Zinc and 4-Hydroxy-2-Nonenal Mediate Lysosomal Membrane Permeabilization Induced by H₂O₂ in Cultured Hippocampal Neurons

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Lysosomal membrane permeabilization (LMP) is implicated in cancer cell death. However, its role and mechanism of action in neuronal death remain to be established. In the present study, we investigate the function of cellular zinc in oxidative stress-induced LMP using hippocampal neurons. Live-cell confocal microscopy with FluoZin-3 fluorescence showed that H₂O₂ exposure induced vesicles containing labile zinc in hippocampal neurons. Double staining with LysoTracker or MitoTracker disclosed that the majority of the zinc-containing vesicles were lysosomes and not mitochondria. H₂O₂ additionally augmented the 4-hydroxy-2-nonenal (HNE) adduct level in lysosomes. Intracellular zinc chelation with TPEN [tetrakis(2-pyridylmethyl)ethylenediamine] completely blocked both HNE accumulation and neuronal death. Interestingly, within 1 h after the onset of H₂O₂ exposure, some of zinc-loaded vesicles lost their zinc signals. Consistent with the characteristics of LMP, a lysosomal enzyme, cathepsin D, was released into the cytosol, and cathepsin inhibitors partially rescued neuronal death. We further examined the possibility that HNE or zinc mediates H₂O₂-triggered LMP. Similar to H₂O₂, exposure to HNE or zinc triggered lysosomal zinc accumulation and LMP. Moreover, isolated lysosomes underwent LMP when exposed to HNE or zinc, but not H₂O₂, supporting the direct mediation of LMP by HNE and/or zinc. The appearance of zinc-containing vesicles and the increases in levels of cathepsin D and HNE, were also observed in hippocampal neurons of rats after kainate seizures. Thus, under oxidative stress, neuronal lysosomes accumulate zinc and HNE, and eventually undergo LMP, which may constitute a key mechanism of oxidative neuronal death.

Key words: lysosome; oxidative stress; cathepsin; Alzheimer's disease; neurotoxicity; kainic acid; seizure

Introduction

Accumulating evidence suggests that the aberrant generation of reactive oxygen species (ROS) and reactive nitrogen species underlies neuronal degeneration in diverse neurological conditions (Love and Jenner, 1999; Butterfield et al., 2001; Perez-Pinzon et al., 2005; Smith et al., 2007). Elucidation of the mechanism of oxidative neuronal death is critical to improve our understanding of the pathogenesis of neurodegenerative conditions.

Zinc, a potential endogenous trigger of oxidative stress in the brain, is abundant in glutamatergic vesicles of the forebrain association pathways, and released with neuronal activity or depolarization (Frederickson et al., 2005). Several studies show that exposure of neuronal cultures to zinc (in micromolar concentrations) triggers oxidative cell death (Kim et al., 1999;

Noh et al., 1999; Park and Koh, 1999; Noh and Koh, 2000; Kim and Koh, 2002). Endogenous zinc is a recognized mediator of neuronal death after ischemia, trauma, or seizures (Koh et al., 1996; Choi and Koh, 1998; Suh et al., 2000; Frederickson et al., 2005).

To clarify the mechanism of zinc-induced neuronal death, it is essential to understand the mechanism of regulation of intracellular zinc levels. To date, a number of zinc transporters have been identified (Kambe et al., 2004; Cousins et al., 2006), but little is known about zinc homeostasis. Recently, nonsynaptic vesicles containing labile zinc were identified in yeast (Devirgiliis et al., 2004; Eide, 2006), astrocytes (Varea et al., 2006), and neurons (Sensi et al., 2003; Colvin et al., 2006). It is speculated that zinc-containing vesicles function as a reservoir for zinc to mitigate its potential toxic effect. In astrocytes, zinc-containing vesicles are possibly derived from lysosomes (Varea et al., 2006), whereas zinc-containing vesicles in neurons are stained with markers for endosomes (Danscher and Stoltenberg, 2005; Colvin et al., 2006). However, Sensi et al. (2003) demonstrated that mitochondria dynamically take up and release zinc. Thus, it appears that zinc-containing vesicles are derived from various organelles.

In some cases, intracellular organelles, such as mitochondria and lysosomes, contribute to oxidative cell death. Mitochondria are the main generators of ROS, which induce mitochondrial

Received Oct. 16, 2007; revised Feb. 4, 2008; accepted Feb. 6, 2008.

This work was supported by the Korea Science and Engineering Foundation through the National Research Laboratory Program (M10600000181-06J0000-18110), by the Brain Research Center of 21st Century Frontier Research Program (M103KV010020-06K2201-02010) funded by Korean Ministry of Science and Technology, and by the Korea Research Foundation (Grant MOEHRD, KRF-2005-084-C00026) funded by the Korean Ministry of Education and Human Resources Development.

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DOI:10.1523/JNEUROSCI.0199-08.2008

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Figure 1. Changes in intracellular zinc with H_2O_2 : formation of zinc-containing vesicles from lysosomes and LMP. **A**, Confocal live-cell images with FluoZin-3 fluorescence of cultured hippocampal neurons at the indicated time points after the addition of 100 μM H_2O_2 to the medium. Zinc signals in round organelles (arrowheads) appeared at 10 min and increased with time. Images were taken from z-series collections (25 images of 1 μm thickness). Scale bar, 10 μm . **B**, Confocal live-cell images of 100 μM H_2O_2 -treated hippocampal neurons stained with FluoZin-3 (green) and LysoTracker (red). The merged photo shows overlap of FluoZin-3 and LysoTracker signals (yellow). Images were obtained from a single z-section of 1 μm thickness. Scale bar, 5 μm . **C**, H_2O_2 -treated neurons were stained with FluoZin-3 (green) and MitoTracker (red). The merged photograph discloses little overlap between zinc and mitochondrial signals. Images were taken from a single z-section. Scale bar, 5 μm . **D**, Confocal live-cell images of 100 μM H_2O_2 -treated hippocampal neurons. Images were taken from z-series collections (25 images). Consistent with the possibility that some zinc-containing vesicles lose their membrane integrity, zinc signals were lost at later time points. Zinc-containing vesicles appeared well demarcated at 20 min after 100 μM H_2O_2 exposure (left), but some of zinc-containing vesicles were no longer visible at 40 min (right; arrows). Scale bar, 10 μm .

dysfunction (Trushina and McMurray, 2007). In turn, mitochondrial dysfunction leads to higher ROS production, establishing a destructive cycle. Moreover, mitochondria may actively participate in apoptosis by releasing various regulatory proteins

(Vila and Przedborski, 2003). On the other hand, lysosomes contain various acidic hydrolases, and serve as the main site for macromolecular degradation. Recent studies implicate lysosomal membrane permeabilization (LMP) as a key mechanism in various types of cell death (Kroemer and Jaattela, 2005; Blomgran et al., 2007; Stoka et al., 2007). However, LMP in neurons has not been investigated in detail.

In the present study, we examined whether (1) oxidative stress induced by H_2O_2 alters zinc homeostasis, including zinc-containing vesicle formation; (2) mitochondria or lysosomes are the origin of zinc-containing vesicles; (3) LMP occurs after H_2O_2 exposure; (4) LMP contributes to oxidative neuronal death; and (5) lysosomal zinc accumulation and LMP occur in the rat brain after kainate seizures.

Materials and Methods

Cell culture. Hippocampal neurons were prepared from fetal mice (embryonic days 14–16). Briefly, dissociated hippocampal cells were plated onto a poly-L-lysine and laminin-coated cover glasses or culture wells in a plating medium (Neurobasal medium supplemented with 0.5 mM L-glutamine, B-27 supplement, and 25 mM glutamic acid). Cytosine arabinoside (10 μM) was added 3–4 d after the plating to halt the growth of non-neuronal cells. The cultures were used for experiment at days *in vitro* 7–9. All culture reagents were purchased from Invitrogen (Carlsbad, CA).

Live-cell confocal microscopy. Hippocampal cultures or fractionated lysosomes were stained with 5 μM FluoZin-3-AM, 75 nM LysoTracker Red DND-99, and 0.5 μM MitoTracker Red CM-H₂XROS (Invitrogen) dye in MEM for 5–30 min in a CO₂ incubator, and transferred to HBSS. For nuclear staining, cells were incubated with 5 μM DRAQ-5 (Invitrogen) for 5 min. Live-cell confocal images were obtained using an Ultra View Confocal Live Cell Imaging System (PerkinElmer, Waltham, MA) with a Nikon (Melville, NY) ECLIPSE TE2000 microscope. Fluorescence intensity was measured in arbitrary units.

Quantification of cell death (lactate dehydrogenase release). Neuronal cell injury in mixed cortical cultures was quantitatively assessed by measuring lactate dehydrogenase (LDH) activity released from damaged cells into the culture medium (Koh and Choi, 1987). Each LDH value was subtracted by the mean background value in sister cultures subjected to a sham wash only (=0%) and scaled to the mean value in sister cultures after 18 h exposure to 100 μM H_2O_2 , 30 μM 4-hydroxy-2-nonenal (HNE), or 35 μM zinc (=100%).

Western blots for cathepsin D. For preparation of the released cathepsin D from lysosomes to the cytosol, we extracted cytosolic proteins using digitonin (Sigma-Aldrich, St. Louis, MO). In brief, cytosolic proteins were extracted with extraction buffer (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, and 1 mM EGTA) containing 25 $\mu g/ml$ digitonin by rocking (100 rpm) on ice for 15 min. After the removal of extraction buffer, protein was precipitated with 10% TCA, washed with methanol, and lysed with lysis buffer (20 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM

Figure 2. H_2O_2 enhances immunochemical reactivity to HNE adducts in lysosomes in a zinc-dependent manner. Cell death induced by H_2O_2 is attenuated by TPEN or cathepsin inhibitors. **A–E**, Cultures were immunostained with an antibody specific for HNE adducts. The HNE adduct level was low in control (CTL) neurons (**A**) but substantially increased at 30 min after 100 μM H_2O_2 exposure (**B**). Double staining with anti-Lamp-1 antibody (**C**) revealed that most HNE adduct immunoreactivity was concentrated in or around lysosomes (merged; **D**). The cell-permeant zinc chelator, 500 nM TPEN, blocked the 100 μM H_2O_2 -induced increase in HNE adducts (**E**). Confocal images were taken from a single z-section. Scale bar, 5 μm . **F**, The bars denote percentage LDH release (mean ± SEM; $n = 6$) 18 h after treatment with 100 μM H_2O_2 alone or with addition of 500 nM TPEN, 30 min before or after the onset of H_2O_2 treatment (** $p < 0.01$, difference from H_2O_2 alone). **G**, The bars denote percentage LDH release (mean ± SEM; $n = 5$) 18 h after treatment with 100 μM H_2O_2 alone or with addition of 0.1 μM CBI or 150 nM PA. CBI was added 30 min before, and PA was added 30 min before or after the onset of H_2O_2 treatment (** $p < 0.01$ and * $p < 0.05$, difference from H_2O_2 alone). **H**, **I**, Confocal live-cell images of FluoZin-3-stained hippocampal neurons 30 min after addition of 100 μM H_2O_2 (**H**) or 100 μM H_2O_2 plus 150 nM PA (**I**). Images were taken from z-series collections (25 images). Scale bar, 10 μm .

EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 μM Na_3VO_4 , 1 $\mu g/ml$ leupeptin, and 1 mM PMSF). For Western blots, equal amounts of protein were loaded for electrophoresis on a 10% SDS-PAGE, and transferred to polyvinylidene difluoride membranes. Immunoblots were visualized using enhanced chemiluminescence (Pierce, Rockford, IL) and analyzed by densitometry for quantitative measurement of protein expression intensity. The relative expression levels were presented as the ratio of the cathepsin D density to the corresponding β -actin density.

Immunocytochemistry. Cells were fixed with 4% paraformaldehyde for 1 h and permeabilized with 0.2% Triton X-100 for 15 min. After blocking with bovine serum albumin, immunocytochemistry was performed with anti-HNE (Alpha Diagnostic, San Antonio, TX), anti-lysosome-associated membrane protein 1 (Lamp-1) (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-cathepsin D (Santa Cruz Biotechnology) antibodies, and Alexa Fluor-conjugated secondary antibodies (Invitrogen).

Preparation of lysosomal-enriched fractions. Lysosomal-abundant fractions were prepared from cultured primary hippocampal neurons using

a Lysosome Enrichment kit (product no. 89839; Pierce). In brief, collected cells were centrifuged at $850 \times g$ for 2 min. The supernatant fractions were discarded, and lysosome enrichment reagent A was added to pellets. Next, pellets were vortexed at medium speed for 5 s and incubated on ice for 2 min. Suspensions were sonicated on ice and treated with an equal volume of lysosome enrichment reagent B. The mixtures were gently inverted several times and centrifuged at $500 \times g$ for 10 min at 4°C. Supernatant fractions were collected in a new tube. Gradients (17–30%) were prepared, and a mixture (15%) of saved supernatant fractions and medium was loaded onto the 17% gradient layer, followed by ultracentrifugation at $145,000 \times g$ for 2 h at 4°C. The top band was isolated as a lysosomal fraction for experiments.

Animals and seizure induction. The animal experiment protocol was approved by the Internal Review Board for Animal Experiments of Asan Life Science Institute, University of Ulsan College of Medicine (Seoul, Korea). Adult male Sprague Dawley rats (8 weeks of age; 240–270 g) were maintained under 12 h light/dark cycles. Control and seizures were induced by intraperitoneal injection without or with 10 mg/kg of kainic

Figure 3. Alterations in zinc-containing vesicles after HNE treatment. Live-cell confocal images of FluoZin-3-stained hippocampal neurons at the indicated times after exposure to 300 μ M HNE. Zinc signals in some of zinc-containing vesicles disappeared at 30 min (arrows). Images were obtained from z-series collections (25 images). Scale bar, 10 μ m.

acid (Tocris Bioscience, Bristol, UK) dissolved in normal saline. Two and one-half hours later, the seizures were halted by intraperitoneal injection of Na phenytoin (50 mg/kg). Seizure behavior after kainate injection was staged according to the classification system of Zhang et al. (1997).

Tissue preparation and zinc-specific fluorescence (FluoZin3-AM) staining. Brains were harvested 3 and 8 h after kainate injection, respectively, frozen immediately on dry ice, and stored at -70°C . Coronal brain sections (10 μ m thick) were prepared using a cryostat and mounted onto prechilled glass slides coated with poly-L-lysine. Prepared brain sections were stained with 25 μ M FluoZin-3-AM (Invitrogen) dye in PBS for 30 min at 37°C incubator, washed with PBS, for nuclear staining, restained with Hoechst 33342 (Invitrogen) in PBS for 2 min at room temperature, and washed with PBS for removing the remaining dye. Brain sections were photographed using a confocal microscope (TCS-SP2; Leica, Nussloch, Germany).

Immunohistochemistry. Brain sections were immunostained with anti-HNE antibody (Alpha Diagnostic) or anti-cathepsin D antibody (Santa Cruz Biotechnology). Briefly, brain sections were fixed with 4% paraformaldehyde and blocked with 3% bovine serum albumin and 0.1% Triton X-100 in PBS, pH 7.4. After incubation with the primary antibody at 4°C overnight, the sections were reacted with Alexa Fluor 555-conjugated secondary antibody (Invitrogen). The stained tissues were examined and photographed under a CCD camera (DP70; Olympus, Tokyo, Japan).

Statistics. All data are presented as mean $\pm$ SEM. The paired *t* test was used to analyze differences between two groups. Values of $p < 0.05$ were considered statistically significant.

Results

H₂O₂ induces zinc-containing vesicles, some of which eventually disintegrate

Before H₂O₂ exposure, cytosolic zinc levels were low, and few zinc-containing vesicles were present in control hippocampal neurons (Fig. 1A). Notably, some faint zinc fluorescence was detected in perinuclear Golgi-like structures (Fig. 1A, arrows), consistent with previous reports (Kirschke and Huang, 2003; Chi et al., 2006). After H₂O₂ exposure, round zinc-containing vesicles appeared (arrowheads), accompanied by an increase in zinc fluorescence in the cytosol and nuclei (Fig. 1A). Double staining of H₂O₂-treated hippocampal neurons with FluoZin-3 and LysoTracker revealed that almost all newly formed zinc-containing vesicles were enlarged lysosomes and/or late endosomes (Fig.

1B). In contrast, we observed little overlap between zinc-containing vesicle and mitochondrial fluorescence (Fig. 1C). Interestingly, at ~ 40 min after H₂O₂ exposure, FluoZin-3 fluorescence loss was evident from some of zinc-containing vesicles, indicative of disintegration (Fig. 1D, arrows).

H₂O₂ enhances the HNE adduct level in lysosomes

Because zinc-containing vesicles are mostly lysosomes, their breakdown may be a consequence of LMP. Aldehyde by-products of lipoperoxidation, particularly HNE, cause lysosomal disruption (Kopitz et al., 2004). Accordingly, we examined whether H₂O₂ affects the levels of HNE adducts in hippocampal neurons. Immunocytochemical staining of hippocampal neurons exposed to H₂O₂ disclosed substantially enhanced HNE adduct levels in the cytosol, compared with control neurons (Fig. 2A,B). To determine whether these HNE adducts accumulate in lysosomes,

cultures were double-stained with antibodies against HNE and Lamp-1. Interestingly, we observed a substantial overlap between HNE adduct and Lamp-1 staining (Fig. 2B–D). Consistent with the role of zinc in HNE adduct accumulation in lysosomes, an intracellular zinc chelator, tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), markedly suppressed the HNE adduct levels in H₂O₂-treated neurons (Fig. 2E). Furthermore, TPEN added 30 min before the onset of H₂O₂ treatment, almost completely blocked H₂O₂-induced neuronal death (Fig. 2F), implying that both intracellular zinc and HNE are necessary for H₂O₂-induced neurotoxicity. Consistent with the theory that LMP contributes to H₂O₂-induced neuronal death, pretreatment with blockers of lysosomal enzymes, cathepsin B inhibitor (CBI) and pepstatin A (PA), partially attenuated cell death in the presence of hydrogen peroxide (Fig. 2G). However, TPEN or PA added 30 min after the onset of H₂O₂ treatment, when LMP had started, did not reduce H₂O₂-induced neuronal death (Fig. 2F,G). These results suggest that zinc chelators or lysosomal enzyme inhibitors may be unable to rescue neurons when they are given late in the toxic cascade. Next, we examined whether a cathepsin inhibitor prevents intracellular zinc increase by H₂O₂. Addition of 150 nM PA (cathepsin D inhibitor) did not attenuate the increase in zinc signals in the cytosol of H₂O₂-treated neurons (Fig. 2H,I), ruling out the possibility that cathepsin D causes the rise of cytosolic zinc levels by degrading zinc-binding proteins.

HNE induces zinc-containing vesicles and enhances zinc levels in the cytosol and nuclei

Because levels of HNE adducts increased during H₂O₂-induced neurotoxicity, and aldehydes, such as HNE, can induce LMP, we examined the effects of HNE on zinc-containing vesicles and intracellular zinc levels. Similar to H₂O₂, exposure to HNE induced zinc-containing vesicles in most neurons (Fig. 3) and a simultaneous increase in the cytosolic zinc levels. Consistently, live-cell confocal microscopy with FluoZin-3 and LysoTracker revealed that most zinc-containing vesicles formed by HNE were lysosomes and/or late endosomes, but not mitochondria (data

not shown). At ~ 30 min after HNE exposure, some zinc-containing vesicles exhibited much reduced zinc signals, suggesting disruption or membrane permeabilization (Fig. 3, arrows). However, zinc fluorescence continued to rise in the cytosol.

HNE triggers LMP in hippocampal neurons

We further examined whether HNE exposure is associated with LMP in hippocampal neurons. To determine whether LMP occurs after HNE exposure, lysosomes were examined with LysoTracker fluorescence under a live-cell confocal microscope. The number of lysosomes significantly decreased after 30 min of HNE exposure, clearly suggestive of LMP (Fig. 4A, arrowheads). As observed with H_2O_2 neurotoxicity, direct exposure of hippocampal neurons to HNE rapidly induced the accumulation of HNE adducts in lysosomes (Fig. 4B). In addition, immunocytochemical staining for cathepsin D, a lysosomal acidic hydrolase, was altered from a particulate, organelle-associated pattern to a more diffuse, cytosolic pattern after HNE treatment (Fig. 4C). The release of cathepsin D into the cytosol was further confirmed by immunoblots of the cytosolic fraction (Fig. 4D,E). Similar to H_2O_2 , HNE neurotoxicity was significantly (albeit to a lesser extent) attenuated by TPEN, a zinc chelator [LDH release, $49.2 \pm 0.68\%$ (SEM; $n = 4$) of control; $p < 0.01$]. Moreover, cathepsin inhibitors suppressed HNE-induced neuronal death (Fig. 4F). Our findings collectively imply that intracellular zinc and LMP contribute to HNE-induced neuronal death.

Zinc exposure induces accumulation of zinc and HNE in lysosomes, resulting in LMP

Significant changes in zinc-containing vesicles were detected on direct exposure to zinc (Fig. 5A). After treatment with $500 \mu M$ zinc, the zinc signals were rapidly increased in zinc-containing vesicles, followed by cytosol and nuclei. However, in contrast to HNE and H_2O_2 , zinc treatment additionally triggered dramatic focal swelling of neurites. All swollen neurites contained high levels of zinc. Over time, zinc-containing vesicles rapidly lost their zinc signals (within a 2 min interval) (Fig. 5B, arrow; supplemental movie, available at www.jneurosci.org as supplemental material), but the focal zinc-containing swollen neurites continued to expand (Fig. 5A). Immunocytochemical staining and Western blots with cathepsin D antibody disclosed release of the enzyme to the cytosol after zinc treatment, characteristic of LMP (Fig. 5C,D). Densitometry of Western blots showed that cytosolic levels of cathepsin D, as normalized to β actin, were significantly greater than those in controls [1.35 ± 0.02 -fold (SEM; $n = 3$);

Figure 4. HNE induces LMP. **A**, Confocal images of hippocampal neurons stained with LysoTracker before (left) and 30 min after addition of medium (control; top) or $300 \mu M$ HNE (bottom). Some of the lysosomes (arrowheads) disappeared. Images were obtained from z-series collections. Nuclei were stained with DRAQ-5 (blue). **B**, Images show that treatment with $300 \mu M$ HNE for 30 min enhanced the level of immunoreactivity to HNE adducts in neurons. Double staining with anti-Lamp-1 antibody shows that HNE adducts accumulate mainly in lysosomes. Confocal images were taken from a single z-section. **C**, Release of cathepsin D (Cat-D), a lysosomal enzyme, in neurons after 15 min exposure to $300 \mu M$ HNE. Confocal images were obtained from z-series collections. Scale bars: **A–C**, $10 \mu m$. **D**, Western blots for cathepsin D in the cytosolic fraction. At 15 min after $300 \mu M$ HNE treatment, the cytosolic cathepsin D level was increased, compared with that in the control. β -Actin immunoblots are used as controls. **E**, Western blots were quantified by densitometry. The bars denote the ratio of cathepsin D bands to corresponding β -actin bands, normalized to the ratio in control as 1 (mean $\pm$ SEM; $n = 3$; ** $p < 0.01$, difference from controls). **F**, Percentage LDH release in cultures (mean $\pm$ SEM; $n = 3$) after 18 h exposure to $30 \mu M$ HNE alone or with the addition of $0.1 \mu M$ CBI or 150 nM PA (* $p < 0.05$ and ** $p < 0.01$, difference from HNE).

$p < 0.01$]. Similar to H_2O_2 and HNE, zinc-induced neuronal death was significantly reduced with CBI and PA (Fig. 5E).

Zinc and HNE induce LMP in isolated lysosomal preparations

Next, we examined whether H_2O_2 , HNE, or zinc alone directly disrupts lysosomes *in vitro*. Lysosome-enriched fractions were prepared as described in Materials and Methods, and stained with LysoTracker. The estimated lysosome number was stable for 30 min. Addition of $100 \mu M$ H_2O_2 in solution did not alter the number of lysosomes within 30 min (Fig. 6A,B). In contrast, treatment of isolated lysosomes with HNE led to a gradual decrease in number (Fig. 6C,D). Similarly, lysosomes treated with zinc were decreased in number (Fig. 6E,F). Accordingly, we propose that an increase in zinc and/or HNE levels in lysosomes contributes to LMP in neurons under conditions of oxidative stress.

Figure 5. Exposure to zinc augments the zinc levels in cytosol and lysosomes. **A**, Live-cell confocal micrographs of FluoZin-3-stained hippocampal neurons at the indicated time points after the addition of 500 μ M zinc to the medium. Images were obtained from z-series collections. Scale bar, 10 μ m. **B**, Live-cell confocal micrograph of a hippocampal neuron at 48 and 50 min after the onset of 500 μ M zinc exposure. Note the disappearance of a zinc-containing vesicle (white arrow) and persistence of one next to it (gray arrow). Images were from z-series collections. Scale bar, 5 μ m. **C**, Hippocampal neurons, sham wash control (left) and 60 min after 500 μ M zinc treatment (right), were stained with anti-cathepsin D antibody. Note more diffuse pattern of staining in the zinc-treated neuron. Images were from z-series collections. Scale bar, 5 μ m. **D**, Western blots for cathepsin D in the cytosolic fraction. At 60 min after 500 μ M zinc treatment, cytosolic cathepsin D increased, compared with that in the control. Immunoblots for β -actin are used as controls. **E**, The bar denotes percentage LDH release (mean $\pm$ SEM; $n = 4$) in cultures after 18 h exposure to 35 μ M zinc or zinc plus 0.1 μ M CBI or 150 nM PA (** $p < 0.01$, difference from zinc control).

Kainate seizures induce zinc-containing vesicles in hippocampal neurons *in vivo*

Finally, we examined whether zinc-containing vesicles appeared in an *in vivo* model of acute neuronal injury. For this, we turned to the kainate model for epileptic brain damage. After intraperitoneal kainate injection (10 mg/kg), rats underwent gradual progression to generalized convulsive seizures, as previously described in Materials and Methods. Brains were obtained at 3 and 8 h after the kainate injection. Confocal images of FluoZin-3-stained hippocampal neurons showed that zinc-containing vesicles appeared at 3 h after the kainate injection (Fig. 7A). Later, zinc signals intensified greatly, covering the whole cytosol, but sparing nuclei. In addition, kainate seizures increased levels of cathepsin D and HNE in hippocampal neurons (Fig. 7B,C). Of note, cathepsin D immunoreactivity appeared more diffuse in

neurons of the kainate group. These results suggest the possibility that lysosomal zinc accumulation and LMP may also occur in the brain subjected to acute insults.

Discussion

Although oxidative stress unquestionably underlies cell death in several pathological conditions (Love and Jenner, 1999), the molecular mechanisms involved are complex and variable. For instance, oxidative stress causes DNA damage, which in turn activates poly(ADP-ribose)polymerase (PARP), resulting in NAD/ATP depletion and cell necrosis (Kaundal et al., 2006). Another prominent pathway involves mitochondrial damage, cytochrome *c* release, and caspase-dependent apoptosis (Cheung et al., 2005). Recent evidence suggests that LMP and release of acidic hydrolases into the cytosol may constitute yet another pathway of oxidative cell death (Kroemer and Jaattela, 2005; Blomgran et al., 2007; Castino et al., 2007; Stoka et al., 2007). The first two mechanisms have been extensively investigated in the nervous system, whereas the third has not been analyzed in detail in neurons. One report showed that lysosomal damage occurs in postischemic CA1 neurons (Yamashima et al., 2003).

Results obtained with cultured hippocampal neurons show that LMP occurs in neuronal death induced by H₂O₂ and HNE. On exposure to H₂O₂, lysosomes lose their membrane integrity, as evident from the loss of zinc and lysosomal signals and cytosolic release of cathepsin D, a lysosomal acidic hydrolase. Interestingly, increased levels of proteins modified by HNE, a by-product of lipoperoxidation, were detected in H₂O₂-treated hippocampal neurons, particularly in association with lysosomes.

Accumulating evidence shows that HNE is a key endogenous neurotoxin produced under oxidative stress (Trevisani et al., 2007). Levels of HNE are elevated in Alzheimer's disease brains and amyotrophic lateral sclerosis spinal cords (Pedersen et al., 1998; Volkel et al., 2006; Williams et al., 2006). Exposure of cultured neurons to HNE triggers cell death via LMP (Castino et al., 2007). Moreover, toxic aldehydes, such as HNE, react with various proteins and cause their dysfunction (Yoritaka et al., 1996; Sayre et al., 2006). HNE-modified proteins may accumulate in lysosomes, leading to lysosomal stress and LMP. Hence, it is possible that the production of HNE is a key step in H₂O₂-induced LMP and neuronal death. Consistent with this theory, our results show that exposure of HNE induces LMP. In addition, the levels of zinc increased in lysosomes, as with H₂O₂ exposure. In view of the finding that cathepsin inhibition suppresses HNE-induced neuronal death, we propose that LMP contributes to HNE neurotoxicity.

The key roles of HNE and zinc in LMP were further supported

by experiments on isolated lysosomes *in vitro*. High concentrations of zinc resulted in the disappearance of some lysosomes. Furthermore, exposure to HNE led to lysosome bursting. In contrast, H_2O_2 did not cause LMP in the isolated lysosomal preparation. These *in vitro* data support the cellular finding that accumulation of zinc and HNE in lysosomes stimulates LMP in H_2O_2 -induced neurotoxicity. Although the importance of LMP in cell death is established, the exact mechanism remains unclear. Zinc and HNE accumulation may be a triggering mechanism, at least in primary hippocampal neurons. Additional studies are required to examine this possibility in cancer cells.

LMP causes the release of various acidic hydrolases into the cytosol. Inhibition of these enzymes is often cytoprotective in the case of LMP-related cell death (Kroemer and Jaattela, 2005; Kurz et al., 2006). In our experiments, H_2O_2 and HNE induced LMP and released cathepsin D into the cytosol. Inhibitors of cathepsin B or D partially blocked neuronal death in both cases, consistent with the contribution of cathepsins to neuronal death. However, the role of other acidic hydrolases was not analyzed in the present study. Broad-spectrum lysosomal enzyme inhibitors may provide enhanced protection against LMP-related neuronal death.

It is unclear how zinc accumulation in lysosomes occurs under oxidative stress. As shown by Aizenman et al. (2000), zinc is initially released to the cytosol from oxidation-sensitive zinc-binding proteins, such as metallothioneins. An interesting possibility is that lysosomes also contain some labile zinc-binding proteins that rapidly release the metal cofactor. Alternatively, cytosolic zinc may preferentially be taken up by lysosomes, which function as the first-line defense system for elevated zinc levels. In the latter case, the machinery for zinc entry to lysosomes (transporters or ion channels) requires identification. In fact, our finding that zinc treatment enhancing intracellular levels also induces zinc accumulation in lysosomes favors the latter possibility that zinc enters lysosomes from the cytosol.

HNE, a lysosomotropic toxin (Crabb et al., 2002), accumulates HNE-modified proteins in lysosomes and induces LMP (Marques et al., 2004). Intriguingly, HNE also promotes zinc accumulation in lysosomes before LMP and cell death. Again, HNE may trigger the release of zinc in lysosomes *in situ* or cause zinc entry secondary to the increase in cytosolic zinc levels. Because zinc itself elicits increased HNE levels in lysosomes, this leads to a detrimental cycle. It is difficult to determine whether zinc or HNE is the main cause of LMP, because chelation of zinc inhibits LMP, but also suppresses HNE levels. Moreover, either zinc or HNE was sufficient in inducing LMP at least in a fraction of isolated lysosomes.

In view of the increasing evidence supporting a role of endog-

Figure 6. HNE and zinc directly induce LMP in isolated lysosomes. *A*, Confocal live-cell images of isolated lysosomes stained with LysoTracker. The treatment with 100 μM H_2O_2 did not alter the lysosomal number. *B*, The bars denote the percentage changes in number of lysosomes against control before and 30 min after the addition of 100 μM H_2O_2 , respectively (mean $\pm$ SEM; $n = 4$). *C*, Isolated lysosomes before and 30 min after the addition of 300 μM HNE. Note the disappearance of certain lysosomes (arrows). *D*, The bars signify the percentage changes in number of lysosomes before and after the addition of 300 μM HNE (mean $\pm$ SEM; $n = 4$; ** $p < 0.01$). *E*, Isolated lysosomes before and 30 min after the addition of 500 μM zinc. Some of the lysosomes disappeared (arrows). *F*, The bars represent percentage changes in number of lysosomes before and 30 min after the addition of 500 μM zinc (mean $\pm$ SEM; $n = 4$; ** $p < 0.01$). Scale bars: *A*, *C*, *E*, 10 μm .

enous zinc in pathological neuronal death, thorough understanding of the underlying mechanism may facilitate the development of effective neuroprotective measures. Previous studies show that protein kinase C (PKC), Erk-1/2 (extracellular signal-regulated kinase 1/2), NADPH oxidase, and PARP are involved in oxidative neuronal death by zinc (Koh, 2001), and p75^{NTR}/NADE (p75^{NTR}-associated death executor) induction as well as AIF (apoptosis-inducing factor) release from mitochondria participate in zinc-induced neuronal apoptosis (Mukai et al., 2000; Park et al., 2000). In addition to these events, the present study provides evidence that toxic zinc exposure enhances the zinc levels in lysosomes, cytosol, and nuclei, increases HNE levels in lysosomes, and induces LMP. Additional studies are required to determine how HNE is produced on toxic zinc exposure. Zinc toxicity evidently involves oxidative stress and lipid peroxidation (Kim and Koh, 2002; Hao and Maret, 2006). PKC activation and NADPH oxidase activation may be one mechanism leading to oxidative stress, and possibly HNE production (Park and Koh, 1999; Noh and Koh, 2000).

Figure 7. The appearance of zinc-containing vesicles in rat hippocampal neurons after kainate seizures. **A**, Confocal live-cell images of FluoZin-3-stained rat hippocampal sections (showing representative CA3 pyramidal neurons) taken at the indicated time points after the intraperitoneal injection of 10 mg/kg kainate. Zinc-containing vesicles (green; arrows) appeared at 3 h after the kainate injection. The cytosolic zinc signals greatly increased at 8 h after the kainate injection. Nuclei were stained with Hoechst 33342 (blue). Scale bar, 10 μm. **B**, **C**, Immunohistochemistry with anti-cathepsin D antibody (**B**), and anti-HNE antibody (**C**) of rat hippocampal sections (showing representative CA3 pyramidal neurons) obtained from sham operated control (CTL) and 8 h after the kainate injection. Scale bar, 10 μm.

Recently, several groups have reported the existence of zinc-containing vesicles designated “zincosomes” in various cells, such as yeast (Eide, 2006), as well as cultured murine neurons and astrocytes. However, the identity of these zinc-containing vesicles is a controversial issue. Zinc-containing vesicles are detected in Golgi and endoplasmic reticulum in yeast (Devirgiliis et al., 2004; Eide, 2006), lysosomes in astrocytes (Varea et al., 2006), and late endosomes/lysosomes in neuron synaptic vesicles (Danscher and Stoltenberg, 2005; Smith et al., 2007). Interestingly, Sensi et al. (2003) showed that mitochondria may function as zinc-containing vesicles, especially on challenge with increased cytosolic zinc levels or oxidative stress. In contrast, our results clearly demonstrate that most zinc-containing vesicles in hippocampal neurons under conditions of oxidative stress or zinc overload are lysosomes. To determine whether lysosomes function as a buffer against the toxic increase in cytosolic zinc levels, the entry mechanism of zinc into lysosomes needs to be investigated.

Although lysosomal disruption has been demonstrated in primate CA1 neurons undergoing ischemic necrosis (Yamashima et al., 2003), the possible role of zinc and HNE in LMP of central neurons has not been studied *in vivo*. Hence, another new and significant contribution of the present study is the demonstration of the formation of zinc-containing vesicles in hippocampal neurons *in vivo* after kainate seizures. In addition, kainate seizures induced the increase of cathepsin D and HNE levels in neurons that had undergone generalized seizures. Although additional studies may be needed, these results seem to suggest the possibility that lysosomal zinc accumulation, increases in HNE adduct levels, and LMP may be a relevant mechanism of oxidative neuronal death *in vivo*.

Zinc is an endogenous metal with many essential functions.

However, as in the case of calcium, zinc dyshomeostasis is detrimental to cells, particularly neurons (Capasso et al., 2005). Movement of zinc across neurons and between intracellular compartments may play a significant role in neuronal death (Revuelta et al., 2005).

The present results demonstrate that alterations in zinc levels in lysosomes are an important step in causing oxidative neuronal death that occurs in diverse conditions of acute brain injury. Thus, measures to inhibit LMP may be useful in reducing oxidative stress- or zinc-triggered neuronal death.

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