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**Part I**

**Special lectures**

Moderator: Richard J. Traystman

## Zinc toxicity in the ischemic brain

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Growing evidence indicates that the brain's heightened vulnerability to ischemia, in large part, reflects a propensity for its intrinsic cell–cell and intracellular signaling mechanisms, normally responsible for information processing, to turn lethal under ischemic conditions. The most extensively studied example of such a signaling mechanism is that mediated by the excitatory neurotransmitter glutamate. In health, glutamate mediates most fast excitatory neurotransmission, but, under ischemic conditions, glutamate floods out from both neurons and astrocytes, building up in the extracellular space and becoming a killer that facilitates excess calcium entry into neurons, contributing to their demise.

Two Princeton Conferences ago, that is in Memphis in 1996, I presented then emerging evidence from my laboratory supporting the idea that another neurotransmitter released from excitatory nerve terminals might become a killer in the ischemic brain: the metal zinc. Besides the “transmitter killer” parallel to glutamate, I noted that there was also a parallel between zinc and calcium, in that both were divalent cation metals mediating ischemic neuronal death via excess influx across the plasma membrane [1].

A substantial body of evidence suggests that zinc is a neurotransmitter/neuro-modulator [reviewed by refs. 2–4], although this possibility has had, to date, a rather low profile within the scientific community. The central nervous system contains a pool of relatively free zinc, separate from the zinc tightly bound to metalloenzymes and transcription factors in all cells. This free central nervous system zinc is concentrated in vesicles within central nerve terminals throughout the telencephalon, largely colocalized (albeit in distinct vesicles) with transmitter glutamate. Consistent with a neurotransmitter role, it is released upon membrane depolarization in a calcium-dependent fashion and then taken back up.

Little is currently known about the functional significance of the zinc neurotransmitter system, probably reflecting a paucity of directed studies. An experience reported a quarter of a century ago in the neurology literature suggests that acute zinc depletion through oral chelation can produce profound reversible changes in

mentation [5]. Certainly, there are many candidates for relevant target actions of synaptically released zinc at the micromolar concentrations that it may well reach [6], as these zinc concentrations can modify the behavior of many important membrane proteins including transmitter receptors, channels and transporters. Of particular relevance, given its systematic colocalization with glutamate, extracellular zinc reduces *N*-methyl-*D*-aspartate (NMDA) receptor activation by both a voltage-independent reduction of channel opening frequency, and a voltage-dependent channel block [7]. Modulation of zinc release, therefore, may provide a mechanism for modifying the relative proportion of NMDA vs.  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) or kainate receptors activated by glutamate. It can also enter postsynaptic neurons (see below), whereupon it may modify signaling, metabolism or gene transcription in a lasting fashion. We have recently found evidence that brief exposure to non-toxic levels of extracellular zinc can activate mitogen-activated protein (MAP) kinase and Src family kinase signaling in neurons, the latter leading to phosphorylation of the NMDA receptor subunits (NR2A and NR2B) and consequent enhancement of NMDA receptor activity [8]. Thus normal zinc actions on the NMDA receptor may be biphasic: an initial direct inhibition followed by more lasting kinase-mediated upregulation.

After transient global ischemia, chelatable  $Zn^{2+}$  translocates from nerve terminals into cell bodies of vulnerable neurons, not just in the hippocampus, but also in the cortex, striatum, amygdala and thalamus [9]. This translocation precedes neuronal degeneration, and its interruption by the intracerebroventricular injection of a chelator, ethylenediaminetetra-acetic acid saturated with equimolar  $Ca^{2+}$  (CaEDTA), reduces subsequent neuronal death. Exposure to the high micromolar concentrations of zinc likely to occur in brain extracellular space after synchronous cellular depolarization is sufficient to kill cultured neurons, especially if the neurons are depolarized, a state that facilitates toxic entry of zinc across the plasma membrane through several routes. Most prominent among these depolarization-facilitated entry routes are L-type voltage-gated calcium channels, but we now have evidence for participation of N-type voltage-gated calcium channels, agonist-gated calcium channels (especially calcium-permeable AMPA receptors when present, for example, on GABAergic neurons (GABA is  $\gamma$ -aminobutyric acid)) and exchanger-mediated transport (exchanged for sodium, presumably via the sodium-calcium exchanger) [10]. Lower levels of toxic zinc exposure induce apoptosis sensitive to deletion of the *bax* gene or inhibition of caspases; higher levels induce explosive necrosis associated with fulminant cell swelling [11,12].

Using mag-fura-5 initially, and later using the lower affinity, albeit non-ratiometric, indicator dye Newport Green, my colleagues and I have estimated levels of intracellular free zinc attained in neurons subjected to toxic levels of extracellular zinc, and found them to be on the order of 200 to 300 nM [13]. This is a tremendous concentration of free zinc, many orders of magnitude above the affinity of

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intracellular binding sites on metallothioneins and other metalloproteins. It is plausible that many metabolic disturbances might result from such extreme elevations in zinc availability within the intracellular milieu, but an especially consequential disturbance may be caused by a reduction in glycolysis, secondary to inhibition of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [14]. Rather than occurring by direct interaction with zinc, this inhibition appears to reflect depletion of oxidized nicotinamide-adenine dinucleotide ( $\text{NAD}^+$ ) by some catabolic process sensitive to inhibition by benzamide. Administration of benzamide, niacinamide or pyruvate increases  $\text{NAD}^+$  levels, restoring GAPDH function and neuronal ATP levels, and attenuating zinc-induced death. Interestingly, the neuroprotective effects of niacinamide and benzamide in brain ischemia have already been established by studies motivated by considering the ability of these substances to enhance ATP synthesis [15] or inhibit poly(ADP-ribose) polymerase activity [16].

In addition to the contribution of zinc toxicity to selective neuronal loss after transient global ischemia, recent observations from our laboratory spearheaded by Jin-Moo Lee have suggested that it may contribute to the development of cerebral infarction after mild transient focal ischemia. Adult male Long-Evans rats subjected to middle cerebral artery occlusion for 30 minutes followed by reperfusion, developed delayed cerebral infarction reaching completion 3 days after the insult. One day after the insult, many degenerating cerebral neurons exhibited increased intracellular zinc, some labeling with an antibody against activated caspase-3. Intracerebroventricular administration of CaEDTA 15 minutes prior to ischemia attenuated subsequent zinc translocation into the cortical neurons, and reduced infarct volume measured 3 days after ischemia. Although the protective effect of CaEDTA at this end-point was substantial (about 70% infarct reduction), it was lost when insult severity was increased from 30 to 60 minutes of arterial occlusion, or when infarct volume was measured 14 days after ischemia. These observations suggest that toxic zinc translocation may accelerate the development of cerebral infarction after mild transient focal ischemia. Our preliminary studies have not demonstrated any protective effects of intracerebroventricular CaEDTA in more traditional models of focal ischemia, using longer periods of reversible ischemia or permanent ischemia, in which infarction develops more rapidly (complete within a matter of hours after insult).

Why might zinc contribute more prominently to neuronal loss after global ischemia than after focal ischemia? Further studies will be needed to answer this important question, but as a working hypothesis, I am inclined to consider that two related factors are especially influential. First, it is clear from the work of many laboratories that NMDA receptor-triggered, calcium-mediated excitotoxicity is a larger component of focal ischemic injury than global ischemic injury. My colleagues and I have called this form of excitotoxicity “rapidly triggered” to emphasize how quickly it can occur; in cortical neuronal cell cultures, 3 to 5

minutes of sustained NMDA receptor activation is sufficient to destroy most neurons [17]. In contrast, AMPA receptor-triggered, calcium-mediated excitotoxicity, a larger component of global ischemic injury, typically occurs more slowly, requiring hours of sustained receptor activation to induce lethal injury in the same cell cultures. Thus one could imagine that zinc-mediated injury might have more opportunity to lead to cell death after global ischemia; whereas after focal ischemia, more fulminant NMDA receptor-triggered, calcium-mediated injury might supervene and render zinc-mediated injury largely invisible to therapeutic interference.

Second, and probably in part responsible for the first factor (the greater involvement of NMDA receptors in focal ischemic injury as compared with global ischemic injury), extracellular pH in brain tissue does not fall as much in the penumbra of focal ischemia as it does in global ischemia, where it may reach values in mid to upper 6s. Not only does this extracellular acidity selectively downregulate NMDA receptor activation and NMDA receptor-mediated injury [18–20], but it appears to shift L-type voltage-gated calcium channels toward a zinc-preferring mode. In recent experiments using whole cell clamp physiology to measure currents through high voltage-activated calcium channels on cultured cortical neurons, we confirmed earlier studies that indicated that lowering the pH to 6.4 reduced calcium currents through these channels, but we were surprised to see that the same pH manipulation markedly enhanced the zinc current through presumably the same channels [21].

The implication of zinc in the pathogenesis of neuronal loss after ischemic insults raises consideration of several novel therapeutic strategies. In broad categories, these would include:

- 1 Reduction of presynaptic zinc stores. For example, through acute reduction of dietary zinc intake, coupled with oral chelation, as a prophylactic measure before high-risk surgery or other anticipated ischemic stress.
- 2 Reduction of zinc release. Can this be accomplished independent of altering glutamate release?
- 3 Extracellular chelation.
- 4 Block of postsynaptic entry routes, such as voltage-gated calcium channels, calcium-permeable AMPA receptors or the sodium–calcium exchanger. Weak neuroprotective effects of dihydropyridines and other L-type voltage-gated calcium channel blockers have been observed in previous studies with both experimental models and human patients. Could these suggestions of benefit reflect the reduction of zinc toxicity in addition to the intended reduction of calcium overload? To attain higher levels of neuroprotection, it may be necessary to concurrently block multiple pathways of zinc entry.
- 5 Enhancement of zinc buffering, sequestration or export via plasma membrane transporters.

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- 6 Elevation of intracellular NAD<sup>+</sup> or ATP levels.
- 7 Blockade of zinc-induced apoptosis.

The most logical clinical setting to begin testing anti-zinc strategies for neuroprotective effect would be that of hospitalized patients resuscitated after cardiac arrest. No effective neuroprotective treatments are currently available for global ischemia in humans, and the natural history of cerebral degeneration has a well-defined relationship to arrest duration. Patients sustaining longer periods of global ischemia at a normal body temperature prior to effective restoration of cerebral blood flow inevitably develop serious neurological morbidity due to delayed selected neuronal death. These patients could be treated immediately with all the resources of the inpatient setting, and ethical risk/benefit considerations would justify relatively aggressive experimental approaches. Other settings where toxic zinc translocation has been identified in animal models and hence, where anti-zinc approaches might be of clinical value, would include head trauma or sustained seizures [22–24].

Lastly, I will speculate that anti-zinc approaches could find a place in the treatment of stroke, in settings where the ischemic insult is limited. Even if these approaches cannot by themselves prevent infarction from ultimately occurring, perhaps they might be useful in buying time, increasing the temporal therapeutic window for other approaches.

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## Central nervous system ischemia: diversity among the caspases

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### Introduction

Ischemic neurons die acutely by osmotically driven rupture of cellular and subcellular membranes by a process called necrosis, but may also die in a delayed manner, dependent on the activation of a family of cysteine proteases named caspases. Caspases are synthesized as inactive proenzymes containing three subunits, an N-terminal prodomain, a large (~20kDa) and a small subunit (~10kDa), which form heterotetromers on cleavage and activation. Family members show a near absolute specificity for cleavage at the N-terminal of aspartate residues. At least 14 caspases have been identified to date, designated 1 to 14. Caspases -1, -2, -3, -7, -8 and -9 are constitutively expressed in the brain. In the spinal cord, caspases -2, -3 and -8 are constitutively expressed. Caspases -1, -4 and -5 (caspase-1 family members) promote cytokine maturation and mediate inflammation whereas caspases -2, -3, -6, -7, -8 and -9 (caspase-3 family members) promote apoptotic cell death. On activation, caspase-11, which is found only in mice, promotes both cytokine maturation and apoptosis.

In this review, we will briefly summarize the evidence implicating caspases in cerebral and spinal cord ischemia. Caspase-driven cell death may have important therapeutic implications for ischemia as well as for other acute and chronic central nervous system (CNS) conditions in which cell death is prominent.

### Global ischemia

Early evidence for the involvement of caspases in global ischemia came from two studies showing upregulation of caspase-1 mRNA by reverse transcriptase-polymerase chain reaction [1] and in situ hybridization [2] beginning 24 hours after forebrain ischemia in the gerbil (see also Table 2.1). The protein was found at 48 hours [1]. Upregulation of caspase-3 mRNA (in situ hybridization) plus a

**Table 2.1.** Literature overview on caspases in ischemic brain injury  
 Global ischemia

Reference	Ischemia (min)	Caspase	Species	Model	Finding
8	5	Caspase-3	Gerbil	BCAO	–
2	5 or 10	Caspase-1	Gerbil	BCAO	+
1	7	Caspase-1	Gerbil	BCAO	+
9	10	Caspase-9	Dog	Cardiac arrest	+
3	10	Caspase-3	Rat	Cardiac arrest	+
53	12	Caspase-3	Rat	4VO	+
4	15	Caspase-3	Rat	4VO	+
6	15	Caspase-3	Rat	4VO	+
7	15	Caspase-3	Rat	BCAO/hypotension	+
5	30	Caspase-3	Rat	4VO	+

**Focal ischemia (MCAO)**

Reference	Ischemia	Caspase	Species	Model	Finding
18	Permanent	Caspase-1	Mouse (KO)	Filament	+
20	Permanent	Caspase-3	Rat	Filament	+
54	Permanent	Caspase-3	Mouse	Distal	+
26	Permanent	Caspases -3, -8	Rat	Distal	+
19	Permanent	Caspase-11	Mouse	Filament	+
17	3 hours	Caspase-1	Mouse	Filament	+
21	2 hours	Caspase-3	Mouse	Filament	+
22	30 min	Caspase-3	Mouse	Filament	+

*Notes:*

BCAO, bilateral carotid artery occlusion; 4VO, four-vessel occlusion; MCAO, middle cerebral artery occlusion; KO, knockout.

*Source:* From ref. 53.

two-fold increase in DEVD (Asp-Glu-Val-Asp) cleaving activity was identified at 24 hours in rat CA1 hippocampal neurons after 10 minutes of cardiac arrest [3]. These findings were confirmed [4–7], along with additional evidence for increased caspase-3 protein and enzyme activity within the hippocampus after global ischemia. One report, however, failed to show active caspase-3 after global ischemia in the gerbil by immunohistochemistry, although the constitutive proform was widely expressed in the CA1 hippocampus [8]. More recently, caspase-9 release from mitochondria was documented by electron microscopy and fluorescence microscopy after canine cardiac arrest [9]. In vitro, caspase-9 forms a complex (apoptosome) with apoptosis activating factor-1, cytochrome *c* and deoxy-adenosine

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triphosphate, thereby promoting downstream caspase cleavage and activation. Because cytochrome *c* release was detected in hippocampal neurons up to 2 hours after a global ischemic insult [10–12], formation of a mitochondrial death complex might play a role in delayed neuronal death after global ischemia.

The importance of caspases and cell death in global ischemia was further established by pharmacological evidence showing enhanced resistance to ischemic injury after caspase inhibition (Table 2.2). Himi et al. [13] injected into the gerbil hippocampus an irreversible pancaspase inhibitor, benzyloxycarbonyl-Asp-CH<sub>2</sub>-dichlorobenzene (zD), and achieved near-complete rescue of CA1 neurons after 8 days. Performance on memory tests was better and cleavage of a caspase-3 substrate, poly(ADP-ribose) polymerase, was inhibited. Several other groups confirmed these findings [4,6,14]. For example, Chen et al. [4] and Gillardon et al. [6] showed that cell death was decreased by 30% to 85% in the CA1 region after inhibition of caspase-3. However, Li and colleagues [15] injected zVAD.FMK or zDEVD.FMK, both as a pre- and post-treatment, but did not find protection, possibly because a 10-fold lower dose ( $2 \times 200$  ng vs.  $3 \times 1.5$   $\mu$ g) was used in their global ischemia model compared with the previous studies.

### Focal ischemia

Early evidence for the significance of caspases in focal ischemia came from a preliminary study using repeated administration of a pancaspase inhibitor, z-VAD, before and after permanent focal ischemia in the rat. Twenty-four hours later, a 50% reduction of total infarct volume was observed [16] (Table 2.2). Hara et al. [17] confirmed these findings in models of transient focal ischemia (2 hours) in mice and rats and showed 25% protection 24 hours after zDEVD.FMK injection, a more selective caspase inhibitor without inhibition of interleukin-1 $\beta$  formation. The same group demonstrated the importance of caspase-1 by showing neuroprotection (45% decrease of infarct volume) using transgenic mice expressing a dominant negative inhibitor of caspase-1 [17]. A similar infarct reduction ( $-50\%$ ) was also found in caspase-1-deficient animals [18]. However, these data might be difficult to interpret because caspase-1 null mice do not express caspase-11 [19], which is also cleaved and activated during cerebral ischemia and seems to be an upstream modulator of caspase-1 (see below).

Caspase-3 has been implicated in focal ischemic brain damage as evidenced by increased rat caspase-3 mRNA 1 hour after the induction of permanent ischemia [20]. Upregulation of murine caspase-3 protein in neurons plus increased enzyme activity in homogenates was shown by Namura et al. [21] and Fink et al. [22], respectively, after severe and mild focal ischemia. After a more severe reversible ischemia (2 hours of occlusion), caspase-3 was maximally active shortly after