Zinc-Induced Cortical Neuronal Death: Contribution of Energy Failure Attributable to Loss of NAD^+ and Inhibition of Glycolysis

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Excessive zinc influx may contribute to neuronal death after certain insults, including transient global ischemia. In light of evidence that levels of intracellular free Zn^{2+} associated with neurotoxicity may be sufficient to inhibit glyceraldehyde-3-phosphate dehydrogenase (GAPDH), experiments were performed looking for reduced glycolysis and energy failure in cultured mouse cortical neurons subjected to lethal Zn^{2+} exposure. As predicted, cultures exposed for 3–22 hr to 40 μM Zn^{2+} developed an early increase in levels of dihydroxyacetone phosphate (DHAP) and fructose 1,6-bisphosphate (FBP) and a progressive loss of ATP levels, followed by neuronal cell death; furthermore, addition of the downstream glycolytic substrate pyruvate to the bathing medium attenuated the fall in ATP and neuronal death.

However, an alternative to direct Zn^{2+} inhibition of GAPDH was raised by the observation that Zn^{2+} exposure also induced an early decrease in nicotinamide-adenine dinucleotide (NAD^+) levels, an event itself capable of inhibiting GAPDH. Favoring this indirect mechanism of GAPDH inhibition, the neuroprotective effects of pyruvate addition were associated with normalization of cellular levels of NAD^+, DHAP, and FBP. Zn^{2+}-induced neuronal death was also attenuated by addition of the energy substrate oxaloacetate, the activator of pyruvate dehydrogenase, dichloroacetate, or the inhibitors of NAD^+ catabolism, niacinamide or benzamide. Acetyl carnitine, α-keto butyrate, lactate, and β-hydroxy-butyrate did not attenuate Zn^{2+}-induced neurotoxicity, perhaps because they could not regenerate NAD^+ or be used for energy production in the presence of glucose.

Key words: pyruvate; niacinamide; energy depletion; PARS; ATP levels; GAPDH inhibition

Zn^{2+} is an essential ion in mammalian cells. It is incorporated into the active site of many metalloenzymes (Vallee and Falchuk, 1993; Berg and Shi, 1996) and is probably used in the CNS as a neurotransmitter or neuromodulator (Frederickson, 1989). It is stored within vesicles in presynaptic boutons (Frederickson et al., 1983; Danscher et al., 1985) and is released together with glutamate by membrane depolarization in a Ca^{2+}-dependent manner (Assaf and Chung, 1984; Howell et al., 1984; Charton et al., 1985). Although its precise role in neural signaling has not been defined, it may regulate neurotransmission by altering the function of several receptors and channels, including NMDA receptors, GABA receptors, glycine receptors, ATP receptors, and voltage-gated Na^+ and Ca^{2+}channels (Harrison and Gibbons, 1994; Smart et al., 1994). Zn^{2+} may also contribute to neuronal death in disease states, such as transient global ischemia or prolonged seizures (Choi and Koh, 1998). Brain ischemia triggers the translocation of presynaptic Zn^{2+} (over the ensuing 1–24 hr) into the soma of selectively vulnerable hippocampal CA1 neurons, as well as other vulnerable neurons in cortex, amygdala, striatum, and thalamus that later go on to die (Tonder et al., 1990; Koh et al., 1996). Both this translocation and subsequent selective neuronal cell death can be blocked by the administration of an extracellular chelator CaEDTA (Koh et al., 1996). The extracellular concentration of Zn^{2+} after intense neuronal activity may reach several hundred micromolar (Assaf and Chung, 1984), concentrations that are neurotoxic to cultured cortical neurons (Yokoyama et al., 1986; Choi and Koh, 1998).

It is presently unknown why exposure to high concentrations of extracellular Zn^{2+} can induce neuronal death. A critical first step appears to be entry across the plasma membrane, mediated by several routes, including voltage-gated calcium channels, agonist-gated calcium channels, and reverse operation of the sodium-calcium exchanger (Choi and Koh, 1998). We have used mag fura 5 to detect elevations in neuronal intracellular free Zn^{2+} ($[Zn^{2+}]_i$) associated with zinc exposure under several conditions (Sensi et al., 1997). Although mag fura 5 is ratiometric and suitable for detecting early increases in $[Zn^{2+}]_i$, its high affinity (K_D of $\sim 10^{-12}$) may limit its ability to detect peak concentrations. Therefore we have recently used a low affinity (K_D of ~ 1 μM Zn^{2+}) non-ratiometric Zn^{2+}-selective dye, Newport Green (Haugland, 1996). A 5 min exposure to 300 μM Zn^{2+} under depolarizing conditions - an insult that triggers widespread cortical neuronal death over the next hr (Yokoyama et al., 1986) –resulted in $[Zn^{2+}]_i$ reaching 400–600 nM as assessed with Newport Green (Canzoniero et al., 1999; Sensi et al., 1999). At this concentration, Zn^{2+} can inhibit the key glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in solution, with 400 nM Zn^{2+} producing 50% inhibition of purified GAPDH (Krotkiewska and Banas, 1992). Zn^{2+} has also been reported to inhibit phosphofructokinase in solution, with an IC_{50} of 1.5 μM in the presence of 60 μM fructose 6-phosphate (Ikeda et al., 1980).

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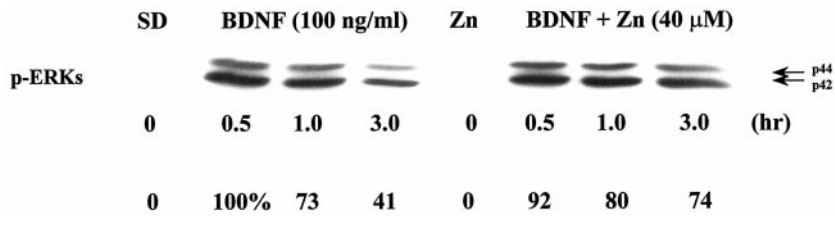

Figure 1. Toxic levels of extracellular zinc do not block BDNF-induced signaling. Neuronal cultures were deprived of serum and treated in the absence (*SD*) or presence of 40 μM Zn^{2+} (*Zn*) with 100 ng/ml BDNF for the indicated times. Cell extracts were prepared, and activation of the MAP kinase pathway was determined by Western blotting using antibodies that detect the phosphorylated form of ERK1 and ERK2 (*p44* and *p42*, respectively). We have quantified the bands using the 0.5 hr plus BDNF, minus zinc as 100%.

The purpose of the present study was to test the hypothesis that neurotoxic Zn^{2+} exposure leads to inhibition of GAPDH, resulting in a buildup of the upstream substrates dihydroxyacetone phosphate (DHAP) and fructose bisphosphate (FBP) and a fall in neuronal ATP levels. Furthermore, if GAPDH inhibition contributed importantly to the neurotoxic effects of Zn^{2+}, we postulated that the administration of suitable downstream energy substrates might be neuroprotective against Zn^{2+} exposure.

Parts of this work have been published previously in abstract form (Sheline and Choi, 1997).

MATERIALS AND METHODS

Cell culture and toxicity studies. Near-pure neuronal cultures were prepared from embryonic day 15 (E15) mouse cortices as described previously (Sheline and Choi, 1998). Dissociated cortical neurons were taken from E15 Swiss–Webster mice and plated in Eagle's minimal essential medium (MEM) (Earle's salts, glutamine-free) containing 21 mM glucose, 5% fetal bovine serum, and 5% horse serum at a density of 5 hemispheres per plate onto poly-D-lysine–laminin-coated plates. At 3 d *in vitro* (DIV), cytosine arabinoside was added to 10 μM to inhibit glial growth. Chronic toxicity studies were initiated by washing cultures four times with MEM containing 21 mM glucose, followed by exposure to $ZnCl_2$ in the same media supplemented with 1 μM (+)-5- methyl-10,11-dihydro-5H-dibenzo [a,d] cycloheplen-5,10-imine maleate (MK-801) and 100 ng/ml of neurotrophin-4 (NT-4) or BDNF. MK-801 was included to prevent wash-induced activation of NMDA receptors and was not itself toxic over the ensuing 24 hr, and NT-4 or BDNF were included as a needed survival factor (serum could not be used because it chelates Zn^{2+}). Acute toxicity studies were initiated by washing cultures four times with HEPES-buffered salt solution, followed by exposure to $ZnCl_2$ in the same media supplemented with 1 μM MK-801 and 100 ng/ml NT-4 or BDNF as a survival-promoting activity in the presence or absence of 60 mM KCl for 5 or 15 min. The exposure was terminated by washing three times with MEM containing 21 mM glucose, the cultures were put back into the same media supplemented with 1 μM MK-801 and 100 ng/ml NT-4 or BDNF as a survival-promoting activity, and cell death was assayed 24 hr later. Near-pure neuronal cultures were washed seven times using salt solution (same as in MEM) without glucose but in the presence of 1× amino acids before testing the use of different energy substrates at 6 mM in the same glucose-free solution plus MK-801 and NT-4 for 24 hr. These same substrates were tested against 40 μM Zn^{2+} exposure, as were the effects of late addition of pyruvate. Cell death was estimated by phase-contrast microscopy after staining with 0.01% trypan blue for 60 min at 37°C and assessed quantitatively by measuring lactate dehydrogenase (LDH) efflux (Koh and Choi, 1987) or propidium iodide fluorescence (Sheline and Choi, 1998) and comparing it with the complete neuronal death induced by exposure to 20 μM A23187 for 24 hr.

Determination of dihdroxyacetone phosphate, lactate, and ATP levels. Near-pure neuronal cultures (8–9 DIV) were used for the ATP measurements. Cultures were lysed by addition of 0.1 M NaOH–1 mM EDTA at the indicated time points. After centrifugation at 13,000 × *g*, the supernatant was neutralized and protein was precipitated by addition of 100 μl of 0.5 M perchloric acid. ATP was measured by the luciferin–luciferase luminescence assay and was normalized to sham-washed controls and to protein content as determined by the bichichonic acid assay (Lust et al., 1981). DHAP, FBP, and extracellular lactate measurements were made on neuronal cell lysates or the bathing medium (for extracellular lactate) prepared in a similar manner, but were lysed by addition of 6% perchloric acid and protein precipitated by addition of potassium carbonate to pH 3.5. DHAP was measured by its enzymatic conversion to glycerol-3-phosphate by glycerol-3-phosphate dehydrogenase, and the concomitant oxidation of nicotinamide-adenine dinucleotide, reduced form (NADH) was measured spectrophotometrically. Glyceraldehyde 3-phosphate was subsequently measured in a linked reaction by its enzymatic conversion to DHAP by triosephosphate isomerase, followed by addition of aldolase to measure FBP levels (Michal, 1974). Extracellular lactate concentration was measured by its conversion to pyruvate in the presence of excess hydrazine, nicotinamide-adenine dinucleotide (NAD^+), and LDH to drive the production of NADH (Gutmann and Wahlefeld, 1974). Other metabolites were also measured by their enzymatic conversion and the concomitant oxidation or reduction of NADH, NAD^+, or nicotinamide-adenine dinucleotide phosphate ($NADP^+$).

Determination of NAD^+ and NADH levels. Neuronal cultures (8–9 DIV) were used for the NAD^+ and NADH measurements. For the NAD^+ and NADH measurements, cultures were lysed by addition of 75% ethanol–0.05 M K2HPO4 after a 4 hr 40 μM Zn^{2+} exposure. Protein was precipitated by addition of $ZnCl_2$ to 20 mM and centrifuged at 13,000 × *g*, and the supernatant was assayed for NAD^+ and NADH levels (Tilton et al., 1991). NAD^+ in the supernatant was measured after its enzymatic conversion to NADH by alcohol dehydrogenase, resulting in an increase in the fluorescence spectrum between 400 and 600 nm after an excitation at 340 nm using a Perkin-Elmer (Emeryville, CA) LS 50B. NADH was measured by the difference in the fluorescence spectrum between 400 and 600 nm before and after treatment of the supernatant with lactate dehydrogenase (Sander et al., 1976).

Whole-cell lysates. Neuronal cultures (8 DIV) were serum-deprived for 1 hr and then exposed as indicated to 100 ng/ml BDNF in the presence or absence of 40 μM Zn^{2+}. The cells were then washed twice with ice-cold PBS and resuspended in cold buffer A (1% NP-40, 20 mM Tris-Cl, pH 7.5, 10 mM EGTA, 40 mM β-glycerophosphate, 2.5 mM $MgCl_2$, 2 mM orthovanadate, 1 mM dithiothreitol, 1 mM phenyl-methyl-sulfonylfluoride, 20 μg/ml aprotinin, and 20 μg/ml leupeptin) for 15 min.

Lysates were centrifuged at 15000 × *g* for 5 min, and supernatants were retained for analysis. Protein concentrations were determined by the bicinchoninic acid method (Pierce, Rockford, IL) using bovine serum albumin as standard.

Western blotting. For Western blots, 25 μg of total cell protein were resolved in 8% SDS-PAGE gels, transferred to nitrocellulose membranes (MSI, Westboro, MA), and incubated with antibodies specific for phospho-ERKs [anti-active mitogen-activated protein kinase (MAPK) antibodies; New England Biolabs, Beverly, MA]. Bound antibodies were detected by the enhanced chemiluminescence method (Amersham Pharmacia Biotech, Arlington Heights, IL).

Reagents. Most reagents were from Sigma (St. Louis, MO). The neurotrophins NT-4 and BDNF were the kind gift of Amgen (Thousand Oaks, CA).

RESULTS

Zinc exposure did not block neurotrophin signaling

A previous study in PC12 cells suggested that Zn^{2+} can alter the conformation and biological activities of several neurotrophins (Ross et al., 1997). Arguing against the possibility that Zn^{2+}-induced neuronal death is mediated by an acute loss of neurotrophin influence, ATP depletion and neuronal death occurred more quickly after Zn^{2+} exposure than after serum deprivation (data not shown; see Figs. 3, 5). Furthermore, 40 μM Zn^{2+} did not prevent 100 ng/ml BDNF or NT-4 from initiating the TrkB-mediated signaling cascade resulting in phosphorylation of the ERK family of protein kinases (Fig. 1).

Table 1. Zinc exposure selectively increased DHAP and FBP levels, and this increase was reversed by addition of pyruvate or niacinamide

(Nanomoles/plate ± SEM)	Control	40 μM Zinc	Zinc + pyruvate	Zinc + niacinamide	Staurosporine
Glucose-6-phosphate	4.2 ± 0.6	7.6 ± 1.0	N.P.	N.P.	N.P.
Fructose-6-phosphate	1.6 ± 0.2	3.2 ± 0.4	N.P.	N.P.	N.P.
DHAP	5.2 ± 0.6	22.4 ± 0.8*	5.2 ± 0.6#	5.0 ± 0.6#	3.2 ± 2.2
Fructose bisphosphate	5.2 ± 0.8	51.4 ± 5.0*	5.0 ± 0.8#	11.2 ± 1.4#	5.8 ± 0.6
2-Phospho-glycerate	6.25 ± 0.5	11.7 ± 0.5*	N.P.	N.P.	N.P.
Phospho-enolpyruvate	5.75 ± 1.7	8.2 ± 4.7	N.P.	N.P.	N.P.
Pyruvate	14.2 ± 2.2	20 ± 4.5	N.P.	N.P.	N.P.

Near-pure cortical neuronal cultures were sham-washed or exposed to 40 μM Zn^{2+} in the presence or absence of 4 mM pyruvate or 1 mM niacinamide. The cells were then harvested and assayed for levels of the indicated glycolytic intermediates (results were pooled from three separate experiments; $n = 5–8$ cultures per condition). Exposure to 100 nM staurosporine for 4–6 hr, sufficient to trigger widespread apoptosis, did not mimic the ability of Zn^{2+} to elevate DHAP or FBP. N.P. indicates that the experiment was not performed. * signifies difference from sham-washed controls, and # signifies difference from Zn^{2+}-treated cultures at $p < 0.05$ by one-way ANOVA, followed by a Bonferroni test.

Zinc exposure increased neuronal levels of DHAP and FBP

Near-pure cortical neuronal cultures, 8–9 d *in vitro*, were exposed to 40 μM Zn^{2+} for 4 hr, an insult duration that did not cause cellmembrane failure as measured by LDH efflux to the bathing medium (see below) or staining with trypan blue or propidium iodide (data not shown). At that time, cells were lysed, and the lysate was assayed for DHAP, FBP, fructose 6-phosphate, glucose 6-phosphate, pyruvate, phosphoenolpyruvate, and glycerate 2-phosphate. Both DHAP and FBP levels were increased several-fold, whereas other measured metabolites (except 2-phospho-glycerate) were unchanged (Table 1). Glyceraldehyde 3-phosphate was undetectable in either control cultures or cultures exposed to Zn^{2+}, most likely because it was preferentially converted into DHAP (Lehninger et al., 1993).

In contrast, when the neuronal cultures were exposed to 100 nM staurosporine for 6 hr, an exposure capable of triggering programmed cell death (Koh et al., 1995; Weil et al., 1996), no changes were seen in the above metabolites (Table 1).

Zinc-induced neuronal death was attenuated by pyruvate

Increasing the duration of 40 μM Zn^{2+} exposure to 24 hr resulted in widespread neuronal degeneration, most marked initially in processes, and later accompanied by trypan blue staining (Fig. 2) and release of LDH to the bathing medium (Fig. 3). Addition of 2–6 mM pyruvate to the bathing medium during this toxic Zn^{2+} exposure resulted in a concentration-dependent reduction in neuronal death, with near-complete preservation of neurons produced by 6 mM pyruvate (Figs. 2, 3*A*). Pyruvate (4 mM) attenuated the neuronal death induced by 24 hr exposure to Zn^{2+} concentrations between 20 and 100 μM (Fig. 3*B*), even if added in a

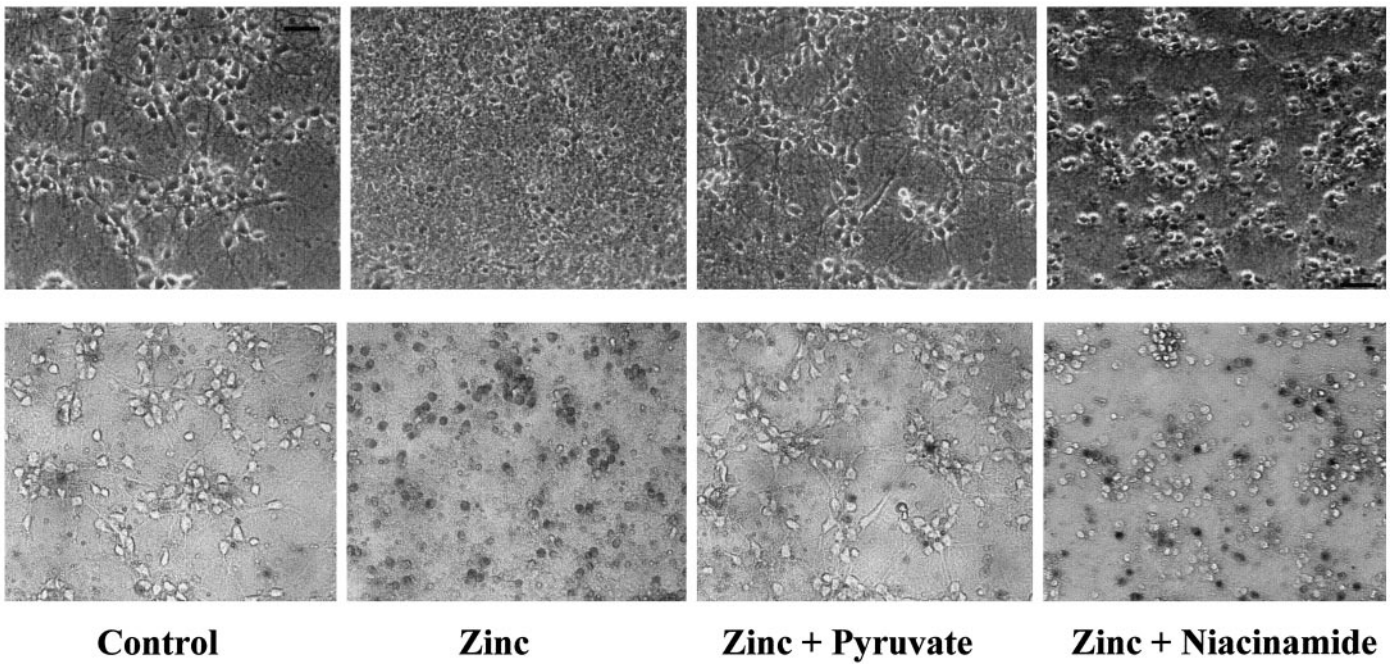

Figure2. Zinc-induced neuronal death is attenuated by pyruvate. Phase-contrast (*top*) and matched bright-field fields after staining with trypan blue (*bottom*) were taken in near-pure neuronal cultures 24 hr after exposure to sham wash (*Control*), 40 μM Zn^{2+}, or 40 μM Zn^{2+} in the presence of 4 mM pyruvate or 1 mM niacinamide. Scale bar, 50 μm.

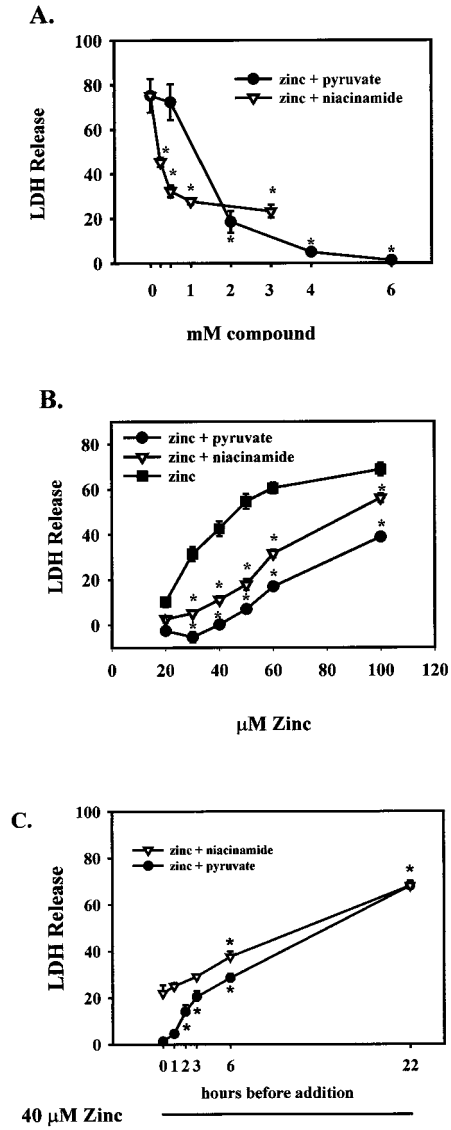

Figure 3. Inhibition of slow zinc toxicity by pyruvate or niacinamide. *A*, Neuronal cultures were exposed to 40 μM Zn^{2+} for 24 hr in the presence of the indicated concentrations of pyruvate and niacinamide, and cell death was assessed by LDH release to the bathing medium (mean + SEM, $n = 9–12$ cultures per condition), scaled to the level associated with near-complete neuronal death (produced by exposure to 20 μM A23187 for 24 hr, 100%). $^*p < 0.05$ indicates difference from Zn^{2+} exposure alone by one-way ANOVA, followed by a Bonferroni test. *B*, Neuronal cultures were exposed to the indicated concentrations of Zn^{2+} for 24 hr in the presence or absence of 4 mM pyruvate or 3 mM niacinamide. $^*p < 0.05$ indicates difference from Zn^{2+} exposure alone at the same time point. *C*, Zn^{2+} (40 μM) was added to the bathing medium at time 0, and afterwards, at the indicated times, 4 mM pyruvate or 3 mM niacinamide were added. Cell death was measured at 22 hr. $^*p < 0.05$ signifies difference from addition at time 0.

delayed manner 3–6 hr after the onset of Zn^{2+} exposure (Fig. 3*C*). The neuroprotective effect of pyruvate addition was well maintained for at least 48 hr after the onset of Zn^{2+} exposure.

The more fulminant form of Zn^{2+} toxicity induced by brief (5–15 min) exposure to high concentrations of Zn^{2+} (100–400 μM) in the presence of 60 mM K^+ [to depolarize cells and enhance entry through voltage-gated calcium channels (Weiss et al., 1993; Sensi et al., 1997)] could also be partially attenuated by 10 mM pyruvate or niacinamide (Fig. 4).

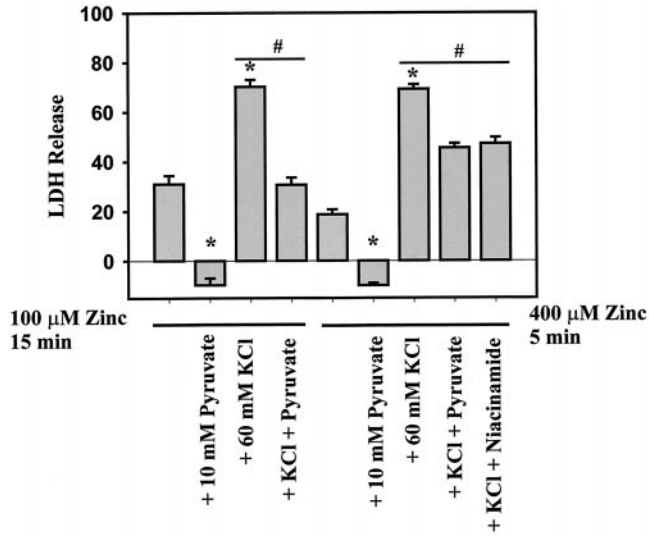

Figure 4. Inhibition of fast zinc toxicity by pyruvate or niacinamide. Neuronal cultures were exposed to 100 μM Zn^{2+} for 15 min or 400 μM Zn^{2+} for 5 min in the presence or absence of 60 mM KCl, with 10 mM pyruvate or 10 mM niacinamide present 1 hr before, during, and 24 hr afterwards as indicated. Neuronal death was determined 24 hr after the onset of Zn^{2+} exposure by LDH efflux ($n = 9–12$ cultures per condition). $^*p < 0.05$ signifies difference from Zn^{2+} exposure alone. $\#p < 0.05$ signifies difference associated with pyruvate or niacinamide addition on neuronal death induced by Zn^{2+} plus KCl.

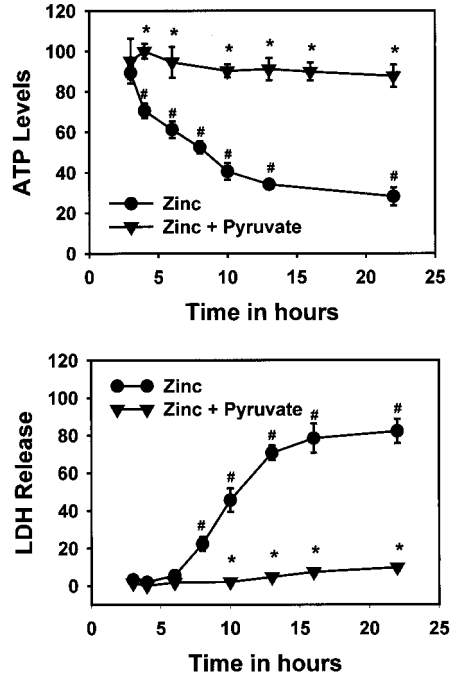

Figure 5. Time course of neuronal cell death and ATP depletion during exposure to zinc with or without pyruvate. Neuronal cultures were exposed continuously to 40 μM Zn^{2+} in the presence or absence of 4 mM pyruvate, and cellular ATP levels (*top*) or LDH release (*bottom*) were determined at the indicated times. Cell death was determined by LDH release to the bathing medium (scaled to signal associated with complete neuronal death induced by 24 hr exposure to 20 μM A23187, set as 100). ATP loss was determined by comparison with levels measured in sham-washed controls. Error bars in both parts are SEM; $n = 9–12$ cultures per condition, pooled from three independent experiments. $^*p < 0.05$ signifies difference from Zn^{2+} exposure alone by two-way ANOVA, followed by a Bonferroni test. $\#p < 0.05$ signifies difference from sham-washed controls by two-way ANOVA, followed by a Bonferroni test.

Table 2. Zinc exposure decreased NAD^+, and this effect was reversed by addition of pyruvate or niacinamide

Condition	NAD^+ (nmol/plate)	NADH (nmol/plate)	Lactate (nmol/plate)
Control	4.63 ± 0.31	0.58 ± 0.052	2020 ± 130
Control + 4 mM pyruvate	5.62 ± 0.59*	0.20 ± 0.016*	2930 ± 110*
Control + 1 mM niacinamide	6.170 ± 0.36*	0.66 ± 0.058	2650 ± 230*
40 μM Zinc	1.36 ± 0.16*	0.83 ± 0.14	2470 ± 80*
40 μM Zinc + 4 mM pyruvate	4.86 ± 0.22#	0.042 ± 0.027#	3600 ± 140#
40 μM Zinc + 1 mM niacinamide	4.38 ± 0.40#	0.89 ± 0.059	3270 ± 170#

Neuronal cultures (6×10^6 cells per plate) were sham-washed (Control) or exposed to 40 μM Zn^{2+} in the presence or absence of 4 mM pyruvate or 1 mM niacinamide for 4 hr, after which the cells were harvested and assayed for levels of NAD^+, NADH, or extracellular lactate (results are pooled from three separate experiments; $n = 5–8$ cultures per condition). * signifies difference from sham-washed controls at $p < 0.05$ by two-way ANOVA, followed by a Bonferroni test. # signifies difference from Zn^{2+}-treated cultures at $p < 0.05$.

Zinc-induced ATP depletion preceded cell death, and both were sensitive to pyruvate

Neuronal cultures were exposed to 40 μM Zn^{2+} for 3–22 hr, with LDH efflux and ATP levels assessed at intermediate time points. ATP loss was detectable after 4 hr, whereas cell death measured by LDH release was not detectable until after 8 hr (Fig. 5). Cell death assessed by propidium iodide staining was not detectable until after 6 hr of Zn^{2+} exposure (data not shown). The addition of 4 mM pyruvate to the bathing medium blocked both ATP loss and cell death (Fig. 5).

Table 3. Effect of selected energy substates, NAD^+ catabolism inhibitors, or cofactors for pyruvate dehydrogenase against zinc neurotoxicity or glucose deprivation-induced neuronal death

% Cell death	40 μM Zinc	Glucose deprivation
No addition	67.7 ± 2.8%	89.6 ± 4.7%
+3 mM Pyruvate	0.3 ± 1.9%*	5.2 ± 2.8%*
+3 mM Pyruvate + 3 mM CIN	61.2 ± 4.1%#	76.5 ± 5.9%#
+3 mM Pyruvate + 20 mM oxamate	47.9 ± 2.7%#	25.7 ± 2.8%#
+3 mM Oxaloacetate	1.6 ± 0.9%*	17.4 ± 1.7%*
+6 mM Malate	37.3 ± 1.8%*	67.6 ± 4.3%*
+6 mM Succinate	48.8 ± 2.4%*	77.4 ± 2.1%*
+6 mM Lactate	67.8 ± 3.5%	3.4 ± 2.3%*
+6 mM β-Hydroxy-butyrate	73.1 ± 4.9%	12.9 ± 1.5%*
+6 mM α-Keto-butyrate	61.3 ± 3.4%	90.3 ± 4.3%
+2 mM FBP	59.1 ± 5.1%	68.6 ± 1.4%*
+2 mM DHAP	62.3 ± 4.1%	30.7 ± 4.8%*
+6 mM Acetyl-carnitine	75.0 ± 4.0%	99.0 ± 2.8%
+3 mM Niacinamide	15.1 ± 1.6%*	86.9 ± 1.4%
+3 mM Benzamide	21.1 ± 2.9%*	77.9 ± 3.9%
+3 mM 3-Aminobenzamide	24.0 ± 3.7%*	83.8 ± 1.7%
+6 mM Dichloroacetate	21.4 ± 1.6%*	79.0 ± 3.6%
+2 mM Riboflavin	65.4 ± 3.5%	83.3 ± 2.0%
+6 mM Thiamine	67.0 ± 1.7%	84.4 ± 1.7%
+0.05 mM Lipoic acid (reduced)	69.4 ± 2.4%	105.8 ± 1.7%
+0.25 mM Lipoic amide	60.2 ± 2.4%	89.8 ± 1.4%

Near-pure neuronal cultures were exposed to 40 μM Zn^{2+} for 24 hr or to glucose deprivation for 24 hr alone or in the presence of the indicated compounds (titrated to optimal concentrations; data not shown), and cell death was determined by LDH efflux or propidium iodide fluorescence. $n = 9–12$ cultures per condition. * indicates difference from no addition and # indicates difference from exposure plus pyruvate at $p < 0.05$. CIN, cinnamic acid.

Zinc-induced decrease in NAD^+ was attenuated by pyruvate or niacinamide

Although the above observations were consistent with the hypothesis that direct inhibition of GAPDH by Zn^{2+} contributed importantly to the neurotoxic effects of the latter, unexpectedly, inclusion of 4 mM pyruvate during Zn^{2+} exposure abolished the buildup of DHAP and FBP found in cultures exposed to Zn^{2+} alone (Table 1).

Exposure to 40 μM Zn^{2+} for 4 hr also induced a several-fold decrease in NAD^+ levels without a compensatory increase in NADH levels, as well as an increase in lactate. The inclusion of 4 mM pyruvate to the Zn^{2+} exposure abolished the decrease in NAD^+, perhaps at the expense of NADH, and further increased lactate (Table 2). In addition, inclusion of 1–3 mM niacinamide, benzamide or 3-aminobenzamide, competitive inhibitors of NAD^+-catabolizing enzymes (for review, see Szabo and Dawson, 1998), blocked the drop in NAD^+ levels and associated neuronal death (Tables 2, 3; data not shown).

Effect of other energy substrates, NAD^+ catabolism inhibitors, or pyruvate dehydrogenase cofactors on zinc-induced neuronal death or glucose deprivation-induced death

Other energy substrates, NAD^+ catabolism inhibitors, and pyruvate dehydrogenase (PDH) cofactors [pyruvate, oxaloacetate, malate, succinate, lactate, β-hydroxy-butyrate, α-keto-butyrate, FBP, DHAP, acetyl-carnitine, niacinamide, benzamide, 3-aminobenzamide, dichloroacetate (DCA), riboflavin, thiamine, lipoic acid, and lipoic amide] were tested at optimal concentrations (concentration titration data not shown) for their ability to reduce Zn^{2+}-induced or glucose deprivation-induced neuronal death. At these concentrations, none of the compounds were found to be toxic or to induce gross changes in cell volume. Oxaloacetate, niacinamide, benzamide, and 3-aminobenzamide were nearly as effective as pyruvate at attenuating Zn^{2+}-induced neuronal death, but of these, only pyruvate and oxaloacetate were used as energy substrates (Table 3). Niacinamide, benzamide, and 3-aminobenzamide competitively inhibit all NAD^+-catabolizing enzymes; niacinamide is also a precursor for NAD^+ synthesis (for review, see Szabo and Dawson, 1998). In addition, DCA, which functions as an activator of the PDH complex (inhibiting the kinase that inhibits the complex), partially attenuated Zn^{2+} neurotoxicity without serving as an energy substrate. However, the other PDH complex cofactors, thiamine or lipoic acid, were ineffective against Zn^{2+} neurotoxicity. The effect of DCA was

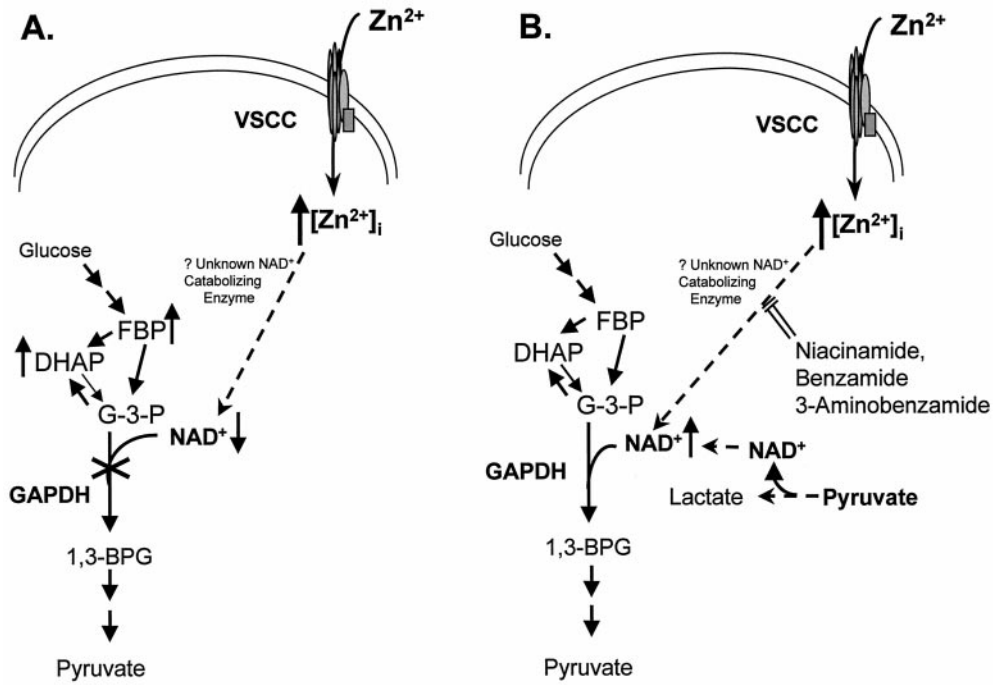

Figure 6. Summary model for Zn^{2+}-induced neuronal death *in vitro*. *VSCC*, Voltage-sensitive calcium channel; *G-3-P*, glyceraldehyde-3-phosphate; *1,3-BPG*,1,3-bisphosphoglycerate. *A*, The model for Zn^{2+}-induced changes. *B*, The protective mechanisms for niacinamide, 3-aminobenzamide, and benzamide involve inhibition of an unknown NAD^+-catabolizing enzyme, thereby maintaining NAD^+ levels; the protective mechanism for pyruvate involves maintaining NAD^+ levels through its conversion to lactate.

synergistic with low levels of pyruvate (data not shown). The protective effect of pyruvate was attenuated by cinnaminic acid, an inhibitor of the monocarboxylate transporter (Schurr et al., 1997), and by oxamate, a competitive inhibitor of lactate dehydrogenase (Wong et al., 1997). These results are summarized in the proposed model for Zn^{2+}-induced neurotoxicity (Fig. 6). Compounds that could not be converted to pyruvate or serve as energy substrates or PDH complex cofactors in neuronal cultures did not attenuate Zn^{2+}-induced neuronal death (Table 3). Two substrates differed in their ability to protect against glucose deprivation-induced death versus Zn^{2+}-induced death. Lactate and β-hydroxy-butyrate protected against the former but not the latter.

DISCUSSION

We observed that neurotoxic levels of Zn^{2+} exposure induced in cortical neurons an early increase in glycolytic intermediates preceding GAPDH, followed by a progressive loss of ATP levels and neuronal cell death. Addition of the downstream glycolytic substrate pyruvate to the bathing medium attenuated both the fall in ATP and neuronal death. Simple chelation of Zn^{2+} by pyruvate is unlikely because the log stability constant for Zn^{2+} pyruvate is 1.3 (Martell and Smith, 1995), and reduction of neuronal death was also achieved by adding another downstream energy substrate, oxaloacetate, or the activator of pyruvate dehydrogenase, dichloroacetate. These findings are consistent with our initial hypothesis that a key mechanism of Zn^{2+} neurotoxicity is energy loss attributable to inhibition of glycolysis at GAPDH, an event that could be mediated directly by Zn^{2+} (see above). In nondividing bacterial cells, GAPDH has the largest control strength of all the glycolytic enzymes for metabolic regulation (Poolman et al., 1987). We have demonstrated previously that chemical inhibition of GAPDH with α-monochlorohydrin can induce cultured cortical neurons to undergo apoptosis (Sheline and Choi, 1998), an event induced by levels of Zn^{2+} exposure (20–35 μM) comparable with those used in the present experiments (D. Lobner and D. W. Choi, unpublished observations) (Y. H. Kim et al., 1999a).

However unexpectedly, Zn^{2+} exposure also induced an early fall in NAD^+ levels, an event itself capable of inhibiting GAPDH (Rovetto et al., 1975; Tilton et al., 1991). Favoring indirect inhibition of GAPDH caused by loss of NAD^+, the neuroprotective effects of pyruvate addition were associated with normalization of cellular levels of NAD^+ and glycolytic intermediates preceding GAPDH. The latter event would not be expected to occur if GAPDH was directly inhibited by Zn^{2+}. Also favoring a central role for Zn^{2+}-induced depression of NAD^+ levels in triggering neuronal death, the fall in NAD^+ and neuronal death induced by Zn^{2+} was attenuated by the NAD^+ catabolism inhibitors niacinamide, benzamide, or 3-aminobenzamide (for review, see Szabo and Dawson, 1998).

One postulated mechanism of Zn^{2+} neurotoxicity is inhibition of mitochondrial electron transport (Skulachev et al., 1967; Link and von Jagow, 1995; Manev et al., 1997); this inhibition would decrease neuronal NAD^+ levels. However, there was not the expected compensatory increase in NADH levels (Franke et al., 1976; Pryor et al., 1992) (Table 2). Alternative attractive possibilities would be inhibition of NAD^+ synthesis or activation of NAD^+ catabolism. Zn^{2+} has been shown to both activate and inhibit different NAD^+-catabolizing enzymes depending on the cell type and the conditions (Larsen et al., 1982; Kukimoto et al., 1996; Jorcke et al., 1997). A prominent NAD^+-catabolizing enzyme is poly (ADP-ribose) synthetase (PARS), an enzyme likely involved in the detection and repair of single-stranded DNA breaks (Wang et al., 1995). We demonstrated here that three competitive inhibitors of PARS [niacinamide, benzamide, and 3-aminobenzamide (for review, see Szabo and Dawson, 1998)] all attenuate Zn^{2+} neurotoxicity (Fig. 2, Table 3), although these inhibitors also inhibit NAD^+ glycohydrylase, another enzyme that breaks down NAD^+ (Ziegler et al., 1996). PARS has been implicated as a mediator of neuronal damage after glutamate exposure (Zhang et al., 1994) or hypoxic insults (Eliasson et al.,

1997; Endres et al., 1997), consistent with a model in which glutamate receptor overactivation leads to the formation of nitric oxide and other reactive oxygen species (ROS), causing DNA strand breakage and PARS activation (for review, see Szabo and Dawson, 1998).

Cellular Zn^{2+} overload itself has been suggested to enhance ROS production (E. Y. Kim et al., 1999; Y. H. Kim et al., 1999a,b). We have found that the powerful ROS scavenger C3-polar regioisomer buckminsterfullerene (Dugan et al., 1997) was relatively ineffective at attenuating Zn^{2+}-induced cortical neuronal death (L. L. Dugan and D. W. Choi, unpublished observations), and a recent study in cortical cultures did not see a general increase in cytosolic ROS in the first hour after toxic Zn^{2+} exposure (Sensi et al., 1999). Furthermore α-keto butyrate, which blocks H_2O_2 hydroxyl radical-induced neuronal death (Desagher et al., 1997) by chemically inactivating H_2O_2 (Holleman, 1904; Desagher et al., 1997), was inactive against Zn^{2+} neurotoxicity in the present study (Table 3). Regardless of whether Zn^{2+} promotes ROS formation, the possibility that Zn^{2+} might somehow activate PARS is consistent with the recent observation that the high-affinity Zn^{2+} chelator *N*, *N*′,*N*′-tetrakis (2-pyridylmethyl) ethylenediamine inhibits PARS (Virag and Szabo, 1999) (although this observation could simply reflect a baseline requirement of PARS for a Zn^{2+} cofactor because it has a Zn^{2+} finger DNA binding domain).

The present proposal that indirect inhibition of GAPDH leading to energy failure is an important mediator of Zn^{2+}-induced neuronal death does not exclude the possibility that Zn^{2+} may have other death-promoting actions. Direct inactivation of BDNF or NT-4/5 may be considered (Ross et al., 1997) but is unlikely in the present system because Zn^{2+} exposure did not block the phosphorylation of ERK1 and ERK2, downstream effectors of the TrkB neurotrophic signaling cascade. Activation of an extracellular acid sphingomyelinase producing the apoptotic signaling molecule ceramide (Schissel et al., 1996) is another possible toxic mechanism, although this enzyme is inactive at the physiological pH used in the present experiments.

The observed ability of pyruvate to restore NAD^+ to control levels at the expense of NADH provides a plausible explanation for its ability to counteract each of the stated effects of Zn^{2+} exposure on neuronal cultures (Table 2). The increase in extracellular lactate associated with pyruvate addition and the inhibition of pyruvate-induced neuroprotection by oxamate, an inhibitor of lactate dehydrogenase, are consistent with pyruvate generating NAD^+ from NADH through its conversion to lactate. However, niacinamide was not as effective as pyruvate in attenuating Zn^{2+}-induced cell death (Fig. 2, Table 3), although it also restored NAD^+ levels (Table 2). Pyruvate also appeared markedly better than niacinamide in protecting neuronal processes (Fig. 2), raising a possibility that Zn^{2+} toxicity in processes may differ from that in soma. It is easily possible that pyruvate may have additional beneficial effects on Zn^{2+}-injured neurons, such as enhancement of mitochondrial membrane potential (Kauppinen and Nicholls, 1986) or enhancement of PDH activity, which is reduced after global ischemia *in vivo* (Kobayashi and Neely, 1983; Zaidan and Sims, 1997).

The inability of lactate and β-hydroxy-butyrate to protect against Zn^{2+}-induced death compared with their ability to substitute for glucose fits with the observation that they are used predominantly after prolonged periods of fasting (Owen et al., 1967; Robinson and Williamson, 1980), whereas Zn^{2+} may induce too rapid a block in glycolysis to allow the uptake and use of alternative substrates. Also, Zn^{2+}-induced NAD^+ deficiency would be expected to inhibit the conversion of lactate to pyruvate by LDH and to inhibit the mitochondrial use of β-hydroxybutyrate. In contrast, oxaloacetate is effective at preventing Zn^{2+}-induced neuronal death, perhaps because it can be converted to pyruvate (Lehninger et al., 1993).

The present observation that several energy substrates or modulators of energy pathway enzyme activity can attenuate Zn^{2+}-induced neuronal death suggests several new approaches to ameliorating this death in the context of certain disease states.

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