Chelating Intracellularly Accumulated Zinc Decreased Ischemic Brain Injury Through Reducing Neuronal Apoptotic Death

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Background and Purpose—Zinc has been reported to possess both neurotoxic and neuroprotective capabilities. The effects of elevated intracellular zinc accumulation following transient focal cerebral ischemia remain to be fully elucidated. Here, we investigated whether removing zinc with the membrane-permeable zinc chelator, N,N,N⁺,N⁺-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN), would decrease the intracellular levels of zinc in the ischemic tissue, leading to reduced brain damage and improved neurological outcomes.

Methods—Rats were pretreated with TPEN or vehicle before or after a 90-minute middle cerebral artery occlusion. Cerebral infarct volume, neurological functions, neuronal apoptosis, poly(ADP-ribose) polymerase activity, and cytosolic labile zinc were assessed after ischemia and reperfusion.

Results—Cerebral ischemia caused a dramatic cytosolic labile zinc accumulation in the ischemic tissue, which was decreased markedly by TPEN (15 mg/kg) pretreatment. Chelating zinc lead to reduced infarct volume compared with vehicle-treated middle cerebral artery occlusion rats, accompanied by much improved neurological assessment and motor function, which were sustained for 14 days after reperfusion. We also determined that reducing zinc accumulation rescued neurons from ischemia-induced apoptotic death by reducing poly(ADP-ribose) polymerase-1 activation.

Conclusions—Ischemia-induced high accumulation of intracellular zinc significantly contributed to ischemic brain damage through promotion of neuronal apoptotic death. Removing zinc may be an effective and novel approach to reduce ischemic brain injury. (Stroke. 2014;45:1139-1147.)

Key Words: brain ischemia • infarction, middle cerebral artery • poly(ADP-ribose) polymerases • zinc

Zinc (Zn⁺⁺) is the second most abundant transition metal in the human body. It is an essential cofactor for many enzymes and transcription factors. In addition, it has roles in neuronal synaptic transmission because high concentrations of Zn⁺⁺ are accumulated in many synaptic vesicles within the central nervous system in response to presynaptic activation. Zinc influx from the extracellular space is a well-demonstrated inducer of injury in cultured neurons. Recent reports have indicated that Zn⁺⁺ plays a major role in the mechanism of brain injury following ischemic stroke. Microdialysis studies have confirmed accumulation of extracellular Zn⁺⁺ in focal and global ischemia models. At high concentration has been consistently shown to be a critical mediator of neuronal death associated with experimental global ischemia. In contrast, the situation with focal ischemia is not as clear. Both neurotoxic and neuroprotective capabilities of zinc have been reported for experimentally induced focal ischemia. The mechanisms of zinc’s action are also not well understood.

In focal ischemia, EDTA calcium has been used to inhibit the brain’s zinc in vivo. However, EDTA calcium is not permeable to the neuronal cell membrane. It reduces only extra-cellular but not intracellular zinc. Apart from vesicular zinc, ≈90% of the total brain Zn⁺⁺ is bound to endogenous proteins. This binding is reversible because oxidative stress has been found to be a key regulator of intracellular zinc homeostasis by interfering with zinc binding to metallothioneins. Such intracellular release has been demonstrated in cultured neurons and ZnT3 knockout animals. Thus, EDTA calcium is not suitable for inhibiting both intracellular and extracellular zinc in the brain. In contrast, N,N,N⁺,N⁺-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN), would decrease the intracellular levels of zinc in the ischemic tissue, leading to reduced brain damage and improved neurological outcomes.
ethylenediamine (TPEN) reduces intra- and extracellular zinc because it can permeate the neuronal cell membrane.11 Injection of TPEN to the hippocampus has been shown to block all staining for zinc in the hippocampus but does not cause neuronal loss in the adult rat brain.11 Using a hippocampal slice model, it was shown that intracellular Zn2+ chelation with TPEN delayed the progression of Ca2+ overload after N-methyl-D-aspartic acid exposure, pointing out a potential dualistic synergism of the ions’ effects.12 Therefore, TPEN is a potential inhibitor of both intracellular and extracellular zinc in the brain. However, there is currently little evidence for such a protective effect of chelating both intra- and extracellular zinc in vivo.

In this study, we investigated whether treatment with zinc chelator TPEN would decrease the intracellular levels of zinc in the ischemic tissue and whether decreased zinc level would lead to reduced brain damage and sustained improvement in neurological outcomes. Furthermore, molecular mechanism of the neuroprotection afforded by chelating zinc was studied.

Methods

Drug Administration and Experimental Groups

TPEN (Sigma-Aldrich) was dissolved in dimethyl sulfoxide and then further diluted in physiological saline to a final concentration of 5 mmol/L in 10% dimethyl sulfoxide. To avoid mechanical damage inflicted by intracerebroventricular drug injection, we administered TPEN (5 or 15 mg/kg) by intraperitoneal injection at 30 minutes before MCAO, 0 or 30 minutes after reperfusion. Saline with 10% dimethyl sulfoxide was used as control.

A total of 216 rats were used in this study and were assigned randomly to 4 groups: vehicle-treated sham-operated group, vehicle-treated ischemic group, and 5 or 15 mg/kg TPEN-treated ischemic groups. Each group was further divided into 6 subgroups according to the different reperfusion time (0, 3, 12, 24, 72 hours, and 14 days).

Behavioral Tests

Three types of behavioral functional tests were performed. The observer was blinded to the experimental conditions. (1) Neurological deficits were scored using a modified scoring system developed by Longa et al.14 (2) Forelimb foot-fault-placing test, which examines forelimb function, was performed as described.15 The number of contralateral forelimb foot faults made by traversing the grid surface was calculated. (3) Open-field tests (locomotor activity) were performed as described.16 The total period of locomotion (in seconds) and rearing times were recorded for 5 minutes. Each animal was tested individually and only once.

Determination of Infarct Volume

After euthanizing the animals, brains were harvested, cut into 2-mm-thick coronal slices, and incubated in 1% 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich Chemical) solution at 37°C for 20 minutes. The stained slices were fixed in 10% formalin solution. The infarct area of each section was measured using the ImageJ Analysis Software. To minimize the error introduced by edema, an indirect method for calculating infarct volume was used.17 The noninfarcted region in the ipsilateral hemisphere was subtracted from that in the contralateral hemisphere. The infarct volume was presented as a percentage of the volume of the contralateral hemisphere.

Tissue Preparation

Immediately after animals were euthanized, brains were snap-frozen in liquid nitrogen and stored at −80°C. Coronal brain sections (20 μm) were obtained using a cryostat (CM1900, Leica) and mounted onto gelatin-coated glass slides.

Staining for Detecting Chelatable Zinc

To detect labile zinc in brain tissue, sections were stained with the zinc-specific membrane-permeable fluorescent dyes Newport Green (NG, N7990, Invitrogen) or TSQ (N-(6-methoxy-8-quinolyl)para-toluenesulfonamide, M688, Invitrogen).1,6 Air-dried sections were washed in saline and incubated with NG (10 μmol/L in PBS; pH, 7.4) for 3 minutes or TSQ (4.5 μmol/L in 140 mmol/L sodium barbital and 140 mmol/L sodium acetate buffer; pH, 10.0) for 90 seconds in the dark. After washing briefly in saline, images were captured by a fluorescence microscope (Nikon 80i, Japan).

Examination of Cell Death

A standard terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) procedure for frozen tissue sections was performed (In Situ Cell Death Detection Kit, POD, Roche Applied Science). Histological images were acquired using Nikon 80i microscope and NIS-Elements BR 3.0 software. Standardized photographic settings were used throughout.

Costaining of Cytosolic Labile Zinc With Cell Death

Colocalization experiment with TSQ and TUNEL was performed to determine the relationship between Zn2+ accumulation and cell death. Slices were first incubated with TSQ for 90 seconds in dark. After rinsing in PBS, the sections were fixed with 4% paraformaldehyde. TUNEL staining was then performed.

Western Blotting Analysis for Measuring Poly(ADP-Ribose) Polymerase-1 Activation and Cleavage

The entire ischemic hemisphere tissue was homogenized on ice in lysis buffer. The total protein content was determined with the bicinchoninic acid protein assay (Thermo Scientific). One hundred micrograms of total protein were electrophoresed in 8% SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA). Western blotting was performed as described. Antipoly(ADP-ribose) polymerase-1 (PARP-1) antibody (1:1000 dilution, Cell Signaling Technology) and antiarabbit antibody (1:1000; Santa Cruz Biotech, Santa Cruz, CA) were used as primary and secondary antibodies, respectively. The membranes were developed with the SuperSignal West Pico horseradish peroxidase substrate kit (Pierce, Rockford, IL). To control sample loading and protein transfer, the membranes were stripped and reprobed with β-actin antibody (1:4000; Sigma). Quantitative results were obtained by measuring the optical density and were expressed as the ratio of each targeted protein to β-actin expression.
Measurement of PARP-1 Activity by Poly(ADP-Ribosyl)ation Immunohistochemistry

Levels of protein-conjugated PAR were detected as a measure of PARP-1 activity. After fixing the cells with 4% paraformaldehyde in PBS, the immunohistochemistry was performed as described. Mouse monoclonal antibody (10 H) raised against PAR (Axchora, LLC, San Diego, CA, diluted to 10 μg/mL) and biotinylated horse antimouse IgG secondary antibody were used as the primary and second antibody. 3,3’-diaminobenzidine was used as a color substrate. The number of PAR-positively stained cells was computed blindly by using Nikon 80i microscope and NIS-Element BR 3.0 software. The number of PAR-positively stained cells within each field was counted.

Statistical Analysis

Results are reported as mean±SD. The difference between means was assessed by the ANOVA and post hoc least significant difference/Tamhane T2 tests for multiple comparisons, with P<0.05 considered statistically significant.

Results

Chelating Zinc Reduces Infarct Volume in Ischemic Rats

We first investigated whether zinc is involved in ischemic brain damage. Zinc chelator TPEN was used to remove cytosolic labile zinc. MCAO produced ipsilateral cerebral infarcts, whereas no lesions were observed in sham control rats (Figure 1A). The MCAO-induced cerebral infarct was reduced by administration of 5 mg/kg TPEN 30 minutes before MCAO, although the change was not statistically significant. When the animals were treated with 15 mg/kg TPEN, significant reduction of the infarct volume was observed 12, 24, and 72 hours after reperfusion (Figure 1A and 1B). This marked and sustained reduction in infarct volume could not be attributed to hypothermia or other alterations of physiological parameters because these parameters were essentially unchanged between animal groups (Table I in the online-only Data Supplement). More importantly, when we expanded the study to include TPEN administration at 0 and 30 minutes after reperfusion with neurological assessments ≤14 days, the reduction in infarct volume was maintained (Figure 1C and Figure I in the online-only Data Supplement). Furthermore, TPEN treatment dramatically increased the animal survival rate observed at 14 days (Figure 1D). These results suggest that chelating zinc is neuroprotective, indicating that zinc is involved in ischemic brain damage.

Chelating Zinc Improves Neurological Assessment and Motor Function

Next, we investigated neurological outcome of chelating zinc in the ischemic rats using 3 independent methods. Compared

Figure 1. N,N,N’,N’-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) treatment significantly reduced the cerebral infarct volume. TPEN or vehicle was injected intraperitoneally 30 minutes before middle cerebral artery occlusion (MCAO; TPEN-pre), or 0 (TPEN-0 minutes) or 30 minutes (TPEN-30 minutes) postreperfusion, and the infarct volume was measured at indicated times following reperfusion. A, Representative 2,3,5-triphenyltetrazolium chloride-stained coronal sections. B and C, Quantitative analysis of infarct volume. Data are means±SD (n=9 for B; n=4 for C); *P<0.05 vs vehicle-treated MCAO group. D, Rat survival rate at 14 days after reperfusion.
with vehicle-treated MCAO rats, treatment with 5 mg/kg TPEN had little effect on neurological deficit scores, whereas 15 mg/kg TPEN significantly decreased it (Figure 2A). Because 5 mg/kg TPEN showed limited effects on both infarct volume and neurological deficit score, further experiments used only 15 mg/kg TPEN.

The second neurological assessment was foot-fault-placing test, which revealed that the ischemia/reperfusion rats experienced severe functional deficits of the left forelimb compared with the sham group when assessed ≤72 hours (Figure 2B). Treatment with TPEN 30 minutes before MCAO markedly improved the behavioral deficits and enhanced the functional recovery when compared with vehicle-treated MCAO group. The third assessment, open-field activity, is associated with ipsilateral cerebral hemisphere volume and the motor function. Open-field activities (locomotion and rearing behavior) demonstrated major deficits in the MCAO group compared with the sham group (Figure 3A and 3B). Chelating zinc significantly reversed the altered locomotion and rearing behavior. Again, when we expanded the study to include TPEN administration at 0 and 30 minutes after reperfusion with neurological assessments ≤14 days, the recovered functions were sustained (Figures 2C, 2D, 3C, and 3D). These results suggest that zinc plays a major role in ischemia-induced behavioral dysfunction.

Cytosolic Labile Zinc Accumulates Following MCAO, Which Could Be Removed by TPEN

To quantify the cytosolic labile zinc accumulation in brain tissue following cerebral ischemia, selective zinc-specific fluorescence indicator NG was used. There was none or few NG-fluorescent cells in the sham group, whereas a drastic reperfusion time-dependent increase of NG-fluorescent cells was observable in the penumbra of MCAO rat (Figure 4A and 4B). The NG-fluorescent cells increased with time, indicating that cytosolic labile zinc level kept on increasing after ischemia, at least ≤24 hours. TPEN treatment reduced the NG-fluorescent cells by >50%. Similar results were obtained by using another zinc-specific fluorescent dye, TSQ (Figure II in the online-only Data Supplement). Double-staining experiments showed that NG-positive cells were largely colocalized with NeuN-positive cells, but not with astrocytes (Figure 4C). These findings indicate that cytosolic labile zinc accumulated time dependently in neurons following ischemia and that TPEN treatment could reduce the zinc level in the ischemic brain.

Cytosolic Labile Zinc Accumulation Is Associated With Neuronal Death

To elucidate the mechanism of zinc-induced brain damage, we evaluated the effect of zinc on cell death by the TUNEL...
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There was no TUNEL staining observed in the brain section of sham group, but TUNEL-positive cells were abundant in the MCAO group (Figure 5A and 5B). Chelating zinc with TPEN markedly decreased TUNEL-positive cells, indicating that cytosolic labile zinc accumulation contributed to neuronal death.

The causal relationship between zinc accumulation and neuronal death was further investigated through double-staining study. Figure 5C shows that the majority of zinc-stained cells displayed TUNEL positive, and treatment with TPEN considerably decreased the zinc and TUNEL colocalized cells. Figure 5D shows that TUNEL-positive cells were largely colocalized with NeuN-positive cells, not with astrocytes. These results suggest that ischemia-induced cytosolic labile zinc accumulation promoted neuronal death.

Chelating Zinc Reduces PARP-1 Activity and Cleavage

PARP-1 is an important protein in cell death regulation. During apoptosis, PARP-1 is cleaved by caspase-3 into 2 fragments (24 and 89 kDa). Thus, PARP-1 cleavage is a marker for apoptotic cell death. To further investigate the effect of zinc on apoptosis, the cleaved PARP-1 (89 kDa) fragment level was measured. Figure 6A shows that cleaved PARP-1 level was significantly increased time dependently after ischemia. TPEN pretreatment largely inhibited ischemic-induced PARP-1 cleavage (Figure 6B). These results are consistent with our finding using TUNEL staining (Figure 5B), indicating that zinc played a significant role in ischemia-induced neuronal death.

During ischemia, PARP-1 is activated by DNA breaks. The activated PARP-1 repairs DNA damage through the formation of PAR. Thus, accumulation of PAR is considered a surrogate marker of PARP-1 activation. We investigated the effect of chelating zinc on ischemia-induced activation of PARP-1. PAR-positive cells were not detectable in sham animals, but PAR-positive cells increased markedly with time in the ischemic tissue (Figure 6C and 6D). Treatment with TPEN reduced PAR accumulation, suggesting that zinc contributed to ischemic brain damage through upregulating PARP-1 activity.

Discussion

This study investigated the role of zinc in ischemic neuronal injury in vivo. We demonstrated that focal ischemia induced abnormal Zn²⁺ accumulation in the neurons (Figure 4), which occurred together with neuronal apoptosis (Figure 6). Treatment with zinc chelator TPEN reduced the accumulation of intracellular zinc (Figure 4), leading to a significant reduction in infarction volume, which was sustained up to ≥14 days (Figure 1), indicating that removing zinc likely spared brain tissues, rather than delayed tissue loss. This is further supported by the dramatic improvement in the survival rate. Furthermore, rescuing the brain tissue with zinc chelation resulted in much improved functional outcomes (Figures 2 and 3). These findings underscore zinc accumulation as an
important causal factor for the development of neuronal death after stroke.

Because TPEN removes both intracellular and extracellular free zinc and attenuates zinc-induced apoptotic death in different types of cultured cells, including neurons, it is a desirable chelator for investigating the role of zinc in ischemic brain injury in vivo. We found that ischemia-induced neuronal zinc accumulation decreased remarkably after TPEN pretreatment (Figure 4), demonstrating that TPEN was able to pass through the blood–brain barrier and the neuronal membrane, leading to successful intracellular zinc chelation. This finding is consistent with the report that repeated subcutaneous administration of TPEN significantly reduced the level of labile zinc throughout the entire brain. Our study also found that the intracellular unbound/loosely bound Zn\(^{2+}\) continued to increase during the course of 24 hours after ischemia (Figure 4), which may be attributable to continuing translocation of presynaptic Zn\(^{2+}\) into postsynaptic neuronal cells or Zn\(^{2+}\) release. Treatment with TPEN reduced the zinc level markedly at every measured time points after ischemia. These
Figure 5. N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) treatment attenuates neuronal cell death in penumbral tissue. 

A, Representative immunostaining for terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) staining at 12 and 24 hours after middle cerebral artery occlusion (MCAO). Arrows indicate TUNEL-positive cells. 

B, Quantification of TUNEL-positive cells. Data are means±SD (n=5). *P<0.05 vs vehicle-treated MCAO group. 

C, Colocalization of N-(6-methoxy-8-quinolyl)-para-toluenesulfonamide (TSQ)–stained and TUNEL-stained cells. White arrows indicate cells labeled with TSQ, TUNEL, or double labeled with both. 

D, Colocalization of TUNEL (arrows) and neuron-specific NeuN or astrocyte-specific glial fibrillary acidic protein (GFAP). Bars=25 μm. DAPI indicates 4′,6-diamidino-2-phenylindole; and TPEN-pre, TPEN injected intraperitoneally 30 minutes before MCAO.
results suggest that zinc chelation could be a potentially effective way for ischemic stroke treatment. Moreover, because the accumulation of zinc is a gradual process, the time window for treatment with zinc removal may be relatively longer than other neuroprotective approaches, which is demonstrated by the fact that TPEN administration at 30 minutes after reperfusion is effective (Figures 1, 2, and 3).

There is currently little in vivo evidence to support that zinc participates in neuronal apoptosis. It remains uncertain whether labile cytosolic zinc accumulation is a cause or an effect of neuronal apoptosis. Our present study showed that zinc-specific fluorescent (TSQ) neurons were also stained with TUNEL (Figure 5C), indicating that cytosolic accumulation of labile zinc is closely associated with neuronal death in ischemia/reperfusion pathogenesis. TPEN administration decreased both zinc and TUNEL staining, indicating that chelation of intracellular zinc decreases neuronal death. Therefore, our study provides the first in vivo evidence that endogenous labile zinc causes neuronal death following transient focal ischemia.

The mechanisms underlying Zn\textsuperscript{2+} toxicity in vivo are not well understood. An array of potential modes of action has been suggested (reviewed in Weiss et al\textsuperscript{3}). The present study extends our understanding of the mechanism by focusing on Zn\textsuperscript{2+}-induced PARP-1 cleavage and activation. Besides acting as a DNA repair enzyme, PARP-1 is also a key modulator of cell death in response to various stress stimuli. Zn\textsuperscript{2+} causes PARP-1 activation and nicotinamide adenine dinucleotide (NAD\textsuperscript{+}) depletion, and PARP-1 blocker was shown to attenuate delayed Zn\textsuperscript{2+}-induced injury to cultured neurons.\textsuperscript{22} Our in vivo results indicate that cerebral ischemia induced an increase in PARP-1 cleavage and the accumulation of PAR polymers in the ischemic tissue (Figure 6). More importantly, this effect was attenuated by chelating zinc, suggesting that Zn\textsuperscript{2+} participates in the activation and cleavage of PARP-1, thereby promoting neuronal death. This finding is supported by our earlier cellular study that PARP-1 modification is a key factor in zinc-induced hypoxic cell death.\textsuperscript{23} Together, these observations suggest an important role for PARP-1 activation in the mechanism of zinc-induced neuronal death following cerebral ischemia. Interestingly, based on our results (Figure 6), the majority of PARP-1 after MCAO is the 89-kDa inactive form, indicating that PARP-1 is largely cleaved and would lose activity after ischemia, whereas PARP-1 activation (Figure 6D) continued to increase at 3, 12, and 24 hours after MCAO. It is possible that PARP-2 may contribute toward the observed PAR accumulation, which will require further investigation.

In summary, our results show that cerebral ischemia resulted in abnormally high intracellular zinc accumulation.
Chelating zinc with TPEN not only reduced the zinc level, but also led to decreased brain damage and much improved neurological functions. It is likely that high level of zinc promoted neuronal apoptosis through PARP-1 activation. These findings provide a novel mechanism explaining ischemic brain damage.

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Disclosures
None.

References
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Supplementary Table I  Physiological parameters were not changed by TPEN treatment

<table>
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<th>Groups</th>
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<th>Rectal temperature (°C)</th>
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<td>5mg/kgTPEN</td>
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<td>15mg/kgTPEN</td>
<td>97.82 ± 7.65</td>
<td>36.94 ± 0.25</td>
<td>328.45 ± 12.55</td>
</tr>
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</table>

TPEN was administered to the rats 30 min before 90 min MCAO, and the physiological parameters were recorded during MCAO. Data are shown as mean ± SD, n = 9 per group. There were no statistically significant differences in any of the parameters between the groups.
**Supplementary Figure I** TPEN post-treatment significantly reduces the cerebral infarct area after focal ischemia/reperfusion in rats. Representative TTC-stained coronal sections from brains. TPEN or vehicle was injected intraperitoneally 0 min or 30 min post reperfusion after a 90 min MCAO, and the infarct volume was measured at 72 h and 14 d after MCAO.
Supplementary Figure II TPEN pre-treatment reduces cytosolic labile zinc accumulated in ischemic brain tissue. (A) Representative TSQ staining of brain sections at 0, 3, 12 and 24 h after MCAO. White arrows show representative TSQ-stained cells. Scale bar = 50 μm. (B) Quantitative analysis of positively stained cells using the fluorescent Zn$^{2+}$ indicator TSQ. Data are means ± S.D. (n = 5). *P < 0.05 versus vehicle-treated MCAO group.