Accumulation of Zinc in Degenerating Hippocampal Neurons of ZnT3-Null Mice after Seizures: Evidence against Synaptic Vesicle Origin

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In several brain injury models, zinc accumulates in degenerating neuronal somata. Suggesting that such zinc accumulation may play a causal role in neurodegeneration, zinc chelation attenuates neuronal death. Because histochemically reactive zinc is present in and released from synaptic vesicles of glutamatergic neurons in the forebrain, it was proposed that zinc translocation from presynaptic terminals to postsynaptic neurons may be the mechanism of toxic zinc accumulation. To test this hypothesis, kainate seizure-induced neuronal death was examined in zinc transporter 3 gene (ZnT3)-null mice, a strain that completely lacks histochemically reactive zinc in synaptic vesicles. Intraperitoneal injection of kainate induced seizures to a similar degree in wild type and ZnT3-null mice. Staining of hippocampal sections with a zinc-specific fluorescent dye, N-((6-methoxy-8-quinolyl)-p-carboxybenzoylsulfonamide, revealed that zinc accumulated in degenerating CA1 and CA3 neurons in both groups, indicating that zinc originated from sources other than synaptic vesicles. Injection of CaEDTA into the cerebral ventricle almost completely blocked zinc accumulation in ZnT3-null mice, suggesting that increases in extracellular zinc concentrations may be a critical event for zinc accumulation. Arguing against the possibility that zinc accumulation results from nonspecific breakdown of zinc-containing proteins, injection of kainate into the cerebellum did not induce zinc accumulation in degenerating granule neurons. Taken together, these results support the existing idea that zinc is released into extracellular space and then enters neurons to exert a cytotoxic effect. However, the origin of zinc is not likely to be synaptic vesicles, because zinc accumulation robustly occurs in ZnT3-null mice lacking synaptic vesicle zinc.

Key words: TFL-Zn; neuronal degeneration; zinc transporter; kainate; cerebellum; CaEDTA

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After kainate injection, seizures were halted by intraperitoneal injection of sodium phenytoin (50 mg/kg). Features of each seizure stage are as follows: stage 1, hypoactivity; stage 2, sedation; stage 3, hyperactivity; stage 4, scratching; stage 5, loss of balance control; stage 6, tremors and generalized convulsions; and stage 7, death.

MATERIALS AND METHODS

Animals. ZnT3-null mice and their wild-type (WT) littermates were bred and maintained in the facility of University of Ulsan College of Medicine. Animals were allowed free access to food and water at 24 ± 0.5°C and exposed to 12 hr light/dark cycles. All animal experiments were performed according to the Guidelines for Laboratory Animal Care and Use (University of Ulsan). Before all experiments, genotyping for ZnT3 was performed using the PCR method as described previously (Cole et al., 1999).

Induction of seizures and scoring of seizure severity. Five WT and ZnT3-null mice were injected intraperitoneally with 40 mg/kg kainate (Tocris Cookson, Bristol, UK) dissolved in 0.9% normal saline. Separately, 1 μl of 50 mM kainate or a mixture of 100 mM ZnCl₂ and 50 mM kainate was injected into the cerebellum of ZnT3-null mice. To determine the effect of zinc chelation on kainate-induced seizure and neuronal cell death, 2 μl of 300 mM CaEDTA in saline was given stereotaxically into the lateral ventricle under anesthesia with halothane in a 1:3 mixture of O₂ and N₂O, beginning 30 min before kainate injection. For 2 hr after kainate injection, seizure severity was behaviorally estimated according to the classification of Peng et al. (1997). Two hours after kainate injection, seizures were halted by intraperitoneal injection of sodium phenytoin (50 mg/kg) (Lee et al., 2000). Features of each seizure stage are as follows: stage 1, hypoactivity; stage 2, sedation; stage 3, hyperactivity; stage 4, scratching; stage 5, loss of balance control; stage 6, tremors and generalized convulsions; and stage 7, death.

Tissue preparation and zinc-specific fluorescence staining. 24 hr after kainate injection, brain was harvested, immediately frozen in dry ice, and stored at -70°C. Coronal brain sections (10 μm thick) including the hippocampus were prepared using the cryostat and mounted on pre-chilled glass slides coated with poly-L-lysine. Unfixed brain sections were stained with N-(6-methoxy-8-quinolyl)-p-carboxybenzoylsulfonamide (TFL-Zn; Kₘ 20 μM; Calbiochem, La Jolla, CA) dissolved in Tris buffer (0.1 mM, pH 8.0) for 90 sec (Budde et al., 1997; Lee et al., 2000). After washing with saline, TFL-stained sections were examined under a fluorescence microscope (excitation, 355–375 nm; dichroic, 380 nm; barrier, 420 nm; Olympus, Tokyo, Japan) and photographed.

RESULTS

Consistent with previous studies (Frederickson et al., 1987; Koh et al., 1996), TFL-Zn staining of the hippocampus of WT mice showed that chelatable zinc was present densely in the mossy fiber terminals and sparsely in other presynaptic fibers, including stratum radiatum of CA1 (Fig. 1A). But knocking out the ZnT3 gene resulted in complete disappearance of zinc in synaptic vesicles throughout the brain (Fig. 1D), whereas it does not affect other nonvesicular zinc pools in the mouse (Cole et al., 1999).

In the present study, WT and ZnT3-null mice (n = 5 each) were intraperitoneally injected with 40 mg/kg kainate, a dose sufficient to produce severe seizures in all mice. Because ZnT3-null mice...
are more sensitive than WT mice to seizures induced by kainate (Cole et al., 2000), we chose this dose to produce comparable seizures. To lessen the mortality (Lee et al., 2000), seizures in both groups were stopped by intraperitoneally injecting sodium phenytoin 2 hr after kainate injection. Using this method, all mice developed seizures with similar time course and severity, as estimated by the behavioral seizure severity scores of Peng et al. (1997) (Fig. 2A).

Staining brain sections of seizure-experienced mice 24 hr later with a zinc-specific fluorescent dye, TFL-Zn, revealed dense zinc accumulation in neuronal cell bodies and somewhat increased TFL-Zn fluorescence in strata radiatum and oriens of not only WT (Fig. 1B) but also ZnT3-null mice (five of five mice for each; Fig. 1E). The TFL-Zn fluorescence of ZnT3-null mice was abolished by treatment with the zinc chelator–remover dithizone (Fig. 1G). Staining with the TUNEL method or with hematoxylin and eosin (Cole et al., 2000) revealed neuronal death in densely TFL-Zn fluorescent cells in both WT and ZnT3-null mice. Interestingly, whereas death of CA3 neurons in ZnT3-null mice was less than that in WT mice, death of CA1 neurons in ZnT3-null mice was markedly enhanced compared with WT mice (Fig. 1C,F). Counting the number of zinc-accumulating neurons and acid-fuchsin-stained neurons in CA1 and CA3 confirmed this impression (Fig. 2B,C).

Next, we examined the possibility that cytosolic zinc in WT and ZnT3-null mice originates from nonspecific release of zinc from degrading zinc-containing proteins. Arguing against this possibility, injection of kainate into the cerebellum resulted in death, but not TFL-Zn staining, of granule neurons in both WT and ZnT3-null mice (Fig. 3A). Only when zinc was given with kainate did TFL-Zn fluorescence appear in most TUNEL(+) cerebellar granule neurons (Fig. 3B). These results are consistent with the previous report that zinc is not implicated in kainate-induced granular cell death in the cerebellum (Frederickson et al., 1989).

To further examine whether toxic zinc accumulation originates from outside the neurons, a cell membrane-impermeant zinc chelator was used. Intraventricular injection of CaEDTA had little effect on kainate-induced seizure severity in ZnT3-null mice (five of five mice; Fig. 2A) but markedly attenuated both zinc accumulation and neuronal death in the hippocampus (Figs. 2, 4), favoring the external origin for toxic zinc accumulation even in ZnT3-null mice.

**DISCUSSION**

Although vesicular zinc is completely absent in ZnT3-null mice, dense TFL-Zn fluorescence develops in degenerating hippocampal neurons after kainate-induced seizures. This result indicates that the main origin of zinc responsible for the TFL-Zn fluorescence after brain injury in ZnT3-null mice is not the histochemically reactive zinc stored in ZnT3-containing synaptic vesicles. Although the present study cannot completely exclude the possibility that certain adaptations in ZnT3-null mice, such as alteration of metallothionein levels or other events related to zinc homeostasis, may underlie the zinc accumulation in degenerating neurons, overall our results suggest the nonsvesicular origin of zinc also in WT animals after brain injuries (Frederickson et al., 1988, 1989; Tonder et al., 1990; Koh et al., 1996; Suh et al., 2000).

Where does zinc accumulating in degenerating neurons of ZnT3-null mice come from, if not synaptic vesicles? The present study does not provide a specific answer to this question. However, overall it suggests the external origin (i.e., from outside of zinc accumulating neurons) of zinc based on the following results. First, injection of kainate into the cerebellum resulted in death of granule neurons but no TFL-Zn fluorescence in them in either WT or ZnT3-null mice, arguing against the possibility that zinc is nonspecifically released from degraded zinc-containing proteins. Only when zinc was given with kainate did the fluorescence appear in the TUNEL(+) granule cells. Corroborating this, Frederickson et al. (1989) reported that zinc is not implicated in kainate-induced neuronal cell death in the cerebellum. These results are consistent with previous findings in cortical culture that excitotoxic, oxidative, or apoptotic injury is not associated with zinc fluorescence unless zinc is added to the exposure medium (Koh et al., 1996). Second, intraventricular injection of a
membrane-impermeant chelator, CaEDTA, blocks zinc accumulation and neuronal death after seizures, ischemia, or trauma (Koh et al., 1996; Cuajungco and Lees, 1998a; Lee et al., 2000; Suh et al., 2000). Also in ZnT3 null mice, CaEDTA nearly completely abolished zinc accumulation and neuronal cell death. These observations favor an external origin of zinc, which implies transient increases in extracellular zinc concentrations.

Although neuronal excitation could release a histochemically invisible, ZnT3-independent pool of zinc, another possibility is that zinc may be released intracellularly and then pumped out by transporters such as zinc-efflux transporter 1 (ZnT1) (Palmiter and Findley, 1995; McMahon and Cousins, 1998), raising local extracellular zinc concentrations. ZnT1 is readily induced in the hippocampus in response to insults such as ischemia (Tsuda et al., 1997). Once released to the extracellular space, the zinc would be available for uptake into vulnerable postsynaptic neurons via open ion channels (Frederickson et al., 1989; Koh et al., 1996). However, although the complete blockade of zinc accumulation and cell death by CaEDTA makes an external origin more likely, the present study cannot completely rule out the alternative possibility that zinc accumulation is an intrinsic event. For example, it seems plausible that hippocampal neurons in vivo may have a mechanism for internal release of zinc (Cuajungco and Lees, 1998b), a feature not shared by cortical neurons in vitro or cerebellar granule neurons in vivo, and that extracellular CaEDTA somehow drives intracellular zinc out of these neurons.

Regardless of which of these possible mechanisms is operational, zinc accumulation is likely a cause rather than a sequel of neuronal death, because its blockade protects against neuronal death. However, contrary to the current zinc translocation hypothesis (Frederickson, 1989; Choi and Koh, 1998; Lee et al., 1999), the present results obtained from ZnT3-null mice suggest that synaptic vesicle zinc may not be the principal source of toxic zinc accumulation. Further studies seem warranted to elucidate the detailed dynamics of zinc homeostasis in brain injury.

REFERENCES


