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CHARACTERIZATION OF EXTRACELLULAR ACCUMULATION OF Zn^{2+} DURING ISCHEMIA AND REPERFUSION OF HIPPOCAMPUS SLICES IN RAT

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Abstract—The mammalian CNS contains an abundance of chelatable zinc that is sequestered in the vesicles of glutamatergic presynaptic terminals and co-released with glutamate. Considerable Zn^{2+} is also released during cerebral ischemia and reperfusion (I/R) although the mechanism of this release has not been elucidated. We report here the real time observation of increase of the concentration of extracellular Zn^{2+} ($[Zn^{2+}]_o$), accompanied by a rapid increase of intracellular free Zn^{2+}concentration, in the areas of dentate gyrus (DG), CA1 and CA3 in acute rat hippocampus slices during ischemia simulated by deprivation of oxygen and glucose (OGD) followed by reperfusion with normal artificial cerebrospinal fluid. A brief period of OGD caused a sustained increase of $[Zn^{2+}]_o$. Subsequent reperfusion with oxygenated medium containing glucose resulted in a further increase of $[Zn^{2+}]_o$. Longer periods of OGD caused greater increases of $[Zn^{2+}]_o$, and subsequent reperfusion caused still further increases of $[Zn^{2+}]_o$, regardless of OGD duration. The Zn^{2+} chelator CaEDTA (10 mM) significantly reduced the increase of $[Zn^{2+}]$ induced by OGD and reperfusion. Significant regional differences of $[Zn^{2+}]_o$ over the areas of the DG, CA1 and CA3 were not observed during I/R. Neither sodium channel blockade by tetrodotoxin (2 μM), perfusion with nominally calcium-free medium nor anatomical disassociation of the DG, CA1 and CA3 regions from one another by lesioning affected the increase of $[Zn^{2+}]_o$. The non-specific nitric oxide synthase (NOS) inhibitor, *N*ω-nitro-L-arginine methyl ester (1 mM), however, blocked the increase of $[Zn^{2+}]_o$ during ischemia and reperfusion. The data indicate the important role of NO in causing the release of Zn^{2+} during I/R and suggest that NOS inhibitors may be used to reduce Zn^{2+}-induced neuronal injury.

Key words: fluorescence, CaEDTA, nitric oxide, TTX.

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Abbreviations: ACSF, artificial cerebrospinal fluid; CaEDTA, ethylenediaminetetraacetic acid disodium-calcium salt; L-NAME, *N*ω-nitro-L-arginine methyl ester; NG, Newport Green; NG-DA, Newport Green–diacetate; NO, nitric oxide; NOS, nitric oxide synthase; OGD, oxygen and glucose deprivation; ROI, region of interest; TTX, tetrodotoxin; $[Zn^{2+}]_i$, concentration of intracellular Zn^{2+}; $[Zn^{2+}]_o$, concentration of extracellular Zn^{2+}.

Zinc, an important trace element, exists either as bound Zn^{2+} or histochemically reactive free Zn^{2+} (chelatable Zn^{2+}) in biological systems. Bound Zn^{2+} makes up the majority of the total zinc in tissues, and is comprised largely of Zn^{2+} bound to metalloproteins and enzymes (Choi and Koh, 1998). This type of Zn^{2+} usually serves as an essential catalytic or structural element of Zn^{2+}-binding proteins. Under oxidative stress, zinc may be released from such proteins (Cuajungco and Lees, 1997; St. Croix et al., 2002) and the pathophysiological role of this Zn^{2+} release is not well understood. Chelatable Zn^{2+} represents up to 10% of the total Zn^{2+} in the brain and is found mostly in synaptic vesicles of a subgroup of glutamatergic neurons distributed throughout the brain, but particularly in dentate granule cells and their projections (Colvin et al., 2000). Chelatable Zn^{2+}, visualized by histochemical stains or fluorescent dyes is released from these nerve terminals by electrical stimulation, where it is translocated into postsynaptic neurons. There it plays an essential role in the induction of long-term potentiation in the CA3 (Colvin et al., 2000; Li et al., 2001a). Released Zn^{2+} may also serve to modulate responses at a number of neurotransmitter receptors, including both excitatory and inhibitory receptors such as the ionotropic glutamate and GABA receptors (Smart et al., 1994; Xie et al., 1994).

Accumulating evidence suggests that Zn^{2+} is involved in excitotoxic neuronal death after head trauma, epilepsy, cerebral ischemia and reperfusion (Choi, 1996; Frederickson et al., 1989; Suh et al., 2000). It has been shown that delayed cell death in certain hippocampal pyramidal neurons after transient global ischemia and reperfusion is accompanied by an accumulation of chelatable Zn^{2+}, specifically in the hippocampal hilus, and CA1 region as well as in the cerebral cortex, thalamus, striatum, and amygdala (Koh et al., 1996). High concentrations of Zn^2 *in vitro* induce neuronal apoptosis and cell death (Kim et al., 1999; Lobner et al., 2000) that can be prevented by the Zn^{2+} chelating agent CaEDTA (ethylenediaminetetraacetic acid disodium-calcium salt; Koh et al., 1996) or metal-binding proteins (van Lookeren Campagne et al., 1999). The appearance of elevated intracellular Zn^{2+} in apoptotic neurons after ischemia and reperfusion that can be reduced by the presence of extracellular CaEDTA has suggested that Zn^{2+} released from presynaptic stores translocates into postsynaptic neurons to cause neuronal death (Lee et al., 2002). A rapid disappearance of Zn^{2+} positive terminals after focal brain ischemia has been observed (Sorensen et al., 1998).

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Although there is evidence suggesting Zn^{2+} translocation in brain ischemic and reperfusion injury, this evidence is based on indirect measurements of terminal vesicle Zn^{2+} loss and post-synaptic concentration of intracellular Zn^{2+} ($[Zn^{2+}]_i$) increase in brain tissue by slicing and staining techniques. Evidence of the presence of concentration of extracellular Zn^{2+} ($[Zn^{2+}]_o$) and a characterization of its accumulation in ischemia and reperfusion is lacking (Koh et al., 1996; Sorensen et al., 1998; Frederickson et al., 2002b). We have shown previously that electric stimulation or high potassium exposure can cause zinc release from nerve terminals and that activation of sodium channels as well as extracellular Ca^{2+} are essential for this release (Li et al., 2001a,b). We do not know, however, the characteristics of zinc release induced by ischemia and reperfusion. Very recently, evidence from other groups suggested that nitric oxide (NO) may be involved in the regulation of intracellular Zn^{2+} concentration (Cuajungco and Lees, 1998; Snider et al., 2000). NO can cause Zn^{2+} release from metallothionein and zinc-finger proteins by *S*-nitrosylation of cysteine clusters that bind Zn^{2+} (Aravindakumar et al., 1999). NO may increase the accumulation of chelatable Zn^{2+} in the hippocampal neuronal perikarya (Cuajungco and Lees, 1998) and cause the release of Zn^{2+} from presynaptic boutons *in vivo* (Frederickson et al., 2002a). Since it is well known that NO is generated during ischemia and reperfusion (Beckman, 1991; Dawson and Dawson, 2000; Moncada and Higgs, 1993), these results suggest that NO may be involved in the release of Zn^{2+}during ischemia and reperfusion.

The goal of this study was to characterize the accumulation of extracellular Zn^{2+} during ischemia and reperfusion. Questions addressed were (1) does ischemia or reperfusion cause an increase of $[Zn^{2+}]_o$; (2) what role does synaptic Zn^{2+} release play in this increase; and (3) what is the role of NO in the accumulation of $[Zn^{2+}]_o$ during ischemia and reperfusion? Acute brain hippocampus slice preparations subjected to brief periods of oxygen and glucose deprivation (OGD) followed by restoration of oxygen and glucose were used to mimic ischemia and reperfusion events *in vivo*. The course of the accumulation of $[Zn^{2+}]_o$ in real-time was determined during a period of ischemia and reperfusion of the rat hippocampal slices using the Zn^{2+}-sensitive fluorescence dye Newport Green (NG) DCF (Thompson et al., 2002).

EXPERIMENTAL PROCEDURES

All animal procedures conformed to all NIH and IBRO guidelines. All efforts were made to minimize the number of animals and their suffering. Male adult Sprague–Dawley rats were anesthetized with ketamine hydrochloride and secobarbital, injected intraperitoneally and decapitated. The brain was quickly removed and immersed in ice-cold (1–4 °C) cutting solution with the composition of (in mM): NaCl, 124; KCL, 1.75; $MgCl_2$ 5; $CaCl_2$, 1.0; KH_2PO_4, 1.25; $NaHCO_3$, 26 and dextrose 10, continuously bubbled with 95% O_2 and 5% CO_2. Transverse hippocampal slices 250 μm in thickness were prepared using a McIlwain tissue chopper (Brinkman Instruments, Westbury, NY, USA). The slices were incubated in artificial cerebrospinal fluid (ACSF) with the composition of (in mM): NaCl, 124; KCl, 1.75; $MgSO_4$, 1.3; $CaCl_2$, 2.4; KH_2PO_4, 1.25; $NaHCO_3$, 26 and dextrose 10, continuously bubbled with 95% O_2 and 5% CO_2 for at least 1 h prior to imaging experiments.

Chemicals and reagents

Cell impermeable NG DCF, dipotassium salt was purchased from Molecular Probes (Eugene, OR, USA). CaEDTA, tetrodotoxin (TTX) and *N*ω-nitro-L-arginine methyl ester (L-NAME) were obtained from Sigma (St. Louis, MO, USA).

Slice ischemia and reperfusion model

Hippocampal slices were transferred from the incubation chamber to a small dish, which was placed on a thermostatically heated stage of an inverted microscope. In the chamber, slices were held in place by a small ring and constantly perfused with 20 μM NG dipotassium salt in ACSF (1.5 ml/min) bubbled continuously with 95% O_2 and 5% CO_2. After a 10 min equilibration, ischemia was induced by switching the ACSF to OGD ACSF (ACSF as described above without glucose, increased NaCl concentration 134 mM to compensate for the loss glucose, and 20 μM NG dipotassium salt equilibrated with N_2 95% and 5% CO_2). Reperfusion was obtained by switching the perfusate back to normal ACSF equilibrated with 95% O_2 and 5% CO_2. The temperature inside the dish was carefully held at 32 °C in a piezo-electrically controlled incubation chamber (Harvard Apparatus, Hollison, MA, USA) throughout the experiments. A series of control experiments were performed to exclude the possibility of artifacts in our measurements: (1) slices were perfused with ACSF without OGD for the duration of most experiments without significant elevation of baseline fluorescence; (2) slices were perfused with OGD ACSF in the absence of a slice without increased or decreased fluorescence; and (3) slices were perfused with OGD ACSF but in the absence of NG without detectible fluorescence at 533 nM from slice autofluorescence or increased transmittance. The experiments were conducted with slices subjected to ischemia alone up to 60 min or 10, 20 or 30 min of simulated ischemia followed by 30 min of reperfusion.

Zn^{2+} imaging

Imaging of $[Zn^{2+}]_o$ was performed using the cell impermeable fluorescence dye NG dipotassium salt as described previously (Li et al., 2001b). In brief, slices were continuously perfused with NG dipotassium (20 μM) in ACSF or glucose free ACSF. NG fluorescence intensity was not affected by OGD or oxygen glucose restoration. (data not shown). For intracellular Zn^{2+} imaging (Li et al., 2001b), the slices were preloaded with 50 μM cellular permeable NG diacetate (NG-DA), 0.1% pluronic acid, and 0.5% dimethyl sulfoxide for 1 h. Extracellular NG-DA was washed out with ACSF. The diacetate ester form of NG enters cells where endogenous esterases hydrolyze the ester to form the membrane impermeable free acid form, effectively trapping the dye inside the cell (Haugland, 1996). All experiments were performed at 32 °C on the stage of an inverted microscope (Axiovert 135; Zeiss, Germany) coupled to a β-Ram light source (Photon Technology International, Lawrenceville, NJ, USA). Images were obtained (excitation, 488 nm; emission, 533 nm) through a 5×0.15 Plan-Neofluar objective with a digital camera (Hammatusu) and a digitized software Open Laboratory 3.3 (Improvision, Lexington, MA, USA). In each experiment, a bright field image of the slice was taken before as well as after fluorescent imaging to confirm the slices had not moved throughout experiment. Images were collected over 1 min at each 2 min interval with four or eight× binning of pixels. Gain was set such that the slice during the equilibration phase of the experiment was just visible enough to distinguish the most obvious features of hippocampal anatomy. All other camera settings were at their lowest levels.

Data analysis

The fluorescent emission intensity was collected from the region of interest (ROI) of each frame, and expressed as ΔF or ΔF/F for the selected ROI. "F" refers to the baseline of fluorescent emission intensity in the selected ROI, and "ΔF" refers to the change of fluorescent emission intensity in the same ROI. "*n*" refers to numbers of animals in each experiment. All measurements are given as means±S.E. Statistical significance was tested using the Student's *t*-test, or paired *t*-test, and $P<0.05$ was considered as significant. ANOVA test were used for comparison of more than two groups.

RESULTS

Increase of $[Zn^{2+}]_o$ induced by ischemia

The hippocampus is exquisitely sensitive to ischemia. Studies have demonstrated that Zn^{2+} accumulates in neurons in the injured regions of the hippocampus after ischemia (Koh et al., 1996; Yin et al., 2002). The present study addresses the effect of ischemia and subsequent reperfusion on the accumulation of extracellular Zn^{2+} in the hippocampus using a cell impermeable form of Zn^{2+}-sensitive dye, NG, to estimate the $[Zn^{2+}]_o$. Ischemia and reperfusion was simulated in hippocampal slices by OGD and subsequent restoration of oxygenated, glucose-containing medium. In controls for autofluorescence, intrinsic fluorescence, increased transmittance from cell swelling, photobleaching and instability of baseline, NG fluorescent intensity was not increased during the course of the experiments ($n=3$, in each) as described in experimental procedures (data not shown). In slices treated with 30 min of OGD-simulated ischemia followed by 30 min of reperfusion, NG fluorescent intensity remained unchanged during a 10 min baseline recording period during which the slice was washed with NG containing ACSF (Fig. 1, panels marked Baseline), while a striking increase of fluorescent intensity was observed throughout the slice during OGD (pseudocolor turned from blue to green, Fig. 1, panels marked Ischemia), and further increased during reperfusion (color turn from green to red, Fig. 1, panels marked Reperfusion). To quantify the increase of NG fluorescent

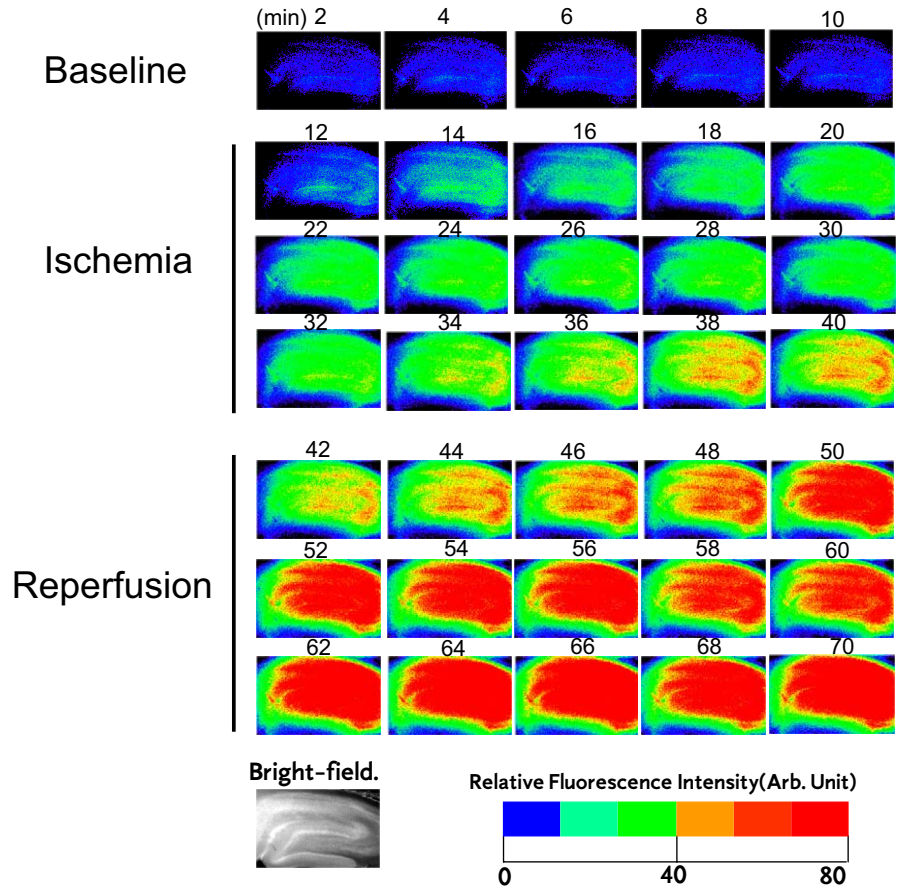

Fig. 1. Fluorescence image of increase of $[Zn^{2+}]_o$ during ischemia and reperfusion in rat hippocampal slices. Rat hippocampus slices were continuously perfused with the cell-impermeable dipotassium salt of NG DCF in ACSF or glucose-free ACSF. Images were taken through a 5×0.15 Plan-Neofluar objective with a digital camera at excitation, 488 nm; emission, 533 nm (see detail in Experimental Procedures). Baseline: fluorescent images of a slice equilibrated in ACSF, 20 μM NG for 10 min. Ischemia: images of the slices subjected to OGD for 30 min (minute 10 to minute 40). Reperfusion: images of the slice reperfused with oxygenated, glucose-containing ACSF for 30 min (minute 40 to minute 70). The images of the hippocampal slice show the changes in fluorescent intensity of $[Zn^{2+}]_o$ in pseudocolor (scale given at lower right corner). A brightfield image of the slice is shown at the lower left corner.

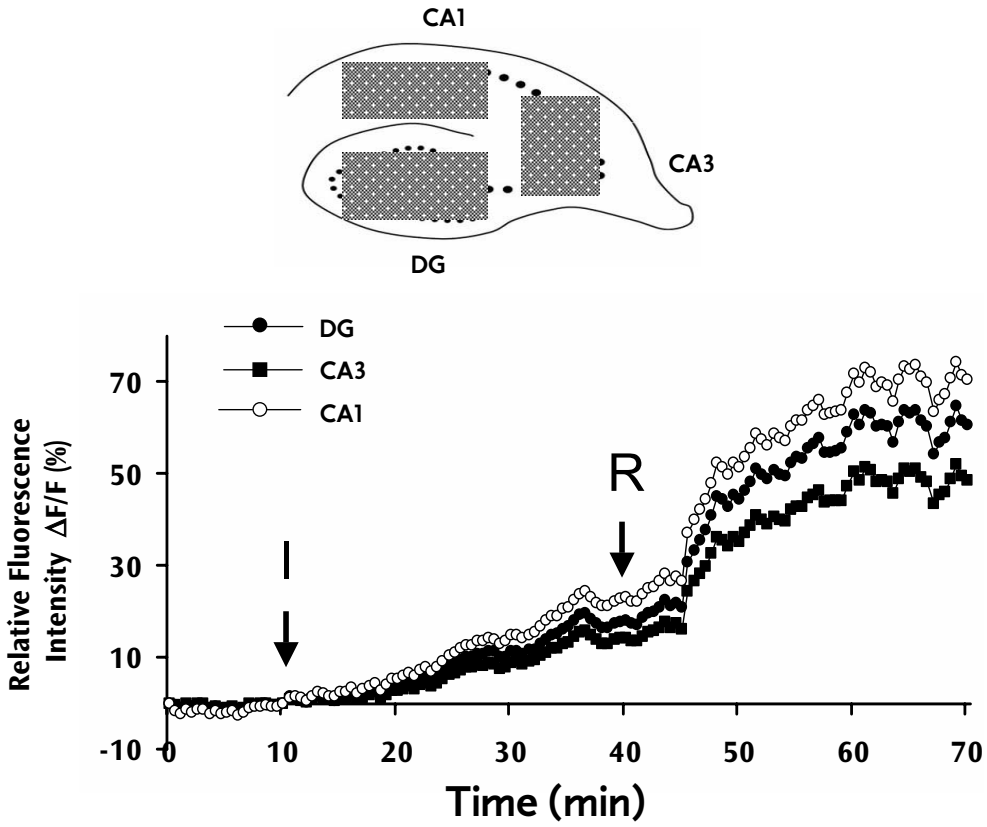

Fig. 2. Typical increase of $[Zn^{2+}]_o$ during ischemia and reperfusion periods in rat hippocampal slices. Top schematic shows the ROIs that were selected on the slice for DG, CA3, and CA1 areas. Graph at bottom shows the increase of relative fluorescence intensity in the hippocampal slice of the ROIs. A hippocampal slice was equilibrated for 10 min with ACSF plus 20 μM NG dipotassium salt and subsequently subjected to 30 min ischemia followed by 30 min reperfusion. Arrows show the start of OGD-simulated ischemia (I) and reperfusion (R).

intensity in the hippocampal slice, ROI in the DG, CA1 and CA3 were selected as shown in Fig. 2 (schematic on top). NG fluorescence intensity in each ROI was typically stable over the 10 min initial perfusion with oxygenated, glucose-containing ACSF (Fig. 2). A gradual increase of fluorescence intensity was observed in the areas of DG, CA3 and CA1 during 30 min OGD-simulated ischemia. An additional marked increase of fluorescent intensity of roughly 50 arbitrary units on average was observed during 30 min reperfusion. There were no significant differences in the increases of fluorescence intensity detected among the areas of DG, CA3 and CA1 (Fig. 2).

Increase of $[Zn^{2+}]_i$ induced by ischemia

To investigate the source of extracellular Zn^{2+} released by OGD and subsequent reperfusion, intracellular Zn^{2+} was imaged using the cell permeable form of NG, NG-DA. There was little fluorescent intensity prior to the initiation of OGD in the hippocampus, although we observed slightly higher background fluorescence intensities in the hilar and CA3 regions compared with other regions of the hippocampal slice (white arrowheads in Fig. 3A). With the initiation of OGD, a rapid rise of fluorescent intensity occurred in all three regions of the hippocampus that reached a maximum at 30 min of OGD (Fig. 3B and C, ROIs selected as illustrated in Fig. 2). During subsequent reperfusion beginning at 40 min, an additional increase of fluorescent intensity was observed. Statistical analysis demonstrated that the fluorescence intensity observed during OGD and reperfusion was significantly higher than that observed during perfusion of ACSF without OGD ($P<0.05$; Fig. 3C). There were no significant differences among the areas of DG, CA3 and CA1 in the effect of OGD and reperfusion on NG fluorescence intensity. Reperfusion with ACSF after OGD did not result in significantly higher fluorescence intensities than that induced by OGD ($P>0.05$).

Effect of ischemia and reperfusion on the increase of $[Zn^{2+}]_o$

To explore the characterization of ischemia and reperfusion on the increase of $[Zn^{2+}]_o$, a series of experiments was set up wherein hippocampal were slices subjected to 10, 20, 30, 40, 50 or 60 min OGD ischemia or 10, 20, 30 min OGD ischemia were followed by 30 min reperfusion. The results show that as little as 10 min of OGD ($n=5$) caused a significant increase of $[Zn^{2+}]_o$ ($P<0.05$) in the DG, CA3 and CA1 compared with controls that were not subjected to OGD ($n=5$). Slices subjected to 20 ($n=5$), 30 ($n=5$) and up to 60 ($n=4$) min of OGD-simulated ischemia

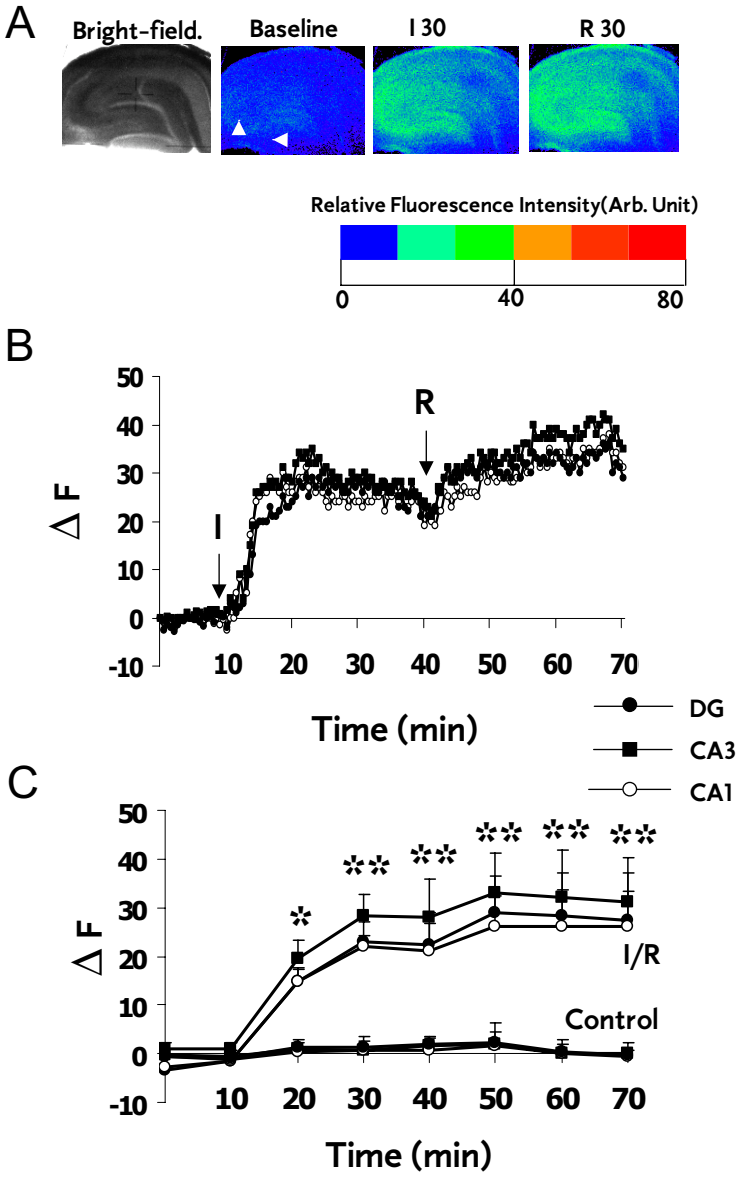

Fig. 3. Increase of $[Zn^{2+}]_i$ during ischemia and reperfusion periods in rat hippocampal slices. Rat hippocampus slices were loaded with 50 μM cell-permeable NG diacetate, 0.1% pluronic acid, and 0.5% dimethyl sulfoxide for 1 h, then the excess extracellular NG was washed out by ACSF. Images were taken through a 5×0.15 Plan-Neofluar objective with a digital camera at excitation, 488 nm; emission, 533 nm (see detail in Experimental Procedures). (A) Slice image of bright field, baseline (arrows indicate of higher contents of Zn^{2+} in DG and CA3 regions), during 30 min OGD ischemia followed by 30 min of reperfusion with ACSF. (B) A representative trace the increase of intracellular Zn^{2+} induce by 30 min OGD ischemia followed 30 min reperfusion (arrows indicate the start of OGD ischemia [I] or reperfusion [R]). (C) Mean increase of the $[Zn^{2+}]_i$ induced by OGD ischemia followed by reperfusion (I/R, $n=5$) is significantly increased compare with no OGD control ($n=3$), * $P<0.05$, ** < 0.01.

each showed a greater accumulation of extracellular Zn^{2+} that reached a maximum after 30 min of OGD (Fig. 4A). Reperfusion with oxygenated, glucose containing medium following 10, 20 and 30 min of OGD, a further increase of $[Zn^{2+}]_o$ was observed in all groups (Fig. 4B). In particular, 30 min of OGD followed by 30 min of reperfusion ($n=5$) caused a significantly higher increase of $[Zn^{2+}]_o$ than slices subjected to 60 min OGD alone ($n=4$, $P<0.05$). We were unable to detect significant regional differences in NG fluorescent intensity increase among DG, CA1 and CA3 ($P>0.05$) in any of the experiment groups.

Effect of CaEDTA on the increase of $[Zn^{2+}]_o$

While NG dipotassium salt is selective for Zn^{2+} among the most abundant metals present in tissue, an artifact of increased fluorescent intensity may occur (Johnson et al., 2000). To obtain further confirmation that the detected increase of NG fluorescent intensity was due to an increase in Zn^{2+}, slices were pretreated with 10 mM CaEDTA for 20 min and then perfused with ACSF or glucose-free ACSF and 1 mM CaEDTA throughout the ischemia and reperfusion periods. The effect of CaEDTA

on fluorescent intensity was evaluated as a percentage of the NG fluorescent intensity observed in paired control slices (not treated with CaEDTA) from the same animal. The relative fluorescent intensity was significantly lower ($P<0.05$, $n=3$; Fig. 4) in the slices treated with CaEDTA compared with the untreated controls. In all of the observed areas (DG, CA3, and CA1) the relative fluorescent intensity in CaEDTA treated slices was only about 20% of that observed in controls during the ischemia period or the combined ischemia-reperfusion period. In aqueous solutions containing 1 mM CaEDTA, 10 μM $Zn^{2+}$and 2.5 μm Fe^{2+} at equilibrium are expected to have 9 pM free Fe^{2+} and 0.2 pM free Zn^{2+} present (www.stanford.edu/~cpatton/webmaxcS.htm). These concentrations of Fe^{2+} and Zn^{2+} are not detectable by NG. The fluorescence that remains after CaEDTA chelation of divalent cations represents the intrinsic fluorescence of NG.

Role of neuronal conduction on the accumulation of $[Zn^{2+}]_o$

To evaluate the role of synaptic release in the accumulation of extracellular Zn^{2+} during ODG and reperfusion, the sodium channel blocker, TTX, was used to prevent release of Zn^{2+} from a depolarization of presynaptic terminal membranes. Slices were pretreated with 2 μM TTX for 20 min before and continuously perfused with 2 μM TTX during the experiment. The relative NG fluorescent intensities during a 30 min period of OGD in TTX-treated slices were 92.0%±9.33; 86.0%±12.2 and 93.7%±13.5 of their parallel, untreated controls in the areas of DG, CA1 and CA3, respectively (Fig. 5A). The relative NG fluorescent intensities in TTX treated slices after both 30 min OGD and 30 min reperfusion treatments were 95.5%±13.9; 87.4%±10.1 and 95.2%±2.4 of their untreated controls in the DG, CA1 and CA3, respectively (Fig. 5). No significant differences in $[Zn^{2+}]_o$ were observed between TTX-treated slices and parallel controls in the observed areas ($P>0.05$), and no significant differences within the TTX-treated slices were observed as well among the areas of the DG, CA3 and CA1 ($P>0.05$).

If synaptic transmission were essential for OGD and reperfusion-induced accumulation of extracellular Zn^{2+}, then a lesioning of the fibers leading from the DG to the CA3 (the mossy fibers) and those leading from the CA3 to the CA1 (the Schaffer collaterals) should prevent the spread of extracellular Zn^{2+} along these fibers. We therefore tested whether lesioning these fibers prevented or reduced the accumulation of extracellular Zn^{2+} in the three regions. Lesioning of the neuronal fibers between the DG, CA1 and CA3 in hippocampus slices was accomplished by cutting them with a sharp knife. The locations of the lesions are showed in Fig. 5 (schematic on top). After the lesions were made, the slices were incubated at room temperature in oxygenated ACSF for at least 1 h before subjecting to 30 min of OGD-simulated ischemia followed by 30 min reperfusion. The results showed that there were no significant differences in $[Zn^{2+}]_o$ in the DG, CA1 and CA3 areas, between lesioned slices and controls ($P>0.05$), and no significant differences were observed between the three areas of the lesioned slices during ischemia and reperfusion ($P>0.05$). These results suggest that impulse conduction via the neuronal connections between the DG, CA1 and CA3 of the hippocampus and any accompanying synaptic release of Zn^{2+} and transmitter do not play a role in the increase of $[Zn^{2+}]_o$ during ischemia and reperfusion (Fig. 5).

Effect of extracellular Ca^{2+} on the increase of $[Zn^{2+}]_o$

Extracellular Ca^{2+} is required for vesicle fusion and neurotransmitter release. A previous study has demonstrated that electrical stimulation-induced Zn^{2+} release from synaptic terminal is blocked in Ca^{2+}-free extracellular medium (Li et al., 2001). To test further whether Zn^{2+} release induced by ischemia and reperfusion depends on synaptic release, slices were subjected to OGD and reperfusion in Ca^{2+}-free or normal Ca^{2+}-containing ACSF. As in normal calcium ACSF (Fig. 5), ischemia induced by OGD in calcium-free ACSF caused an increase of $[Zn^{2+}]_o$, and reperfusion with oxygenated, glucose-containing ACSF caused an additional increase. The relative fluorescent intensities observed during OGD and reperfusion in Ca^{2+}-free medium treated slices in the DG, CA1 and CA3, respectively, were 102.4%±16.1; 109.3%±2.4 and 117.2%±11.7 of the intensities of their normal controls (Fig. 4). No significant differences were observed between Ca^{2+}-free and normal Ca^{2+}–ACSF groups with respect to the $[Zn^{2+}]_o$ increase caused by OGD and reperfusion ($P>0.05$).

Effect of NO synthase (NOS) inhibitor, L-NAME, on the increase of $[Zn^{2+}]_o$

NO can cause an apparent release of Zn^{2+} from presynaptic boutons *in vivo* (Frederickson et al., 2002a) and release from bound protein *in vitro* (Aravindakumar et al., 1999). NO is produced by a NOS-dependent pathway during cerebral ischemia and reperfusion (Cardenas et al., 2000; Kojima et al., 1998; Kumura et al., 1996). To explore the regulation of NO in Zn^{2+} translocation during OGD and reperfusion, we tested whether the non-specific NOS inhibitor, L-NAME, blocks accumulation of $[Zn^{2+}]_o$ during OGD-simulated ischemia and reperfusion. Hippocampal slices were treated with or without L-NAME during OGD and reperfusion. For the L-NAME treatment, slices were pretreated with 1 mM L-NAME in ACSF for 20 min and then continuously perfused with 1 mM L-NAME throughout the OGD and reperfusion periods. The effect of L-NAME on Zn^{2+} release was expressed as a percentage of the fluorescent intensity of the control slice (from the same animal) in the ROI areas of DG, CA3, and CA1. The fluorescent intensity in L-NAME-treated slices was 49.0%±19.4; 49.6%±17.4 and 50.0%±18.9 respectively of their parallel controls during OGD. The increase in $[Zn^{2+}]_o$ was significantly decreased by L-NAME compared with controls ($P<0.05$; Fig. 5A). Similarly, the fluorescent intensity in L-NAME-treated slices was 33.4%±11.9; 36.7%±12.5 and 32.9%±11.6 of their parallel controls in the ROI areas

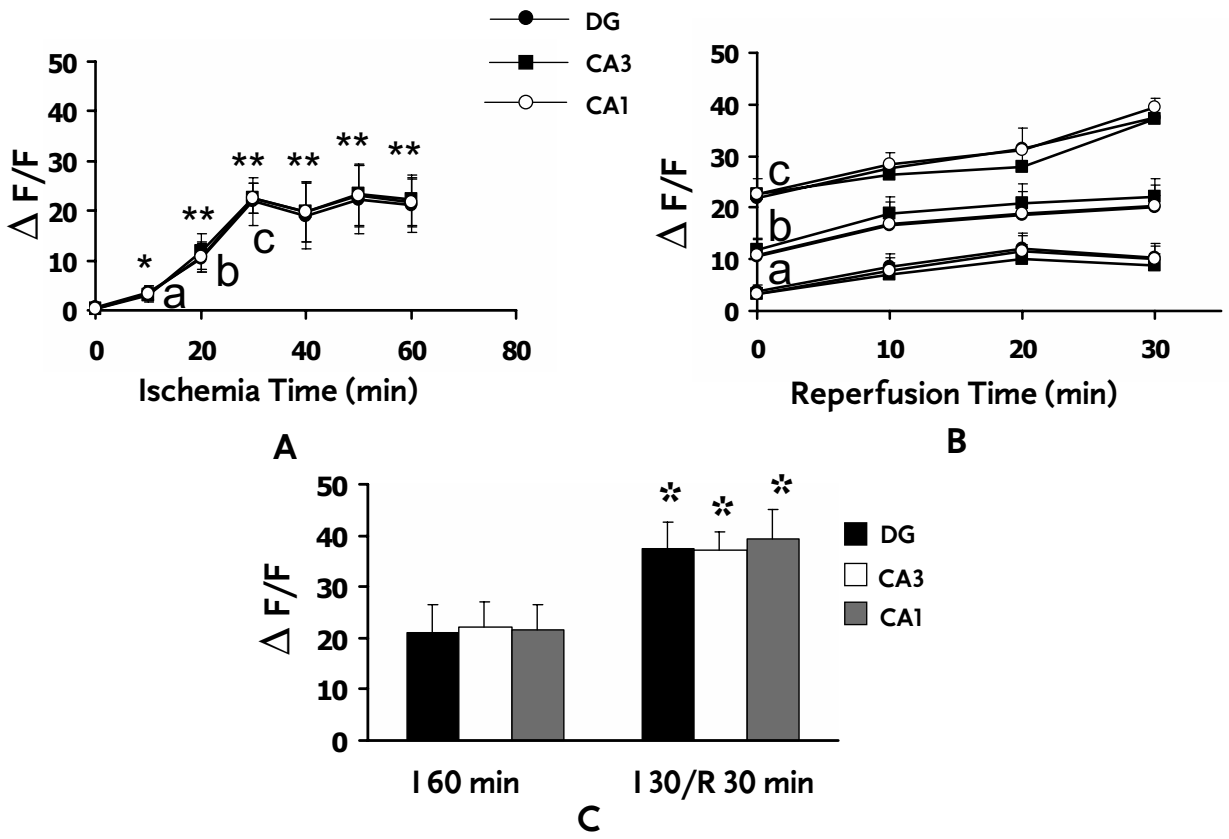

Fig. 4. Time course of the effect of ischemia and reperfusion on increase of $[Zn^{2+}]_o$ in rat hippocampal slices. (A) Slices were subjected to ischemia induced by OGD for the indicated times (n=5). The increase in relative fluorescence intensity in the DG, CA1, and CA3 regions are plotted (mean±S.E.) with the indicated symbols. (B) The slices that were subjected to 10, 20 and 30, min of OGD in A (marked a, b and c, respectively) were also subjected to 30 min of reperfusion (restoration of oxygenated, glucose-containing medium). Relative fluorescence intensity of each region was plotted as in A. (C) Relative fluorescent intensity in each hippocampal area of the groups that were subjected to 60 min of OGD-simulated ischemia alone (n=4) and 30 min ischemia plus 30 min reperfusion (n=5) are plotted in a bar graph. * $P<0.05$, ** <0.01.

when the slices subjected to ischemia followed by reperfusion. This is a marked decrease compared with controls ($P<0.01$; Fig. 5B). These results suggest that accumulation of $[Zn^{2+}]_o$ following ischemia or ischemia and reperfusion is inhibited by the NOS non-specific NOS inhibitor L-NAME.

DISCUSSION

Recently, our awareness of the importance of zinc in biology has expanded dramatically as a result of the development of the new selective indicators of Zn^{2+} (Gee et al., 2002; Sensi et al., 2002; Tsuda et al., 1997). One of these selective indictors of Zn^{2+}, NG, has been shown to produce an enhanced fluorescent intensity with the binding of Zn^{2+}. NG dipotassium salt is a form of cell impermeable Zn^{2+} sensitive dye, which cannot cross the cell membrane. Changes in NG fluorescence intensity detect changes in $[Zn^{2+}]_o$. NG-DA is the uncharged ester form of NG. It crosses the plasma membrane and is trapped intracellularly after impermeable hydrolysis of the ester to the NG form. Intracellular NG can then bind (relatively) free Zn^{2+} in the cytosol to produce an increase in fluorescence intensity. This increase in fluorescence intensity is proportional to $[Zn^{2+}]_i$ (Haugland, 1996).

NG was relatively insensitive to the presence of millimolar concentrations of Ca^{2+}, Mg^{2+} (Thompson et al., 2002). The concentration of $Ca^{2+}$and Mg^{2+} in the ACSF is 2.4 and 1.3 mM, respectively. These Ca^{2+} and Mg^{2+} concentrations had little effect on Zn^{2+} detection in previous studies (Li et al., 2001b). CaEDTA does not appreciably reduce Ca^{2+} or Mg^{2+} concentrations because it is already saturated with calcium (Wang and Quastel, 1990; Westergaard et al., 1995). Although CaEDTA is a cell-impermeable chelator, it is able to deplete intracellular divalent cations from neurons (Frederickson et al., 2002b). Other than Fe^{2+}, Zn^{2+} and Cu^{2+}, most transition metal cations are present in brain tissue at concentrations below 1 nM, which is below the limits of detection by NG (K_d for Zn^{2+} is approximately 1 μM). Between Fe^{2+}, Zn^{2+} and Cu^{2+}, NG fluorescence is enhanced significantly only by Zn^{2+} (Haugland, 1996). Zn^{2+} binds to CaEDTA with 152 times the affinity of Fe^{2+} and is nine times more effective than Fe^{2+} in enhancing NG fluorescence. Thus, the increase in NG fluorescence during OGD-simulated ischemia and reperfusion and the dramatic decrease in fluo-

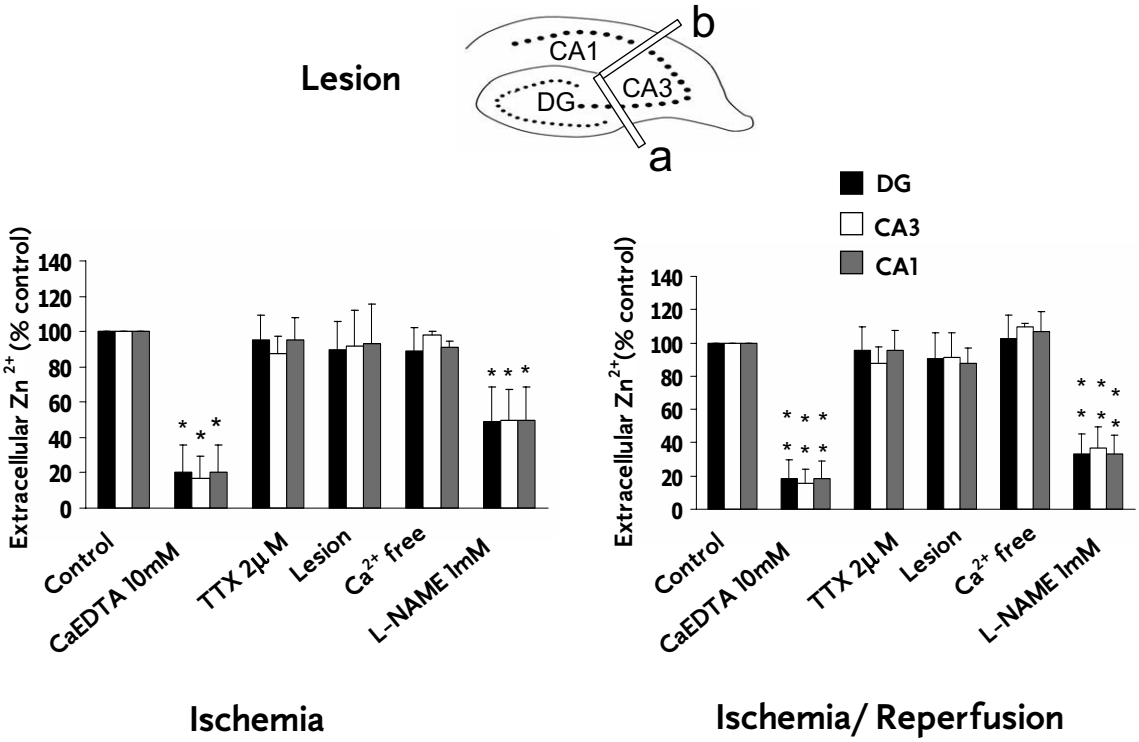

Fig. 5. Effects of neuronal conduction and pharmacological agents on the increase of $[Zn^{2+}]_o$ with ischemia and reperfusion. Slices were treated with either CaEDTA (10 mM pretreatment for 20 min, and 1 mM perfused throughout the experiment, $n=3$), TTX (2 μM, $n=5$) for 20 min pretreatment and throughout the experiment, lesions ($n=5$, the locations of the lesions are show in a schematic the top center of figure), calcium-free ACSF ($n=3$) or the non-specific NOS inhibitor, L-NAME for a 20 min pretreatment and throughout the experiment ($n=7$). The increase of fluorescent intensity relative to parallel controls in each region (DG, CA3, CA1) are plotted as a bar graph. Panel A shows the effect of the treatments on the increase of $[Zn^{2+}]_o$ during the period of 30 min OGD. Panel B shows the effect of the treatments on the increase of $[Zn^{2+}]_o$ during 30 min OGD ischemia followed by 30 min reperfusion. Values plotted are expressed as a percentage of the parallel controls, mean±S.E., * $P<0.05$, ** <0.01.

rescence detected following the addition of CaEDTA indicate the presence of increased concentration of Zn^{2+}. We have shown that a period of OGD, or OGD followed by reperfusion causes a significant increase in $[Zn^{2+}]_o$ in hippocampal slices in the DG, CA1 and CA3, using real-time NG fluorescence imaging. The increase in extracellular Zn^{2+} observed during OGD and reperfusion in our study had the following characteristics: (1) the increase of $[Zn^{2+}]_o$ was accompanied by a rapid increase of $[Zn^{2+}]_i$; (2) continuous OGD caused a gradual increase in $[Zn^{2+}]_o$, and subsequent reperfusion caused a further increase in $[Zn^{2+}]_o$; (3) there were no regional differences in the distribution of the extracellular Zn^{2+} in the areas of DG, CA1 and CA3 during OGD and reperfusion; (4) the blockade of voltage-gated sodium channels by TTX, the anatomical lesioning of the neuronal fibers connecting the DG with the CA3 and the CA3 with the CA1 and the removal of extracellular Ca^{2+} from the medium failed to affect OGD and reperfusion-elicited extracellular Zn^{2+} accumulation; (5) the non-specific NOS inhibitor, L-NAME, blocked the increase in $[Zn^{2+}]_o$ during ischemia and reperfusion.

The discovery of Zn^{2+} depletion from presynaptic terminals following transient global cerebral ischemia has led to the suggestion that the translocation of Zn^{2+} released from synaptic terminals and subsequent elevation in intracellular Zn^{2+} contributes to the neuronal death caused by ischemia. Since then, many studies of the role of Zn^{2+} in the neurotoxicity caused by ischemia have been conducted (Kim et al., 1999; Koh et al., 1996; Matsushita et al., 1996; Sheline et al., 2000; Sorensen et al., 1998). Although these studies have amassed what appears to be considerable evidence supporting the role of presynaptic release of Zn^{2+} and its entry into postsynaptic neurons during transient and global ischemia/reperfusion injury, these studies were based entirely on the disappearance of TSQ staining of chelatable Zn^{2+} from presynaptic terminals after a brief episode of ischemia and reperfusion (Frederickson et al., 1989; Sloviter, 1985). In these studies, the levels of chelatable Zn^{2+} were observed only at a time well removed from the period of ischemia and reperfusion. The $[Zn^{2+}]_o$ during the ischemia and reperfusion period was unknown. Real-time changes in $[Zn^{2+}]_o$ and its characterization had not yet been made possible by the development of fluorescent dyes for Zn^{2+}. We now have been able to show that OGD as well as OGD and reperfusion are both able to induced significant extracellular Zn^{2+} accumulation. These results show the time course of this extracellular Zn^{2+} accumulation.

Chelatable Zn^{2+} occurs primarily in synaptic vesicles of a subset of glutamatergic nerve terminals throughout the mammalian cortex and limbic region. The greatest concentration of zinc-containing terminals is found in the mossy

fiber projections of dentate granule cells of the hippocampus (Weiss et al., 2000). If extracellular Zn^{2+} accumulation during ischemia were the result of synaptic released Zn^{2+} only, then one would expect that OGD-induced accumulation of extracellular Zn^{2+} in the hippocampus would be greatest in the DG and the CA3 stratum lucidum because of the greater concentration of Zn^{2+}-containing terminals there. We did not observe regional differences in OGD-induced $[Zn^{2+}]_o$ increase suggesting that presynaptic terminal Zn^{2+} is not the only source of the Zn^{2+} accumulation. Alternatively, it is possible that spontaneous or treatment-stimulated synaptic transmission from efferent neuronal projections of the hippocampus could obscure non-uniform Zn^{2+} release by OGD and reperfusion. If regional differences in Zn^{2+} release were masked by synaptic transmission, the regional variation in Zn^{2+} release would become apparent when the slices were treated with TTX or the neuronal connections between the hippocampal areas were lesioned. Our results showed, however, that neither TTX treatment nor fiber lesions affected the uniform accumulation of $[Zn^{2+}]_o$, among the three hippocampal regions examined. These results suggest that $[Zn^{2+}]_o$ accumulation during ischemia and reperfusion is unlike electrically stimulated terminal Zn^{2+} release, which is TTX sensitive, and is propagated along nerve fibers.

Moreover, it has been presumed that zinc release from presynaptic terminals is the result of synaptic vesicle fusion with the presynaptic membrane (Colvin et al., 2000). It is well known that extracellular Ca^{2+} is required for vesicle fusion and neurotransmitter release. Previous studies demonstrated that electrical stimulation (Li et al., 2001). In contrast, we have demonstrated in this study that ischemia and reperfusion-induced extracellular Zn^{2+} accumulation cannot be affected by removing calcium from the extracellular medium, indicating a different mechanism for the extracellular accumulation of Zn^{2+} during OGD-simulated ischemia and reperfusion.

It is well known that NOSs are activated during ischemia by calcium dependent and independent pathways (Beckman, 1991; Dawson and Dawson, 2000; Moncada and Higgs, 1993). The calcium dependent pathway is activated by increased intracellular free calcium, which comes from extracellular and intracellular calcium pools (Dawson and Dawson, 2000; Paschen and Doutheil, 1999; Perez-Pinzon et al., 1998; Wang et al., 2002). Calcium released from intracellular pools is of critical importance for ischemia-induced cellular injury (Grondahl and Langmoen, 1996; Paschen, 2000). NO generated from NOS is thought to play an important role in ischemic injury and neuronal degeneration (Schulz et al., 1995; Dawson and Dawson, 1996). Accumulating evidence indicates that NO is involved in the elevation of intracellular Zn^{2+} during ischemia and reperfusion. The infusion of NO donors *in vivo* causes the loss of vesicular Zn^{2+} (Cuajungco and Lees, 1998; Frederickson et al., 2002a) and an accumulation of TSQ staining Zn^{2+} in the perikarya of pyramidal neurons (Cuajungco and Lees, 1998). NO displacement of Zn^{2+} from intracellular Zn^{2+}-binding proteins results in an accumulation of intracellular free Zn^{2+} *in vitro* (Aravindakumar et al., 1999). These observations indicate that NO may be the cause of the increase in intracellular Zn^{2+} release that occurs during ischemia, but it remains to be shown that NO generated during ischemia is required for extracellular Zn^{2+} accumulation. By direct observation in real-time of the accumulation of extracellular Zn^{2+} rather than using the histochemical stains of Zn^{2+} in fixed tissue (Cuajungco and Lees, 1998; Frederickson et al., 2002b), our study shows that $[Zn^{2+}]_o$ increases during OGD-simulated ischemia and reperfusion and that the NOS inhibitor, L-NAME, blocks this increase. The increase in $[Zn^{2+}]_o$ does not require synaptic transmission, membrane depolarization or synaptic vesicle-plasma membrane fusion. Because the blockade of NOS by L-NAME, presumably an intracellular event, also blocked the accumulation of extracellular Zn^{2+}, the source of the extracellular Zn^{2+} increase would have to be the cytosol of cells. Although it is possible that the attack of NO on synaptic vesicle proteins might lead to the loss of vesicular Zn^{2+} stores, these stores would have to pass into the cytosol before appearing in the extracellular space. Thus, the observations demonstrate the importance of NO in mediating Zn^{2+} release following ischemia and reperfusion, and may be important for the development of effective treatments for Zn^{2+}-induced injury during ischemia and reperfusion.

The mechanisms by which Zn^{2+} leases the cell after OGD are unclear. ZnT-1, a cellular Zn^{2+} exporter, is constitutively expressed in neurons (Palmiter and Findley, 1995). Transient (5 min) ischemic insult or exposure of primary hippocampal neurons to high extracellular Zn^{2+} (150 μM) induces a robust increase in expression of zinc transport-1 (ZnT1) mRNA within 1 h (Tsuda et al., 1997). Our results demonstrate that a rapid increase in intracellular Zn^{2+} with OGD treatment is followed by an increase in extracellular Zn^{2+}. ZnT-1 may participate in transporting the increased intracellular Zn^{2+} out of the cell, with increasing in efficiency with time from initiation of OGD, as a result of increased activity or expression. Alternatively, an increase of membrane permeability induced by ischemia and reperfusion may also be involved in the process of Zn^{2+} transfer from intracellular to extracellular space (Bazan and Rodriguez de Turco, 1980).

In summary, this study shows that ischemia and reperfusion both contribute to the accumulation of $[Zn^{2+}]_o$ during ischemia and reperfusion accompanied by a rapid increase of $[Zn^{2+}]_i$. It also shows that accumulation of extracellular Zn^{2+} was similar across all hippocampal regions. Pharmacological studies show that neither neuronal conduction nor extracellular calcium affect the increase of $[Zn^{2+}]_o$, while the NOS inhibitor, L-NAME, inhibits this type of extracellular Zn^{2+} accumulation. We have demonstrated that extracellular Zn^{2+} accumulation in ischemia and reperfusion is mechanistically quite different from calcium-mediated exocytotic release from presynaptic terminals. Our study raises the possibility that ischemia-induced release of Zn^{2+} into extracellular fluid plays a role in the neuronal damage. If that hypothesis is correct, alternative therapeutic approaches to the treatment of neuronal injury

followed ischemia and reperfusion might be derived toward reducing or preventing the ischemia-induced elevation of Zn^{2+}.

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