Zn$^{2+}$ Induces Permeability Transition Pore Opening and Release of Pro-apoptotic Peptides from Neuronal Mitochondria*

Received for publication, September 13, 2001, and in revised form, October 9, 2001 Published, JBC Papers in Press, October 10, 2001, DOI 10.1074/jbc.M108834200

Dongmei Jiang§§, Patrick G. Sullivan§§, Stefano Sensi‡‡**, Oswald Steward¶¶¶, and John H. Weiss§§§§

From the Departments of §Neurology, ¶¶Anatomy and Neurobiology, and §§Neurobiology and Behavior and the §Reeve-Irvine Research Center, University of California, Irvine, California 92697-4292 and the ¶¶¶Department of Neurology, University “G. d’Annunzio,” Chieti 66013, Italy

Rapid entry of Ca$^{2+}$ or Zn$^{2+}$ kills neurons. Mitochondria are major sites of Ca$^{2+}$-dependent toxicity. This study examines Zn$^{2+}$-initiated mitochondrial cell death signaling. 10 nM Zn$^{2+}$ induced acute swelling of isolated mitochondria, which was much greater than that induced by higher Ca$^{2+}$ levels. Zn$^{2+}$ entry into mitochondria was dependent upon the Ca$^{2+}$ uniporter, and the consequent swelling resulted from opening of the mitochondrial permeability transition pore. Confocal imaging of intact neurons revealed entry of Zn$^{2+}$ (with Ca$^{2+}$) to cause pronounced mitochondrial swelling, which was far greater than that induced by Ca$^{2+}$ entry alone. Further experiments compared the abilities of Zn$^{2+}$ and Ca$^{2+}$ to induce mitochondrial release of cytochrome c (Cyt-c) or apoptosis-inducing factor. In isolated mitochondria, 10 nM Zn$^{2+}$ exposures induced Cyt-c release. Induction of Zn$^{2+}$ entry into cortical neurons resulted in distinct increases in cytosolic Cyt-c immunolabeling and in cytosolic and nuclear apoptosis-inducing factor labeling within 60 min. In comparison, higher absolute [Ca$^{2+}$], rises were less effective in inducing release of these factors. Addition of the mitochondrial permeability transition pore inhibitors cyclosporin A and bongkrekic acid decreased Zn$^{2+}$-dependent release of the factors and attenuated neuronal cell death as assessed by trypan blue staining 5–6 h after the exposures.

Neurotoxic effects of the excitatory neurotransmitter glutamate (“excitotoxicity”) contribute to secondary neuronal cell death following ischemia, intense seizures, and traumatic brain and spinal cord injuries (1). Although many studies have focused on the role of Ca$^{2+}$ in excitotoxic injury, recent studies have highlighted likely roles of another divalent cation, Zn$^{2+}$, which is sequestered in synaptic vesicles of many excitatory forebrain neurons (2). Under conditions of intense presynaptic activity, as occurs in epilepsy and ischemia, Zn$^{2+}$ is released along with glutamate into the synaptic space, where it may achieve peak levels of 100–300 μM (2). Observations that Zn$^{2+}$ accumulates in injured postsynaptic neurons and that extracellular Zn$^{2+}$ chelators decrease both Zn$^{2+}$ accumulation and the resultant neurodegeneration support the hypothesis that transsynaptic movement of Zn$^{2+}$ (“Zn$^{2+}$ translocation”) contributes to neuronal injury under these conditions (2).

Studies in simplified systems have provided clues to mechanisms through which Zn$^{2+}$ injures neurons. Upon glutamate receptor activation, it can gain entry into neurons largely through the same routes as Ca$^{2+}$, although with a distinct rank order of permeability: Ca$^{2+}$-permeable AMPA/kainate channels > voltage-sensitive Ca$^{2+}$ channels > NMDA1 channels (3). With particularly rapid entry through highly Zn$^{2+}$- and Ca$^{2+}$-permeable AMPA/kainate channels, resulting in microsomal Zn$^{2+}$, rises, microfluorometric studies have demonstrated abrupt dissipation of the mitochondrial membrane potential (∆ψm) and generation of reactive oxygen species (3). These observations are consistent with findings that with micromolar exposures, Zn$^{2+}$ is taken up into, inhibits respiration of, and induces opening of a nonspecific large conductance channel, the mitochondrial permeability transition pore (mPTP), in isolated non-neural mitochondria (4–6).

Although studies of neurotoxicity caused by less intense Zn$^{2+}$ exposures have not explicitly implicated mitochondria, they have reported effects, including reactive oxygen species generation and reduction of ATP levels (7–10), that could in part reflect a mitochondrial site of action. Also, although observations of DNA fragmentation or attenuation of injury by caspase or protein synthesis inhibitors suggest activation of apoptotic processes, other studies showing swelling of intracellular organelles and demonstrating protection by antioxidants favor necrotic injury mechanisms (7, 9, 11).

An emerging body of evidence supports a central role for mitochondria in mediating both necrotic and apoptotic forms of cell death. Indeed, although acute mitochondrial disruption initiated by Ca$^{2+}$ overload is generally associated with necrotic cell death, under certain conditions, mitochondria release peptide mediators of apoptotic cell death pathways, including cytochrome c (Cyt-c), apoptosis-inducing factor (AIF), and Smac/DIABLO (12, 13). The nature of the release mechanism(s) is at present highly debated, but the release is modulated by members of the Bcl-2 protein family and can occur following mPTP opening (12–14). Based on the lines of evidence outlined above, we hypothesize that as with Ca$^{2+}$, the toxic effects of Zn$^{2+}$ are

* This work was supported by National Institutes of Health Grants NS30884 and AG00836 (to J. H. W.), Grant AG00919 (to S. L. S.), Grant NS30884 and AG00836 (to J. H. W.), Grant AG00919 (to S. L. S.), Grant AG00836 (to O. S.), and Grant 2T32AG00096 (to D. J.); a grant from the Alzheimer’s Association (to J. H. W.); and a NeuTherapeutics fellowship (to P. G. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§§ To whom correspondence should be addressed: Dept. of Neurology, University of California, Irvine, CA 92697-4292. Tel.: 949-824-6774; Fax: 949-824-1668; E-mail: jweiss@uci.edu.

¶¶ Both authors contributed equally to this work.

§§§ Both authors contributed equally to this work.

1 The abbreviations used are: NMDA, N-methyl-D-aspartate; mPTP, mitochondrial permeability transition pore; Cyt-c, cytochrome c; AIF, apoptosis-inducing factor; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline; MOPS, 4-morpholinopropionic acid; HSS, HEPES-buffered salt solution; CsA, cyclosporin A; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid.
substantially mediated through effects on mitochondria. To test this idea, we have compared the abilities of Zn$^{2+}$ and Ca$^{2+}$ to trigger mitochondrial swelling (both in isolated brain mitochondria and in intact neurons) via opening of the mPTP and their efficacies in inducing release of the apoptotic mediators Cyt-c and AIF.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**—Bongkrekic acid was purchased from BIOMOL Research Labs Inc. (Plymouth Meeting, PA). Cyclosporin A, 2,3-dihydroxy-6-nitro-7-sulfamoylbenzozquinoline (NBQX), and staurosporine were purchased from Molecular Probes (Eugene, OR). Tissue culture media and serum were purchased from Life Technologies, Inc. All other chemicals and reagents were obtained from common commercial sources.

**Animals**—Adult male Sprague-Dawley rats were used in the isolated mitochondrial experiments. Marine cortical cultures were prepared from embryonic Swiss-Webster mice. Prior to death, all animals were housed individually on a 12-h light/dark cycle with access to water and food *ad libitum*. All animal procedures were approved by the Institutional Animal Care and Use Committee.

**Mitochondrial Isolation, Swelling Assessment, and Measurement of Cyt-c Release**—Isolated mitochondria were prepared largely as described and swelling rates were assessed in energized conditions as the decrease in absorbance (light scattering) (15). Briefly, 250 μg of mitochondria was suspended in 1 ml of respiration buffer (250 mM sucrose, 20 mM HEPES, 2 mM MgCl$_2$, and 2.5 mM inorganic phosphates, pH 7.2) plus 5 mM glutamate and succinate (energized conditions) or an isotonic buffer (150 mM KCl, 20 mM MOPS, 10 mM Tris, 1 μM rotenone, 1 μM antimycin, and 2 μM iodonitrophenol (to ensure complete equilibration of Ca$^{2+}$ and Zn$^{2+}$ ions across the mitochondrial membrane), pH 7.2) (de-energized conditions). After a 5-min preincubation at 36 °C and base-line measurement, CaCl$_2$ (50 mM/ml of protein for energized conditions or 50 μM for de-energized conditions) and/or ZnSO$_4$ (10 and 100 μM) was added to the cuvette in equal volumes (10 μl) at t = 0 (10 μl of H$_2$O was used as a control). Alamethicin (40 μg/ml) was added at the end of all experiments to induce maximal swelling. When used, bongkrekic acid (5 μM) or ruthenium red (20 μM) was added prior to the 5-min preincubation and was present throughout the experiment. The swelling rate was measured as the decrease in absorbance (540 nm) over 4.5 min following addition of ions. For data analysis, basal (Ca$^{2+}$/Zn$^{2+}$-independent) absorbance changes were subtracted, and swelling was expressed as the loss of absorbance/unit time/μg of mitochondrial protein (arbitrary units). For statistical analysis, the high and low were omitted for each group.

Release of Cyt-c from isolated mitochondria into the media was measured using a Quantikine M cytochrome c enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN). Mitochondria (250 μg) were incubated under energized conditions exactly as described above. Exactly 4.5 min after addition of Ca$^{2+}$ and/or Zn$^{2+}$, the suspension was centrifuged (13,000 × g, 10 min, 4 °C); protease inhibitor mixture (Sigma) was added; and both the supernatant and pellet were processed for assays (all in duplicate) following the manufacturer’s recommendations. For data analysis, basal release in the absence of Ca$^{2+}$ or Zn$^{2+}$ was subtracted from all groups.

**Cortical Culture Preparation and Intracellular Cation (Ca$^{2+}$ or Zn$^{2+}$) Exposures**—Dissociated mixed neocortical cultures were prepared largely as described (16) from 14–16-day-old embryonic Swiss-Webster mice (plated at 1–2 × 10$^5$ cells/cm$^2$) on previously established astrocytic monolayers. Before exposures, 12-15-day-old cultures were transferred to a simplified HEPES-buffered salt solution (HSS) consisting of 120 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl$_2$, 20 mM HEPES, 1.8 mM CaCl$_2$, and 10 mM NaOH, pH 7.4. Entry of Zn$^{2+}$ and/or Ca$^{2+}$ into neurons through voltage-sensitive Ca$^{2+}$ channels was triggered by replacement of HSS with depolarizing solutions containing 50 mM K$^+$ and/or Ca$^{2+}$, the suspension was centrifuged (13,000 × g, 10 min, 4 °C); protease inhibitor mixture (Sigma) was added; and both the supernatant and pellet were processed for assays (all in duplicate) following the manufacturer’s recommendations. For data analysis, basal release in the absence of Ca$^{2+}$ or Zn$^{2+}$ was subtracted from all groups.

In some experiments, rapid Ca$^{2+}$ influx was induced by exposure to HSS containing 100 μM NMMA and 10 μM glycin. An additional exposure (exposure 2) was given in the presence of 25 μM MK-801 and NBQX (both at 10 μM) to prevent influx through glutamate channels. For cell injury and immunocytochemistry experiments, exposures were for 6 min (25 °C) and were terminated by replacing exposure solution with minimal essential medium plus glucose (25 mM) prior to returning the dishes to the 37 °C/5% CO$_2$ incubator. Immunocytochemistry was performed at various time points after the treatments, and trypan blue exclusion assays were performed 5–6 h after treatment. For confocal imaging experiments (described below), treatments were for 10 min, followed by washout into HSS, and the mitochondrial morphology was monitored as described.

**Confocal Fluorescence Microscopy**—Cortical cultures were loaded with the mitochondrial sequestered dye MitoTracker Green FM (200 nm, 30 min, 37 °C), washed, and mounted on the stage of a confocal microscope. Images were obtained with an Olympus Fluovview confocal microscope (100× objective; excitation (argon), 488 nm; emission, 516 nm) at base line and 10, 30, and 50 min following the start of brief exposures (10 min, 25 °C) as described above.

At base line, mitochondria appeared as thin rod-like structures; after K$^+$ or Zn$^{2+}$ buffer exposures, mitochondrial morphological changes were evident, with progressive rounding and increases in diameter. To quantify mitochondrial changes after these exposures, mitochondria in each cell were visually assigned an “swelling index” on a 4-point scale (0, minimal change from base line; 1, evident increased swelling, but of a minority of mitochondria; 2, increased swelling, affecting most mitochondria; and 3, swelling of virtually all mitochondria).

**Immunocytochemistry**—Confocal microscopy was performed using primary antibodies to Cyt-c (BD Pharmingen, San Diego, CA) and AIF (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Briefly, after exposures as described above, cultures were fixed with 4% paraformaldehyde for 30 min; blocked for 1 h at 25 °C in phosphate-buffered saline with 2% horse serum, 0.1% Triton X-100, and 1% bovine serum albumin; and incubated with primary antibody (diluted 1:1000 for Cyt-c and 1:250 for AIF) in blocking solution for 16 h at 4 °C. Cultures were then washed and incubated with biotinylated secondary antibody for 2 h in phosphate-buffered saline with 0.1% Tween 20 and 1% bovine serum albumin prior to reaction with ABC reagent (Vector Labs, Inc., Burlingame, CA) for 1 h and with 3,3'-diaminobenzidine for 5 min. Control cultures processed without primary antibody exhibited no specific staining.

To assess the extent of Cyt-c and AIF release, we estimated the percentage of positive cells by cell counting. For Cyt-c, only cells that showed strong homogeneous staining throughout nearly all of the cytosolic space (although generally sparing the nucleus) were considered positive. For AIF, strong homogeneous staining in the cytosol and/or the nucleus was defined as positive. For both markers, faint, punctate, or blotchy patterns of staining, seen in almost all neurons at base line, were considered negative (Fig. 3).

In each experiment, positive cells were counted in 8–20 random digitally photographed 200× microscope fields from each of one to three glass-bottomed dishes for each condition, with each dish usually containing 50–200 neurons. Values for each condition were calculated as the percentage of positive cells in each field, averaged over all fields counted. Because of some variability in culture preparations in basal levels of release and responsiveness to agonist exposures, significance was determined by paired t tests between each condition with paired K$^+$ buffer conditions.

**Trypan Blue Exclusion**—At 5–6 h after treatments, cells were incubated for 2–5 min in a solution of 0.2% trypan blue in phosphate-buffered saline. In each experiment, nonviable cells (stained blue) and viable cells (colorless) were counted in 5–10 random digitally photographed 100× microscope fields from each of one to three glass-bottomed dishes for each condition, with each dish usually containing between 100 and 400 neurons. As in the immunocytochemistry experiments, values were calculated as the percentage of nonviable cells, and significance was determined by paired t tests with K$^+$ buffer conditions.

**RESULTS**

Zn$^{2+}$ Induces Swelling of Both Isolated Mitochondria and Mitochondria in Intact Cortical Neurons—Prior studies have demonstrated the ability of relatively high concentrations of Zn$^{2+}$ (2–10 μM) to induce swelling of isolated liver mitochondria, indicative of opening of the mPTP (6). Accordingly, our first goal was to compare the degree of swelling of neuronal mitochondria induced by Zn$^{2+}$ versus that induced by Ca$^{2+}$.

In mitochondria isolated from adult rat forebrain, swelling was evidenced by rapid decreases in absorbance after exposure to the divalent cations (Fig. 1A). Under both energized (with oxidative substrates) and de-energized (with iodonitrophenol to ensure complete equilibration of Ca$^{2+}$ and Zn$^{2+}$ ions across the mitochondrial membrane) conditions, mitochondria exhibited...
**Zn$^{2+}$-induced Mitochondrial Release of Cyt-c and AIF**

In isolated rat brain mitochondria, swelling was measured using an enzyme-linked immunosorbent assay (ELISA). As illustrated by representative traces (panels a and c) and in a compilation of seven independent experiments (swelling expressed in arbitrary units (AU)) (panels b and d), addition of Zn$^{2+}$ (10 or 100 nM) initiated rapid swelling. Although Ca$^{2+}$ (energized conditions, 50 nmol/mg of protein; and de-energized conditions, 50 μM) alone had little effect, addition of Ca$^{2+}$ with either 10 or 100 nM Zn$^{2+}$ induced greater swelling than Zn$^{2+}$ alone. (Zn$^{2+}$ + Ca$^{2+}$)-induced swelling was blocked by ruthenium red (RR; 20 μM) under energized conditions (panel b) and by bongkrekic acid (BA; 5 μM) under both energized and de-energized conditions (panels b and d). Alamethicin (ala; 40 μg/ml) was added at the end of all experiments to induce maximal swelling. Error bars represent means ± S.E. Analysis of variance revealed a significant group-dependent difference in swelling under both energized and de-energized conditions (p < 0.0001), with post-hoc analysis by Fisher's post-hoc least square difference test. *p < 0.05 compared with Ca$^{2+}$; #p < 0.05 compared the same concentration of Zn$^{2+}$ alone. B, Zn$^{2+}$-induced Cyt-c release. Cyt-c release from isolated energized forebrain mitochondria was measured using an enzyme-linked immunosorbent assay upon exposures identical to those used to assess swelling (see “Experimental Procedures”). Parallel effects on swelling, addition of Zn$^{2+}$ alone (10 or 100 nM) or with Ca$^{2+}$ (50 nmol/mg) induced far more Cyt-c release than Ca$^{2+}$ alone. Error bars represent means ± S.E. Significance was assessed as described for A. *p < 0.05 compared with 50 μM Ca$^{2+}$; #p < 0.05 compared 100 nM Zn$^{2+}$.

Rapid swelling upon addition of Zn$^{2+}$ (10 or 100 nM) (Fig. 1A). Although addition of Ca$^{2+}$ (energized conditions, 50 nmol/mg of protein, and de-energized conditions, 50 μM) induced little mitochondrial swelling under either condition, addition of Ca$^{2+}$ alone with Zn$^{2+}$ induced greater swelling than Zn$^{2+}$ alone. Higher levels of Ca$^{2+}$ (energized conditions, 100 nmol/mg of protein; and de-energized conditions, 100 μM) induced rapid swelling, whereas <10 nM Zn$^{2+}$ had little effect (data not shown). Inclusion of ruthenium red, an inhibitor of the Ca$^{2+}$ uniporter, blocked Zn$^{2+}$-induced swelling under energized conditions, suggesting that Zn$^{2+}$ gains entry into mitochondria through this route. Addition of the mPTP antagonist bongkrekic acid inhibited mitochondrial swelling under all conditions, confirming that the swelling is due to opening of this high conductance channel (Fig. 1A).

A consequence of mPTP opening is the catastrophic release of mitochondrial proteins into the medium. Release of Cyt-c from isolated energized mitochondria was monitored under conditions identical to those described above (Fig. 1B). In the presence of Ca$^{2+}$ (50 nmol/mg of protein), only a small amount of Cyt-c was released, whereas Zn$^{2+}$ (10 and 100 nM) alone or in combination with Ca$^{2+}$ resulted in a 3–6-fold increase in release.

To assess morphology of mitochondria in intact neurons, neurons were loaded with MitoTracker Green and examined by confocal microscopy (see “Experimental Procedures”). This dye has the advantage of being largely unaffected by loss of Δψm or reactive oxygen species generation in neurons (17). Induction of Zn$^{2+}$ and/or Ca$^{2+}$ entry into neurons was accomplished by exposure to the ions in the presence of K buffer, which depolarizes neurons and permits entry of either divalent cation through voltage-sensitive Ca$^{2+}$ channels. Exposures were carried out in the presence of the glutamate blockers MK-801 and NBQX (each at 10 μM) to prevent effects of endogenously released glutamate. Neurons were imaged at base line and 10, 30, and 50 min after the start of a brief exposure (10 min) to HSS (“sham wash”), to K buffer (triggering entry of Ca$^{2+}$ alone), or to K/Zn buffer (causing entry of both Ca$^{2+}$ and Zn$^{2+}$). At base line, mitochondria appeared as slim, strongly fluorescent, rod-like structures. After K/Zn buffer exposures, there was a remarkable change in mitochondrial morphology as evidenced by a progressive increase in mitochondrial diameter that peaked within 30–50 min of exposure onset. In contrast, little change was noted after exposures either to K buffer alone or to sham wash (Fig. 2). Over 80% of neurons imaged after KZn buffer exposures showed a substantial increase in mitochondrial swelling at 50 min (swelling index of 2 or 3; see “Experimental Procedures”), in contrast to <25% after exposure to K buffer alone (Fig. 2B).

**Zn$^{2+}$-induced Release of Cyt-c and AIF from Mitochondria in Cortical Neurons: Role of mPTP**—The subcellular localization of Cyt-c and AIF in cortical neurons was assessed immunocytochemically after exposures either to sham wash or to induction of Zn$^{2+}$ or Ca$^{2+}$ entry as described above. In untreated (sham-washed) cultures, staining for both AIF and Cyt-c was light and often appeared to be localized in puncta distributed throughout the cytoplasm, consistent with the mitochondrial localization of these peptides.

Following a 6-min exposure to K/Zn buffer (with MK-801 and NBQX, as described above), there was a dramatic increase in immunostaining for both Cyt-c and AIF. Increased cytoplasmic Cyt-c staining was evident within 30 min, appearing initially as clumps of stain; and within 1 h, near-homogeneous cytoplasmic staining, usually sparing the nucleus, was evident in many neurons (Fig. 3). Increases in AIF staining were evident within 1 h of the K/Zn buffer exposures; and unlike Cyt-c, which spares the nucleus, at 3 h after the exposures, many neurons showed strong nuclear AIF accumulation (Fig. 3). Exposure to staurosporine (1 μM, 5 h), a potent inducer of neuronal apoptosis that has been reported to induce both Cyt-c and AIF release, resulted in qualitatively similar patterns of Cyt-c and AIF staining in many neurons (data not shown).

For quantification of Cyt-c or AIF release, a uniform time of assessment (1 h for Cyt-c and 3 h for AIF) and criteria for positive staining (Fig. 3; see “Experimental Procedures”) were established. After KZn buffer exposures, 44 ± 4% of neurons
(n = 12 experiments) were judged to be Cyt-c-positive and 35 ± 3% (n = 10 experiments) to be AIF-positive. In contrast, little Cyt-c or AIF labeling was evident in cultures exposed to sham wash, to 300 μM Zn²⁺, or to K buffer alone (Fig. 4).

Rapid Ca²⁺ entry through NMDA channels has been reported to trigger mitochondrial Ca²⁺ overload with opening of the mPTP and release of Cyt-c (18–22). Thus, to induce toxic Ca²⁺ overload, cultures were subjected to NMDA exposure (100 μM NMDA + 10 μM glycine for 6 min), which induces substantial injury in 24 h. Although the Zn²⁺ exposures employed have been found to induce Δ[Zn²⁺], of several hundred nanomolar (3, 23), the NMDA exposures induce markedly higher (Ca²⁺), rises of >10 μM (24). However, in comparison with Zn²⁺ influx, the NMDA exposures induced markedly less Cyt-c release (Fig. 4A).

The mPTP antagonists cyclosporin A (CsA) and bongkrekic acid were used to examine the possibility that opening of the mPTP is involved in Zn²⁺-induced release of Cyt-c and AIF. CsA (10 μM) or bongkrekic acid (5 μM) was added for 30 min before, during, and after the K/Zn buffer exposures, and Cyt-c and AIF immunoreactivity was examined 1 and 3 h, respectively, after the exposures. We found CsA to attenuate release of both Cyt-c and AIF, and bongkrekic acid to attenuate release of AIF (effect on Cyt-c release not tested) (Fig. 4). Thus, these data support a role for mPTP opening in the Zn²⁺-dependent release of Cyt-c and AIF from mitochondria.

PTP Blockers Attenuate Zn²⁺-induced Neuronal Cell Death—The demonstration that Zn²⁺ can induce opening of the mPTP and release of the apoptotic mediators Cyt-c and AIF into the cytoplasm does not indicate that these mechanisms contribute to the resultant Zn²⁺-dependent neuronal death. Thus, further experiments were carried out using the mPTP blockers to test the possibility that mPTP opening does play a role in cell death resulting after the Zn²⁺ exposures. Cortical cultures were exposed to K/Zn buffer exactly as described

Fig. 2. Zn²⁺ induces mitochondrial swelling in live neurons. A, representative confocal images. After loading with MitoTracker Green, cortical cultures were mounted on the stage of a confocal microscope and exposed to HSS alone (Sham), to K buffer, or to K/Zn buffer. After 10 min, cultures were washed into HSS. Confocal images of cultures exposed to K buffer (left panels) or to K/Zn buffer (right panels) at base line (Basal) and 30 min after the start of exposure are shown. The lower panels show the neurons under differential interference contrast (DIC). Note the rod-like appearance of most mitochondria at base line that persists after K buffer exposure (arrowheads), in contrast to the progressive rounding and swelling of many mitochondria after K/Zn buffer exposure (arrows). B, summary data. Cultures were imaged after exposure to sham wash, to K buffer, or to K/Zn buffer as described for A, and the extent of mitochondrial swelling was compared with that at base line at each time point visually assessed on a 4-point scale (0 (no change from base line)) to 3 (maximal swelling); see “Experimental Procedures”). The stacked bar graph indicates the percentage of neurons in each category after each exposure (n = 17 (K/Zn buffer), 13 (K buffer), and 8 (sham wash)). Note the large majority of neurons showing substantial mitochondrial swelling only after K/Zn buffer exposures.

Fig. 3. Representative appearance of neurons immunostained for Cyt-c or AIF. Cultures were immunolabeled for Cyt-c (upper panels) or for AIF (lower panels) after exposure to sham wash (left panels) or 1 h (for Cyt-c) or 3 h (for AIF) after induction of Zn²⁺ entry by a 6-min exposure to K/Zn buffer in the absence (middle panels) or presence (right panels) of CsA. Black arrows show representative neurons considered to be Cyt-c-negative or AIF-negative. Note the typical faint and/or punctate labeling of neurons in sham wash-exposed cultures as well as of certain neurons after the K/Zn buffer exposures. The red arrows in the upper right panel show neurons with a non-homogeneous (or clumpy) Cyt-c staining pattern. Such neurons, often seen at earlier time points (30 min) or in the presence of mPTP blockers, were also counted as Cyt-c-negative. Red arrows show representative neurons considered to be Cyt-c-positive or AIF-positive. These were most evident after K/Zn buffer exposures (middle panels). Note the homogeneous cytosolic Cyt-c staining (upper panels) and the frequent strong nuclear accumulation of AIF staining (lower panels). The red arrow in the lower right panel shows AIF labeling in a strong homogeneous cytosolic pattern with little nuclear staining. Such neurons, often seen at earlier time points (1 h) and in the presence of mPTP blockers, were counted as AIF-positive. Of note, these high power micrographs show the typical appearance of immunostained cells, but do not accurately represent proportions of immunopositive cells under the different conditions. Bar = 25 μm.
Advances in recent years have established that mitochondria play a critical role in neuronal injury caused by excitotoxic Ca\textsuperscript{2+} overload (25). Mitochondria can buffer large amounts of intracellular Ca\textsuperscript{2+}; and upon Ca\textsuperscript{2+} overloading, several events are set in motion that can mediate neuronal death. These include cessation of mitochondrial function (causing loss of ATP), loss of mitochondrial membrane integrity due to opening of the mPTP, and increased generation of reactive oxygen species. Such acute effects are generally thought to underlie rapidly evolving necrotic forms of cell death characterized by organelar swelling and cell lysis. With less intense glutamate exposures, when energy levels may be relatively preserved, slowly evolving apoptotic cell death pathways may also be triggered (26), in large part after mitochondrial release of certain pro-apoptotic peptides, including Cyt-c, which plays a role in the activation of the apoptotic effector enzyme caspase-3, and AIF, which translocates to the nucleus and induces fragmentation of nuclear DNA (12).

Our findings suggest that Zn\textsuperscript{2+} might initiate neuronal injury through similar mechanisms, but with greater potency than Ca\textsuperscript{2+}. Indeed, in initial studies on isolated neuronal mitochondria, we found that in comparison with Ca\textsuperscript{2+}, Zn\textsuperscript{2+} was remarkably potent, with 10 nM levels causing rapid swelling of both energized and de-energized mitochondria. Although these levels are much lower than those previously reported to induce swelling of isolated liver mitochondria (6), such a disparity in sensitivity might be compatible with the observation that liver cells have high [Zn\textsuperscript{2+}] (12–15\% of total cytosolic Zn; 27). Consistent with a previous report (28), Zn\textsuperscript{2+}-induced swelling of energized mitochondria appeared to depend upon uptake through the Ca\textsuperscript{2+} uniporter. Induction of swelling under de-energized conditions indicates that, provided an entry route (via ionomycin pores), the effect is independent of electron transport and \(\Delta \psi\). Although the specific site of Zn\textsuperscript{2+} effects on mitochondria is uncertain, the swelling appears to result from mPTP opening and is coupled to release of Cyt-c.

This high Zn\textsuperscript{2+} sensitivity carried over to intact neurons, in which depolarizing exposures estimated to induce submicromolar Zn\textsuperscript{2+}, rises (3, 23) caused greater mitochondrial swelling than exposures in the absence of Zn\textsuperscript{2+} that induce far higher [Ca\textsuperscript{2+}], rises (24). Paralleling effects on swelling, the [Zn\textsuperscript{2+}]), rises also caused more release of the apoptotic mediators Cyt-c and AIF. The additional observations that mPTP antagonists attenuated both release of the pro-apoptotic factors and the subsequent neuronal loss suggest that, as in the case of Ca\textsuperscript{2+} overload (12–14), mPTP opening might contribute to both of these downstream events. There are two conspicuous differences between these effects and the present observation in isolated mitochondria. First, although they occurred at estimated intracellular levels below those needed for Ca\textsuperscript{2+} to cause...
similar effects, they were much higher than the 10 nM levels of Zn$^{2+}$ found to trigger mPTP opening in isolated mitochondria. Although further studies will be necessary to fully ascertain the basis for this apparent discrepancy in Zn$^{2+}$ sensitivity, it is likely to reflect the presence of physiological Zn$^{2+}$ buffers and binding sites in intact cells (29). Second, the partial attenuation of pro-apoptotic factor release and injury by mPTP antagonists is in contrast to the virtual complete block of mPTP opening in isolated mitochondria. This is reminiscent of observations of Ca$^{2+}$-dependent release of Cyt-c in intact neurons, where mPTP blockers have often shown poor efficacy in attenuating release. Although it is clear that mPTP opening can result in Cyt-c release, it is presently uncertain whether the incomplete block observed reflects the existence of mPTP-independent release mechanisms (30) or whether sufficiently strong divalent cation loading somehow overwhelms the blocking capability of the mPTP antagonists (13, 14, 31).

In summary, our observations may strengthen the analogies between toxic effects induced by Zn$^{2+}$ and Ca$^{2+}$; both can mediate injury through effects on mitochondria, and both might trigger either acute necrotic or slower apoptotic forms of injury depending largely upon the intensity of the ionic exposure. Parallels between Zn$^{2+}$- and Ca$^{2+}$-dependent injury mechanisms may well extend to human diseases in which excitotoxicity is implicated. Indeed, indices of apoptotic damage, including nuclear fragmentation, Cyt-c release, and attenuation of injury by caspase inhibitors, have been reported in submaximal ischemia and glutamate exposures (32, 33). Such apoptotic signaling might be hypothesized to be of particular importance in vivo under conditions in which only a subset of mitochondria in a cell undergo permeability transition (14), perhaps reflecting subcellular concentrations of Ca$^{2+}$ or Zn$^{2+}$ adjacent to sites of postsynaptic entry. Additional recent studies have raised the possibility that in addition to translocation, under certain conditions, Zn$^{2+}$ release from intracellular stores might injure neurons (34). Whatever the sources, understanding ways in which cytosolic [Zn$^{2+}$]$_i$ rises contribute to necrotic and/or apoptotic neuronal injury is likely to powerfully impact the development of neuroprotective interventions for these conditions.

Acknowledgment—We thank Simin Amindari for expert assistance with the cell cultures.

REFERENCES

1. Choi, D. W. (1988) Neuron 1, 623–634
2. Frederiksson, C. J., Suh, S. W., Dufresne, D., Frederiksson, C. J., and Thompson, R. B. (2000) J. Nutr. 130, 1471S–1483S
3. Sensi, S. L., Yin, H. Z., Carriedo, S. G., Rao, S. S., and Weiss, J. H. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 2414–2419
4. Brierley, G. P., and Knight, V. A. (1967) Biochemistry 6, 3892–3901
5. Skulachev, V. P., Chistyakov, V. V., Jasaitis, A. A., and Smirnova, E. G. (1967) Biochim. Biophys. Acta 149, 175–182
6. Wudarczyk, J., Debska, G., and Lenartowicz, E. (1999) Arch. Biochem. Biophys. 363, 1–8
7. Masiewicz, K., Khurshid, Y., Osztéry, D., Tsunoda, S., and Cagnoli, M. C. (1997) Exp. Neurol. 146, 171–178
8. Kim, E. Y., Koh, J. Y., Kim, Y. H., Sohn, S., Lee, K., and Gwag, B. J. (1999) Eur. J. Neurosci. 11, 327–334
9. Kim, Y. H., Kim, E. Y., Gwag, B. J., Sohn, S., and Koh, J. Y. (1999) Neuro-science 89, 175–182
10. Sheline, C. T., Behrens, M. M., and Choi, D. W. (2000) J. Neurosci. 20, 3139–3146
11. Lohner, D., Canzoniero, L. M., Manzerra, P., Gottron, F., Ying, H., Knudson, M., Tian, M., Dugan, L. L., Korschner, G. A., Sheline, C. T., Korsmeyer, S. J., and Choi, D. W. (2000) Cell. Mol. Biol. 46, 797–806
12. Kroemer, G., and Reed, J. C. (2000) Nat. Med. 6, 513–519
13. Bernardi, P., Petrò, V., De Lisa, F., and Forte, M. (2001) Trends Biochem. Sci. 26, 112–117
14. Crompton, M. (1999) Biochem. J. 341, 233–249
15. Sullivan, P. G., Geiger, J. D., Mattson, M. P., and Scheff, S. W. (2000) Ann. Neurol. 48, 723–729
16. Yin, H. Z., Ha, D. H., Carriedo, S. G., and Weiss, J. H. (1998) Brain Res. 781, 45–55
17. Buckman, J. F., Hernandez, H., Kress, G. J., Voytyakova, T. V., Pal, S., and Reynolds, I. J. (2001) J. Neurosci. Methods 104, 165–176
18. White, R. J., and Reynolds, I. J. (1995) J. Neurosci. 15, 1318–1328
19. Peng, T. I., Jou, M. J., Sheu, S. S., and Greenamyer, J. T. (1998) Exp. Neurol. 149, 1–12
20. Schinder, A. F., Olson, E. C., Spitzer, N. C., and Montal, M. (1996) J. Neurosci. 16, 6125–6133
21. Dubinsky, J. M., and Levi, Y. (1998) J. Neurosci. Res. 53, 728–741
22. Atlante, A., Gagliardi, S., Marra, E., Calissano, P., and Passarella, S. (1999) J. Neurochem. 73, 237–246
23. Canzoniero, L. M., Turetsky, D. M., and Choi, D. W. (1999) J. Neurosci. 19, RC11
24. Hyrc, K., Handran, S. D., Rothman, S. M., and Goldberg, M. (1997) J. Neurosci. 17, 6669–6677
25. Nicholls, D. G., and Budd, S. L. (2000) Physiol. Rev. 80, 315–360
26. Bonfoco, E., Kraine, D., Akrakera, M., Nicotera, P., and Lipton, S. A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7162–7166
27. Brand, I. A., and Kleineke, J. (1996) J. Biol. Chem. 271, 1941–1949
28. Saria, N. E., and Niva, K. (1994) FEBS Lett. 356, 195–199
29. Aeschner, M., Cherian, M. G., Klaassen, C. D., Palmer, R. D., Erickson, J. C., and Bush, A. I. (1997) Toxicol. Appl. Pharmacol. 142, 229–242
30. Andreyev, A. Y., Fahy, B., and Fiskum, G. (1998) FEBS Lett. 439, 373–376
31. Brustovetsky, N., and Dubinsky, J. M. (2000) J. Neurosci. 20, 8229–8237
32. Nicotera, P., and Lipton, S. A. (1999) J. Cereb. Blood Flow Metab. 19, 583–591
33. Lipton, P. (1999) Physiol. Rev. 79, 1431–1568
34. Aizenman, E., Stout, A. K., Hartnett, K. A., Dineley, K. E., McLaughlin, B., and Reynolds, I. J. (2000) J. Neurochem. 75, 1878–1888
Zn^{2+} Induces Permeability Transition Pore Opening and Release of Pro-apoptotic Peptides from Neuronal Mitochondria

Dongmei Jiang, Patrick G. Sullivan, Stefano L. Sensi, Oswald Steward and John H. Weiss

J. Biol. Chem. 2001, 276:47524-47529.
doi: 10.1074/jbc.M108834200 originally published online October 10, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M108834200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 34 references, 11 of which can be accessed free at http://www.jbc.org/content/276/50/47524.full.html#ref-list-1