at 48 or 60 hr after global ischemia chelates extracellular Zn^{2+}, preventing its influx via newly synthesized GluR2-lacking AMPARs, voltage-sensitive Ca^{2+} channels, and/or by reverse operation of the Zn^{2+} antiporter and can chelate intracellular Zn^{2+}, which leaches out of neurons to restore the extracellular Zn^{2+} concentration (Frederickson et al., 2002). In contrast, CaEDTA administered at 3 or 6 hr after ischemia did not afford significant protection (Fig. 2*u*). Thus, CaEDTA affords protection at temporally separated windows of time, consistent with a model in which Zn^{2+} acts via temporally distinct mechanisms to elicit neuronal death.

Early CaEDTA attenuates ischemia-induced suppression of GluR2 mRNA in CA1 neurons

The notion that Zn^{2+} acts at an early time after ischemic insult is consistent with a role in transcriptional changes, which occur at early times after ischemia. Global ischemia activates the restrictive element-1 silencing transcription factor (REST)–neuron-restrictive silencer factor (Calderone et al., 2003), a nine zinc finger transcription factor that suppresses neural-specific target genes including GluR2 (Huang et al., 1999, 2002; Brene et al., 2000; Calderone et al., 2003). Although it is not known how ischemia–hypoxia triggers induction of REST, an attractive hypothesis is that the early rise in calcium during the ischemic episode triggers a calcium-responsive transcription factor, which in turn induces REST expression. To examine a role for Zn^{2+} in the transcriptional changes associated with global ischemia, we administered the Zn^{2+} chelator CaEDTA or saline to gerbils 30 min before global ischemia or sham operation and measured AMPAR mRNA expression in CA1 neurons by *in situ* hybridization at times after reperfusion. In control hippocampus, GluR2 mRNA expression was pronounced throughout the pyramidal cell layers of CA1 and CA3 and the granule cell layer of the dentate gyrus (Fig. 3*a*). Global ischemia induced a marked and long-lasting reduction in GluR2 mRNA expression in the pyramidal cell layer of CA1, evident at 48 and 72 hr (reduction to 41.2% ± 0.5% of control at 48 hr, $n = 3$, $p < 0.01$; to 22.1 ± 2% of control at 72 hr, $n = 4$, $p < 0.01$) (Fig. 3*a*,*c*), times before the onset of neuronal death (Fig. 2). At 5 d, a time when there is extensive cell loss in the CA1 pyramidal cell layer, GluR2 mRNA was reduced to 19.4 ±

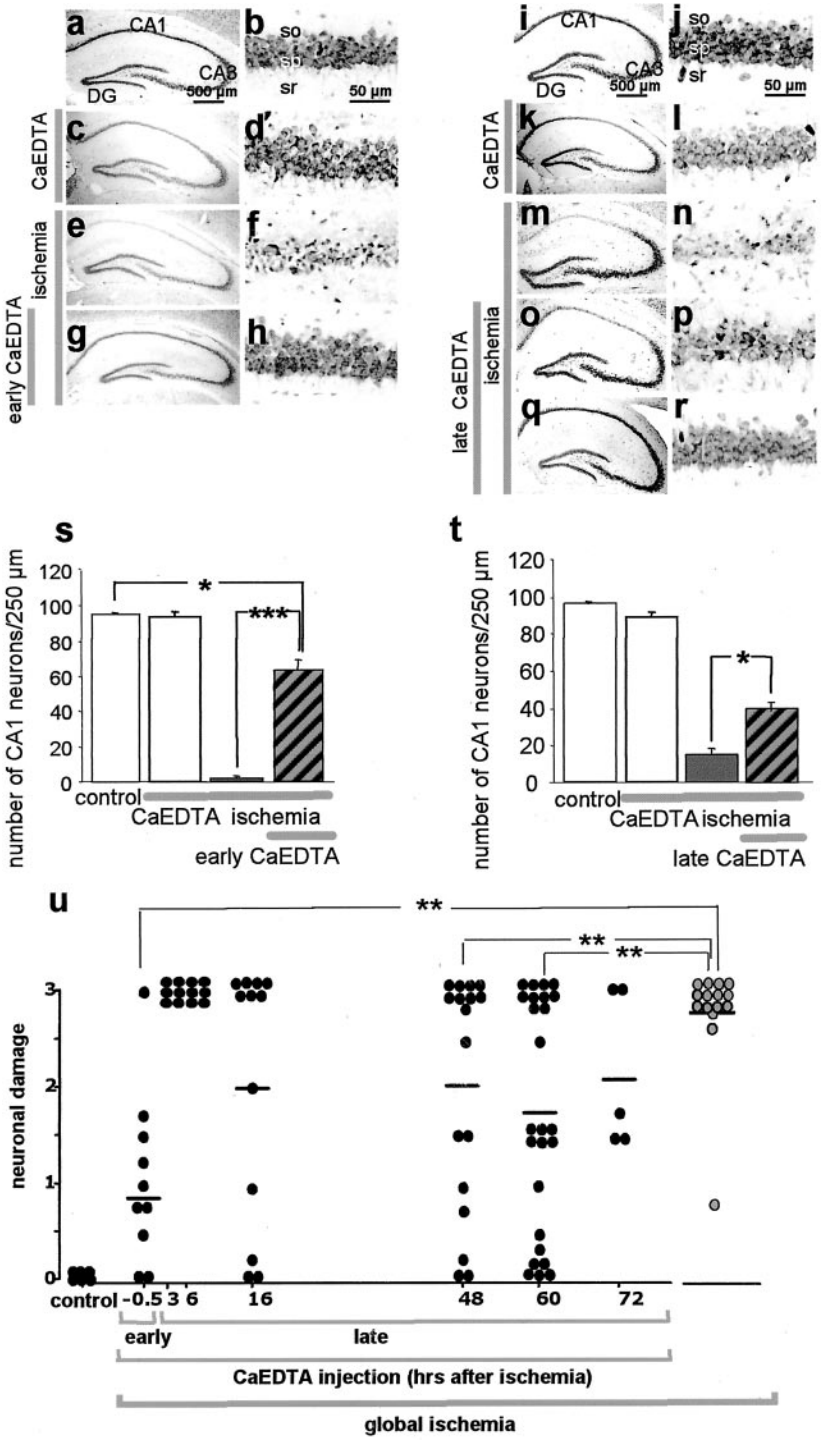


Figure 2. Early and late CaEDTA afford protection against ischemia-induced neuronal death. Toluidine blue-stained coronal brain sections at the level of the dorsal hippocampus from control ($n = 3$; *a*, *b*) and experimental animals subjected to CaEDTA injection (*c*, *d*), global ischemia (*e*, *f*), or to CaEDTA (300 mM, i.c.v.), followed by ischemia (*g*, *h*) and killed 5 d after surgery. Brain sections from sham ($n = 3$; *i*, *j*) and experimental animals subjected to CaEDTA injection (*k*, *l*), ischemia ($n = 4$; *m*, *n*), or to ischemia, followed by CaEDTA at 60 hr after ischemia ($n = 6$; *o*–*r*) and killed 7 d after ischemia. Late CaEDTA afforded robust neuroprotection is some (*q*, *r*), but not other (*o*, *p*), animals. *s*, *t*, Quantitation of cell counts from sections such as those shown in *a*–*h* (*s*) and *i*–*r* (*t*). *u*, Summary data for CaEDTA injections given at times ranging from 30 min before ischemia to 72 hr after ischemia. Global ischemia was induced in gerbils by BCCO (5 min). Animals were killed at 7 d after ischemia, and brain sections at the level of the dorsal hippocampus were scored for neuronal damage according to Pulsinelli and Brierley (1979). CaEDTA afforded neuroprotection in a substantial number of animals when administered 30 min before ischemia or at 48 or 60 hr after ischemia but was ineffective when administered at 3 or 6 hr after ischemia. Statistical significance of changes in neuronal counts (*s*, *t*) was assessed by means of Student's unpaired *t* test and of changes in neuronal damage scores (*u*) by means of ANOVA (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Error bars represent SEM. Scale bars: (in *a*, *i*) *a*, *c*, *e*, *g*, *i*, *k*, *m*, *o*, *q*, 500 μm; (in *b*, *j*) *b*, *d*, *f*, *h*, *j*, *l*, *n*, *p*, *r*, 50 μm. so, Stratum oriens; sp, stratum pyramidale; sr, stratum radiatum.

10% of control at 5 d ($n = 3$; $p < 0.01$ vs control) (Fig. 3*a*,*c*). The reduction in GluR2 expression was cell specific in that it was not altered in CA3 pyramidal cells or dentate gyrus granule cells (Fig. 3*a*). The ischemia-induced alteration in mRNA expression was subunit specific in that GluR1 mRNA was unchanged in all hippocampal subfields at 48 hr, corroborated by Pellegrini-Giampietro et al. (1992) and Gorter et al. (1997).

In contrast, in CaEDTA-pretreated animals, ischemia induced a marked, but transient reduction of GluR2 mRNA in the CA1 (reduction to 44.3% ± 4.4% of control at 48 hr, $n = 4$, $p < 0.01$ vs control; to 46.1 ± 8% of control at 72 hr, $n = 4$, $p < 0.01$ vs control; and to 88.0% ± 2.8% of control at 5 d, $n = 4$, $p < 0.01$ vs control) (Fig. 3*b*,*c*). Whereas at 48 hr after ischemia, the extent of GluR2 mRNA downregulation was virtually the same in CaEDTA- versus saline-injected animals, at 72 hr GluR2 expression was significantly greater in the CaEDTA- versus saline-injected group ($p < 0.01$ vs saline-injected; $p < 0.001$ vs control). By 5 d after global ischemia in CaEDTA-treated animals, GluR2 mRNA had recovered to near control levels (to 93.6 ± 3.4% of control; $p < 0.001$ vs saline-injected; $n = 6$) (Fig. 3*b*,*c*). The transient GluR2 downregulation observed after global ischemia in CaEDTA-treated animals was cell specific in that it was unchanged in CA3 and dentate gyrus (DG) at all times examined. These observations implicate Zn^{2+} in the persistent downregulation of GluR2 mRNA.

Early CaEDTA attenuates ischemia-induced downregulation of GluR2 protein in CA1

Because GluR2 may also be under translational control, we examined the effects of CaEDTA on the ischemia-induced downregulation of GluR2 protein in CA1. Protein samples from the hippocampal CA1 and dentate gyrus of control and experimental animals were subjected to Western blot analysis. Analysis of band densities indicated that global ischemia reduced GluR2 subunit abundance in CA1, evident at 48 and 72 hr [reduction to 72.8 ± 7.1% of control at 48 hr, $n = 4$, not significant (n.s.); and to 54.1 ± 5.5% of control at 72 hr, $n = 3$, $p < 0.01$] (Fig. 3*d*,*f*). The reduction was subfield selective in that ischemia induced only a modest reduction in GluR2 subunit abundance in dentate gyrus, evident at 72 hr (Fig. 3*e*,*g*). CaEDTA significantly attenuated the ischemia-induced downregulation of GluR2 protein in CA1 (ischemic-induced reduction in CaEDTA-

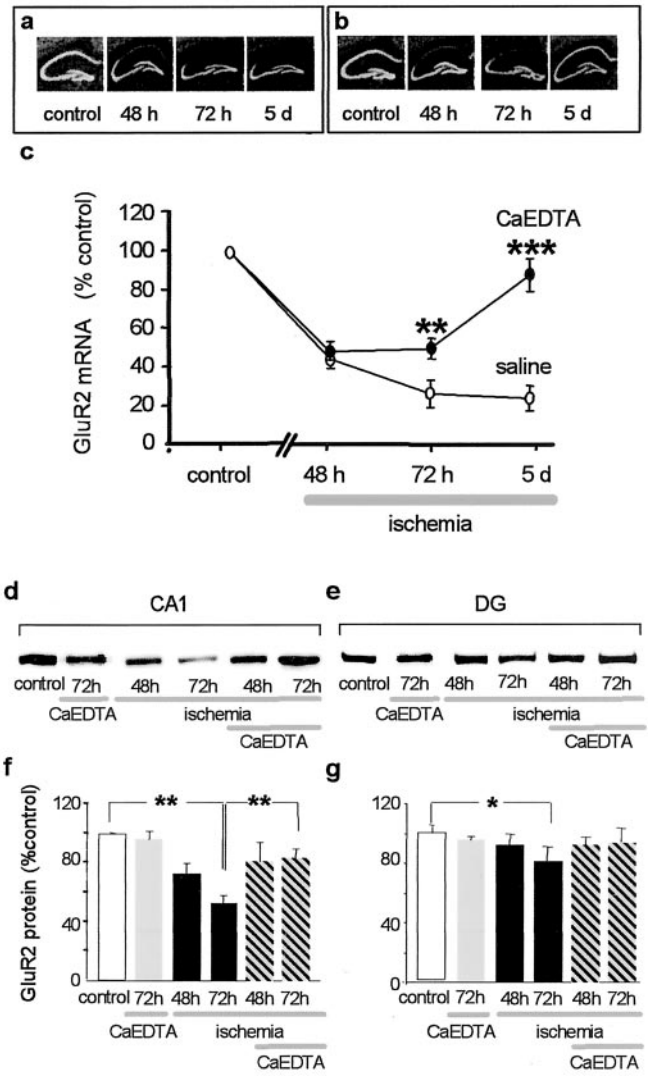


Figure 3. Early CaEDTA attenuates ischemia-induced suppression of GluR2 mRNA and protein in CA1. *a*, *b*, Representative film autoradiograms of GluR2 mRNA expression detected by *in situ* hybridization in the hippocampus of control and experimental animals subjected to global ischemia at 48 hr ($n = 3$), 72 hr ($n = 4$), and 5 d ($n = 3$) after reperfusion (*a*) or to pretreatment with CaEDTA (300 mM, i.c.v.), followed by ischemia at 48 hr ($n = 4$), 72 hr ($n = 4$), and 5 d after reperfusion ($n = 4$) (*b*). *c*, Quantitative analysis of data such as those in *a*, *b*. Mean optical densities were normalized to the corresponding control value for a given region. Ischemia caused a loss of GluR2 mRNA in CA1 (but not DG or CA3), evident at 48 hr. CaEDTA pretreatment did not alter ischemia-induced downregulation of GluR2 mRNA (48 hr) but accelerated its recovery to control values (72 hr and 5 d). *d*, *e*, Representative Western blots probed with a monoclonal antibody against a sequence within the N-terminal domain of the GluR2 subunit. *f*, *g*, Relative GluR2 subunit abundance for protein samples isolated from the CA1 (*f*) and DG (*g*) of control ($n = 4$) and experimental animals at 48 hr after CaEDTA injection ($n = 4$), 48 hr ($n = 4$) and 72 hr ($n = 3$) after ischemia, and at 48 hr ($n = 4$) and 72 hr ($n = 4$) after CaEDTA injection, followed by ischemia. GluR2 abundance was determined from band densities for GluR2 after normalization to the band densities for actin, which served as a loading control. Relative GluR2 subunit abundance was markedly decreased in CA1 (but not DG) at 48 and 72 hr after ischemia. CaEDTA pretreatment attenuated GluR2 downregulation, assessed at 72 hr and 5 d. Bars are means ± SEM. Statistical significance was assessed by means of Student's unpaired *t* test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

treated animals to 81.6% ± 12.9 of control at 48 hr, $n = 4$, n.s. vs control, $p < 0.05$ vs saline-injected ischemic animals; and to 84.1 ± 5.8% of control at 72 hr, $n = 4$, n.s. vs control, $p < 0.01$ vs saline-injected ischemic animals) (Fig. 3*d*,*f*) and prevented the slight decrease in GluR2 abundance in dentate gyrus (Fig. 3*e*,*g*). CaEDTA administered 30 min before sham operation did not detectably alter GluR2 protein abundance in CA1 (Fig. 3*d*,*f*) or DG (Fig. 3*e*,*g*). These findings implicate Zn^{2+} in the duration and extent of GluR2 protein downregulation.

Early CaEDTA blocks the onset of apoptosis in vulnerable CA1 neurons

We next examined a possible role for early Zn^{2+} in the initiation of apoptosis. A major pathway implicated in initiation of the caspase death cascade is the intrinsic (mitochondrial) pathway in which breakdown of the functional integrity of the outer membrane of the mitochondria leads to release of cytochrome *c* (Shi, 2002; van Loo et al., 2002; Zukin et al., 2004). Once in the cytosol, cytochrome *c* assembles with Apaf-1, dATP, and procaspase-9 to form the apoptosome, which activates caspase-9, which in turn activates caspase-3. A second mitochondrial activator of caspases (Smac/DIABLO) is coreleased from the mitochondria with cytochrome *c* (Shi, 2002; van Loo et al., 2002; Zukin et al., 2004). Because the available cytochrome *c* and Smac/DIABLO antibodies recognize rat (but not gerbil) antigens, we used rats for these experiments and for assay of caspase function, which would be expected to correlate with mitochondrial release of cytochrome *c*. The four-vessel occlusion model of global ischemia in rats is a well established model of global ischemia in which a brief (10 min) ischemic episode affords highly delayed, highly selective death of CA1 pyramidal neurons, which is not manifested until >48 hr. Global ischemia did not significantly alter the abundance of either cytochrome *c* (Fig. 4*a*) or Smac/DIABLO (Fig. 4*c*) in the mitochondrial fraction of the CA1 but increased the abundance of cytochrome *c* (Fig. 4*b*) and Smac/DIABLO (Fig. 4*d*) in the cytosol, assessed at 12 hr after reperfusion. These data are in confirmation of other studies (Ouyang et al., 1999; Sugawara et al., 1999; Tanaka et al., 2004). CaEDTA reduced the mitochondrial and cytosolic levels of cytochrome *c* (Fig. 4*a*,*b*) and the cytosolic levels of Smac/DIABLO in the CA1 of control animals (Fig. 4*d*) and completely blocked the ischemia-elicited rise in cytosolic cytochrome *c* (Fig. 4*b*) and Smac/DIABLO (Fig. 4*d*). Together, these findings indicate that CaEDTA strengthens the integrity of the outer mitochondrial membrane in control neurons and prevents the breakdown of the functional integrity of the outer mitochondrial membrane in postischemic neurons. A less likely possibility is that CaEDTA reduces the stability of cytochrome *c* and Smac/DIABLO in the cytosol.

Early CaEDTA attenuates caspase activity and late-stage apoptosis

Caspase-3 is a protease and death-promoting protein implicated in the execution step of apoptosis (for review, see Yuan et al., 2003; Tanaka et al., 2004). Global ischemia induces activation of caspase-3, a step critical to ischemia-induced neuronal death (Chen et al., 1998; Tanaka et al., 2004). To examine expression of procaspase-3 and its proteolytic processing to generate cleaved (activated) caspase-3, we performed Western blot analysis of samples from the CA1 of control and experimental animals. In control animals, abundance of procaspase-3 (32 kDa) (Fig. 5*a*, first lane) and cleaved (activated) caspase-3 (17 kDa) (Fig. 5*b*, first lane) were low in CA1, as assessed by Western analysis. Global ischemia induced a marked increase in procaspase-3 (Fig. 5*a*, second lane) and cleaved caspase-3 in CA1 (Fig. 5*b*, second lane), evident at 24 hr. CaEDTA pretreatment under conditions that afford robust neuroprotection did not detectably alter the ischemia-induced upregulation of procaspases-3 or its proteo-

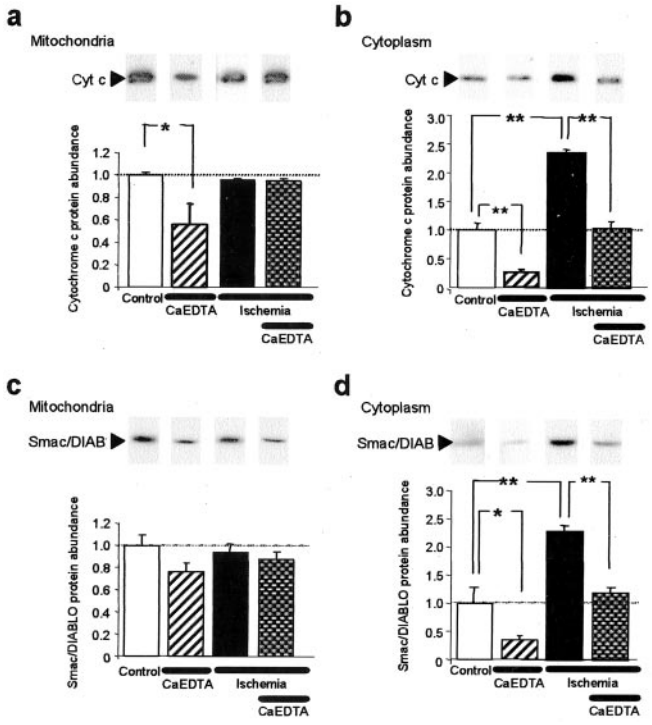


Figure 4. Early CaEDTA blocks the ischemia-induced mitochondrial release of cytochrome *c* (Cyt c) and Smac/DIABLO. Representative Western blots and relative abundance of cytochrome *c* (*a*, *b*) and Smac/DIABLO (*c*, *d*) in the mitochondrial (*a*, *c*) and cytosolic (*b*, *d*) fractions of protein samples isolated from control and experimental animals subjected to CaEDTA injection, ischemia, or CaEDTA (300 mM, i.c.v.; 30 min before ischemia), followed by ischemia and killed at 12 hr after reperfusion. Westerns were probed with anti-cytochrome *c* antibody (*a*, *b*) or anti-Smac/DIABLO antibody (*c*, *d*). A single injection of CaEDTA significantly reduced the mitochondrial and cytosolic levels of cytochrome *c* (*a*, *b*) and cytosolic levels of Smac/DIABLO (*d*) in control animals and greatly attenuated the ischemia-induced increase in cytosolic cytochrome *c* (*b*) and Smac/DIABLO (*d*). Mean band densities for cytochrome *c* or Smac/DIABLO in protein samples from the CA1 of experimental animals were normalized to the corresponding values for samples from control animals. Statistical significance was assessed by ANOVA, followed by Newman–Keuls test (**$p < 0.01$). Error bars represent SEM.

lytic processing to generate cleaved (activated) caspase-3 (Fig. 5*a*,*b*, third lane).

The results thus far demonstrate that global ischemia promotes upregulation of the "terminator" protease caspase-3, but do not distinguish between active and inactive protein. To directly measure caspase-3 functional activity, we took advantage of FAM-DEVD-FMK, a fluorescein-tagged analog of the caspase inhibitor zDEVD-FMK. FAM-DEVD-FMK enters cells and binds irreversibly to caspase-3 and thus provides a fluorescent indicator of the abundance of active caspase-3. In sections of control brain, caspase activity was low (Fig. 5*c*,*d*,*i*). Global ischemia induced a dramatic increase in caspase activity in the hippocampal CA1, evident at 24 hr (Fig. 5*e*,*f*,*i*). The increase in caspase activity was specific in that it was not observed in the resistant CA3 or dentate gyrus. Early injection of CaEDTA caused a marked inhibition of caspase-3 activity, assessed at 24 hr (Fig. 5*g–i*). These findings are consistent with a model in which early Zn^{2+} is critical to caspase-3 activity and onset of apoptosis.

If early CaEDTA indeed arrests caspase activity, it might block apoptotic cascades that occur at late times after ischemia. Global ischemia elicits induction of $p75^{NTR}$, which coincides with the late rise in Zn^{2+} in selectively vulnerable CA1 neurons (Park et al., 2000). To examine induction of $p75^{NTR}$ and DNA fragmentation in neurons undergoing apoptosis, we performed triple labeling of $p75^{NTR}$, TUNEL, and Hoechst 33342 (a nuclear stain) on sections of hippocampus from experimental and control animals 72 hr after global ischemia (Fig. 5*j–u*). $p75^{NTR}$ is a proapoptotic neurotrophin receptor that can trigger apoptotic cell death via a death-receptor-dependent pathway (Roux and Barker, 2002) and provides a marker for neurons undergoing apoptosis in animals subjected to seizures (Roux et al., 1999) or global ischemia (Park et al., 2000; Jover et al., 2002; Tanaka et al., 2004). We used a TUNEL technique in which a terminal deoxynucleotidyl transferase transfers fluorescein "tags" to nicked ends present in fragmented DNA. In sections from control brain, $p75^{NTR}$ immunoreactivity and TUNEL labeling were undetectable in the pyramidal cell layers of the CA1 (Fig. 5*j–l*) and in other subfields of the hippocampus (data not shown). Global ischemia induced marked $p75^{NTR}$ immunoreactivity and TUNEL positivity in CA1 pyramidal neurons, evident at 72 hr ($n = 3$ per treatment group) (Fig. 5*m–r*), in confirmation of our previous results (Jover et al., 2002). These findings indicate that $p75^{NTR}$ expression occurs in postischemic CA1 neurons undergoing apoptosis. Early injection of CaEDTA greatly attenuated $p75^{NTR}$ expression [in confirmation of Park et al. (2000)] and blocked DNA fragmentation in CA1 (Fig. 5*s–u*). These findings demonstrate that early CaEDTA intervenes downstream of proteolytic processing and activation of caspase-3 and upstream of $p75^{NTR}$ expression and DNA fragmentation to protect CA1 neurons and implicate Zn^{2+} in the late stages of apoptotic cell death.

Late CaEDTA prevents the delayed rise in Zn^{2+} and late-stage apoptosis

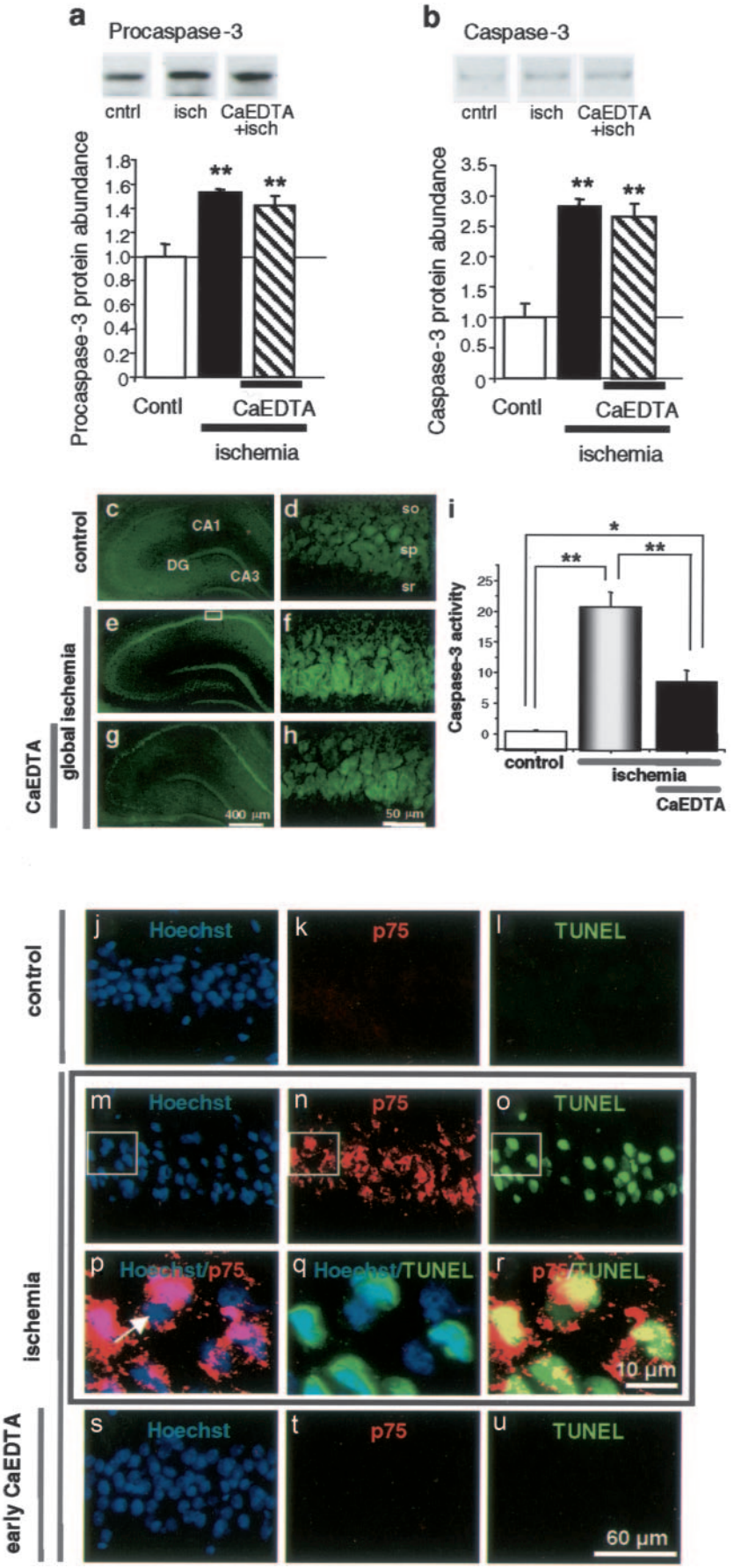
Our findings indicate a substantial delay (48–72 hr) between ischemic insult and Zn^{2+} accumulation in selectively vulnerable neurons. These observations raise the possibility that late injections of CaEDTA should prevent the late Zn^{2+} rise in CA1 neurons. To examine this hypothesis, we subjected animals to global ischemia, followed by administration of CaEDTA at 60 hr, and assessed intracellular Zn^{2+} concentration by TSQ labeling of brain sections at the level of the dorsal hippocampus. As in Figure 1, global ischemia induced a pronounced rise in Zn^{2+} fluorescence in cell bodies of CA1 pyramidal neurons, evident at 72 (Figs. 6*c*, 1*i*,*j*) but not 48 hr (Figs. 6*b*,1*g*,*h*). CaEDTA injected at 60 hr after ischemia, a time when downregulation of GluR2 protein and expression of GluR2-lacking AMPARs are pronounced, substantially attenuated the late rise in intracellular Zn^{2+} in CA1 neurons (similar results were observed in eight of eight animals subjected to ischemia, followed by CaEDTA at 60 hr) (Fig. 6*d–f*). These findings do not, however, distinguish between chelation of extracellular Zn^{2+} before its entry into postsynaptic neurons versus chelation of intracellular Zn^{2+} as it leeches out of degenerating CA1 neurons.

Whereas release of cytochrome *c* and caspase-3 activation occur in the first 3 hr after insult, induction of $p75^{NTR}$ and onset of DNA fragmentation do not occur until ~72 hr after global ischemia (Zukin et al., 2004). We therefore examined the effects of late CaEDTA on these markers of end-stage apoptosis. As in Figure 5, global ischemia induced a marked increase in $p75^{NTR}$ and TUNEL staining in CA1 neurons at 72 hr after ischemia ($n = 3$ per treatment group) (Figs. 6*k*,*l*, 5*m–r*). Late injection of CaEDTA (60 hr after ischemia) greatly attenuated induction of $p75^{NTR}$ expression and DNA fragmentation in CA1 neurons (Fig. 6*m–o*, typical of five of five animals). Thus, late injection of CaEDTA blocks end-stage apoptosis and rescues CA1 neurons from ischemic cell death.

Discussion

Considerable evidence suggests that Zn^{2+} at high intracellular concentrations is a critical mediator of the neuronal death associated with global ischemia, seizures, traumatic brain injury, and other brain disorders (Choi and Koh, 1998; Weiss et al., 2000; Dineley et al., 2003; Zukin et al., 2004). Global ischemia elicits a delayed rise in Zn^{2+} in the cell bodies of selectively vulnerable CA1 neurons before the onset of cell death (Koh et al., 1996; Park et al., 2000). CaEDTA pretreatment prevents late Zn^{2+} accumulation and protects CA1 neurons (Koh et al., 1996; Park et al., 2000). The substantial delay between neuronal insult and rise in toxic Zn^{2+} suggests the opportunity for intervention at late times after ischemia. Here we show the striking finding that CaEDTA administered as late as 60 hr after global ischemia affords robust protection of CA1 neurons in ~50% of animals. In contrast, the selective caspase-3 inhibitor z-DEVD-FMK affords robust protection of neurons against ischemic death when administered before or 2 hr after ischemia (Chen et al., 1998), but not when administered at 6–72 hr after ischemia (our unpublished observations). Thus, diverse protective strategies exhibit different windows of therapeutic intervention. The finding of neuroprotection by late CaEDTA administration has powerful implications for intervention in ischemia-induced neuronal death and supports Zn^{2+} as an important target for future therapeutic approaches.

We also show that Zn^{2+} acts via temporally distinct mechanisms to promote neuronal death. CaEDTA pretreatment attenuates ischemia-induced GluR2 mRNA and protein downregulation (and, by inference, expression of GluR2-lacking Zn^{2+}-permeable AMPARs), the delayed rise in Zn^{2+}, and neuronal death. These findings indicate that Zn^{2+} acts at step(s) upstream from GluR2 gene expression and implicate early Zn^{2+} in transcriptional regulation and/or mRNA stability. In CaEDTA-treated animals, ischemia induces transient GluR2 downregulation, indicating that REST and GluR2 expression return to control levels in protected cells. Early CaEDTA also blocks the release of cytochrome *c* and Smac/DIABLO from the mitochondria, an event that occurs within the first 1–2 hr after insult. These findings are consistent with observations that exposure of isolated mitochondria to Zn^{2+} *in vitro* induces release of cytochrome *c*, apoptosis-inducing factor, and Smac/DIABLO (Jiang et al., 2001). An in-



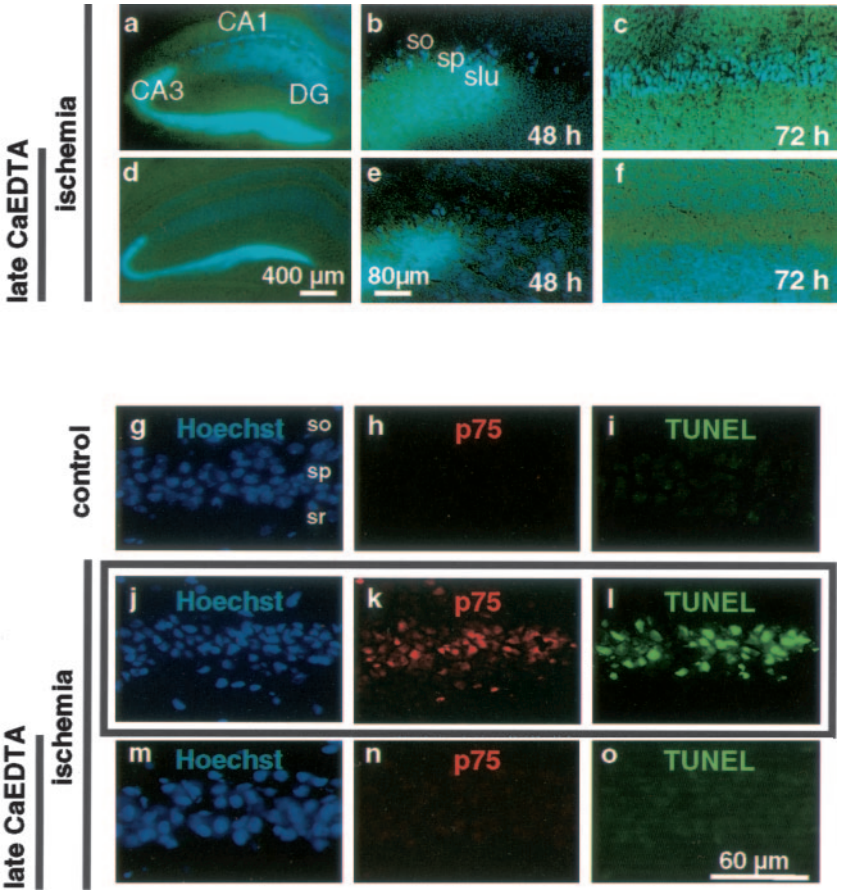


Figure 6. Late CaEDTA blocks the ischemia-induced rise in Zn^{2+} and late-stage apoptosis in CA1 neurons. Zn^{2+} fluorescence in TSQ-stained coronal brain sections from experimental animals subjected to global ischemia (*a–c*) or to global ischemia, followed by CaEDTA (300 mM, i.c.v. at 60 hr) and killed at 72 hr after reperfusion (*d–f*). Global ischemia induced a pronounced increase in Zn^{2+} fluorescence in the cell bodies of scattered hilar neurons (*a*) and CA3 pyramidal neurons (*b*). At 72 hr, Zn^{2+} fluorescence was pronounced in cell bodies of CA1 pyramidal neurons (*c*). Late injection of CaEDTA prevented the rise in Zn^{2+} fluorescence in CA1 neurons. *g–o*, Representative brain sections at the level of the dorsal hippocampus labeled with the nuclear stain Hoechst 33342 (*g*, *j*, *m*), an anti-p75^{NTR} antibody (*h*, *k*, *n*), and TUNEL (*i*, *l*, *o*) from control (*g–i*) and experimental gerbils subjected to global ischemia (*j–l*) or to global ischemia followed by CaEDTA (300 mM, i.c.v. at 60 hr) and killed at 72 hr after reperfusion (*m–o*). p75^{NTR} immunolabeling visualized in red (Texas Red), TUNEL reaction in green (fluorescein), and Hoechst-stained nuclei in blue. so, Stratum oriens; sp, stratum pyramidale; sr, stratum radiatum. Coincidence of p75^{NTR} immunolabeling and TUNEL positivity was observed in neurons throughout the CA1 pyramidal cell layer; CaEDTA injection at 60 hr after reperfusion markedly reduced p75^{NTR} expression and TUNEL. Scale bars: *a*, *d*, 400 μm; *b*, *c*, *e*, *f*, 80 μm; *g–o*, 60 μm.

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Figure 5. Early CaEDTA acts downstream of caspase-3 cleavage to block caspase-3 activity, p75^{NTR} induction, and TUNEL. Representative Western blots and relative abundance of procaspase-3 (*a*) and caspase-3 (*b*) in protein samples isolated from control (Contl) and experimental animals subjected to ischemia or CaEDTA (300 mM, i.c.v.; 30 min before ischemia), followed by ischemia and killed at 24 hr after reperfusion. Westerns were probed with an anti-caspase-3 antibody. Global ischemia induced an upregulation of procaspase-3 (*a*) and promoted its proteolytic processing to generate cleaved (activated) caspase-3 (*b*). CaEDTA did not significantly alter the ischemia-induced upregulation of procaspase-3 (*a*) or the appearance of caspase-3 (*b*). Mean band densities for procaspase-3 and cleaved (activated) caspase-3 in protein samples from the CA1 of experimental animals were normalized to the corresponding values for samples from control animals. Representative brain sections at the level of the dorsal hippocampus from animals subjected to sham operation (control; *c*, *d*), global ischemia (*e*, *f*), or CaEDTA (300 mM, i.c.v.; 30 min before ischemia), followed by ischemia and killed at 24 hr (*g*, *h*), labeled with FAM-DEVD-FMK, a fluorescein-tagged analog of zDEVD-FMK, a potent inhibitor of activated caspases. *c*, *d*, Caspase-3 activity was low in control brain. *e*, *f*, Global ischemia induced caspase-3 activity in the CA1 pyramidal cell layer, evident at 24 hr (*g*, *h*). *i*, Summary of data for animals subjected to sham operation, ischemia, or CaEDTA, followed by ischemia. Scale bars, 400 μm (low magnification); 50 μm (high magnification). Error bars represent SEM. *j–u*, Triple labeling of representative brain sections with the nuclear stain Hoechst 33342 (*j*, *m*, *p*, *s*), an anti-p75^{NTR} antibody (*k*, *n*, *q*, *t*), and TUNEL (*l*, *o*, *r*, *u*) in the CA1 pyramidal layer of control (*j*, *l*) and experimental gerbils subjected to global ischemia (*m–r*) or to CaEDTA (300 mM, i.c.v.; 30 min before ischemia), followed by ischemia and killed at 72 hr (*s–u*). *p* is the superimposition of *m* and *n*. *q* is a superimposition of *m* and *o*. *r* is a superimposition of *n* and *o*. Indicated boxes are enlarged in *p–r*. p75^{NTR} immunolabeling visualized in red (Texas Red), TUNEL in green (fluorescein), and Hoechst in blue. so, Stratum oriens; sp, stratum pyramidale; sr, stratum radiatum. Global ischemia induced p75^{NTR} expression and TUNEL positivity in CA1 pyramidal neurons; CaEDTA pretreatment reduced p75^{NTR} immunolabeling and TUNEL. Scale bars: (in *r*) *p–r*, 10 μm; (in *u*) *j–o* and *s–u*, 60 μm. Statistical significance in *a*, *b*, *i* was assessed by ANOVA, followed by Newman–Keuls test (*$p < 0.05$; **$p < 0.01$).

ferred conclusion is that CaEDTA preserves the functional integrity of the mitochondrial outer membrane. Late CaEDTA, administered at a time when GluR2 is downregulated and newly synthesized Zn^{2+}-permeable AMPARs are expressed (Pellegrini-Giampietro et al., 1997), directly chelates Zn^{2+} as it rises and prevents p75^{NTR} induction and DNA fragmentation, thereby enabling neurons to survive. These findings extend findings by others that CaEDTA administration before ischemia prevents p75^{NTR} and NADE expression (Park et al., 2000) and implicate Zn^{2+} in the end-stages of apoptosis in ischemia-induced neuronal death.

The timing issue

Global ischemia *in vivo* elicits a delayed rise in neurotoxic Zn^{2+} and onset of neuronal death in CA1 neurons 48–72 hr after insult. In contrast, the functional "half-life" of CaEDTA *in vivo* is ~90 min (Frederickson et al., 2002); thus, the duration of direct action of early CaEDTA is extinguished 48–72 hr before the rise in Zn^{2+} and death of CA1 neurons. How then does early CaEDTA prevent the late rise in neurotoxic Zn^{2+}? Findings in the present study implicate early Zn^{2+} at near-physiological concentrations in transcriptional changes and/or mRNA stability, and in the initiation of apoptotic death cascades, and raise the possibility of a causal relationship between these early events and the late rise in toxic Zn^{2+}. The observation that CaEDTA reduces the duration and extent of GluR2 protein downregulation is consistent with an additional role for Zn^{2+} in translational regulation and/or protein stability. By shortening the duration of GluR2 mRNA downregulation and reducing the duration and extent of GluR2 protein downregulation, CaEDTA would impede assembly and insertion of functional GluR2-lacking AMPARs observed at synaptic and extrasynaptic sites of postischemic CA1 neurons at late times after insult (Gorter et al., 1997). Because the delayed rise in Zn^{2+} may occur, at least in part, via GluR2-lacking AMPARs (Yin et al., 2002), CaEDTA would prevent Zn^{2+} entry into CA1 neurons. Although not addressed by the present study, an attractive hypothesis is that early CaEDTA depletes extracellular Zn^{2+} [and intracellular Zn^{2+}, which leeches out of CA1 neurons as extracellular Zn^{2+} is depleted (Li et al., 2001)] and thereby ultimately inhibits activity of REST, a nine zinc finger transcription factor that represses neural-specific targets, including GluR2. By blocking cytochrome *c* release, a key step

in the intrinsic or mitochondrial pathway of caspase activation, CaEDTA could halt the self-amplifying caspase death cascade and thereby block DNA fragmentation and apoptotic cell death.

The source of the late rise in neurotoxic Zn^{2+}

An important unresolved issue is the source of the late rise in Zn^{2+} in CA1 neurons. Early studies proposed that neurotoxic Zn^{2+} translocated from presynaptic to postsynaptic cells in response to high neuronal activity or insult (Koh et al., 1996). This notion was supported by observations that CaEDTA, a membrane-impermeant chelator of extracellular Zn^{2+} (Koh et al., 1996), and 1-naphthylacetylspermine, a selective channel blocker of GluR2-lacking AMPARs, block the ischemia-induced rise in intracellular Zn^{2+} and cell death in *in vitro* (Yin et al., 2002) and *in vivo* models of ischemia (our unpublished observations). Moreover, whereas overexpression of Ca^{2+}/Zn^{2+}-impermeable GluR2(R) channels, using shutoff-deficient Semliki Forest Virus-based vectors, blocks Ca^{2+} and/or Zn^{2+} influx and affords protection of CA1 neurons, overexpression of Ca^{2+}/Zn^{2+}-permeable GluR2 (Q) channels induces neuronal death in otherwise resistant granule neurons of the dentate gyrus (Liu et al., 2004). On the other hand, Zn^{2+} accumulates in degenerating CA1/CA3 pyramidal neurons of ZnT-3-null mice in response to kainate-elicited seizures despite the virtual absence of vesicular Zn^{2+} (Lee et al., 2000). This finding suggests that the Zn^{2+} accumulation in postischemic neurons of wild-type animals originates from sources other than synaptic vesicles as, for example, intracellular stores comprised primarily of Zn^{2+} metalloenzymes such as metallothionein and zinc finger-containing transcription factors. Consistent with this, exposure of neurons in culture to oxidative stress promotes the release of Zn^{2+} from intracellular stores, an event critical to initiation of neuronal apoptosis (Aizenman et al., 2000). Indeed, a more recent evaluation of CaEDTA reveals an ability to chelate both extracellular and intracellular Zn^{2+}, which exhibits significant mobility across neuronal membranes (Frederickson et al., 2002).

Mechanisms by which late Zn^{2+} promotes neuronal death

The molecular mechanisms by which Zn^{2+} mediates neuronal death have begun to be unraveled in the past few years, primarily from studies of isolated mitochondria and neurons in culture. Whereas relatively low concentrations of Zn^{2+} ($\sim$20 μM) elicit neuronal death with the hallmarks of apoptosis, higher concentrations of Zn^{2+} (50–100 μM) elicit neuronal death that is characteristically necrotic (Kim et al., 1999). Mechanisms by which Zn^{2+} elicits neuronal death include production of free radicals, loss of mitochondrial membrane potential, formation of the mitochondrial transition pore, dysregulation of the electron transport chain, disruption of glycolysis, production of reactive oxygen species, and reduction in cellular ATP (Choi and Koh, 1998; Weiss et al., 2000; Dineley et al., 2003; Zukin et al., 2004). The present study extends our understanding of how Zn^{2+} kills neurons in an *in vivo* model of global ischemia: early Zn^{2+} at near-physiological concentrations is necessary for the breakdown of the functional integrity of the mitochondrial outer membrane and maintained caspase activity and is critical to transcriptional changes that promote neuronal death; late Zn^{2+} at high intracellular concentrations promotes induction of the $p75^{NTR}$ death cascade, DNA fragmentation, and end-stage apoptosis.

Zinc chelation as a potential clinical strategy

Perhaps the most compelling finding of this paper is the neuroprotection provided by late CaEDTA. Late CaEDTA attenuates Zn^{2+} accumulation, $p75^{NTR}$ upregulation, and TUNEL activity in virtually all animals, yet when administered at either 48 or 60 hr, protects CA1 neurons in only approximately one-half of the animals. To explain these data, we hypothesize that Zn^{2+} initiates the final stages of death at different times in different animals. Because the action of CaEDTA lasts <90 min, CaEDTA at 48 hr might protect neurons in the subset of animals that are poised and ready to undergo irreversible, Zn^{2+}-mediated damage, but not in animals in which Zn^{2+} damage is relatively more delayed. CaEDTA at 60 hr would afford protection in animals in which damage is relatively delayed but could not rescue neurons in which irreversible damage had already occurred. Given that Zn^{2+} is required for normal function and that excessive chelation is likely to be deleterious, more complex dosage regimens clearly should be investigated. Furthermore, the possibility of buffering Zn^{2+} at different levels should be evaluated. The observation in the present study that late Zn^{2+} chelation protects neurons in a substantial fraction of animals provides a powerful impetus for these studies.

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