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FORUM REVIEW ARTICLE

Redox Regulation of Intracellular Zinc: Molecular Signaling in the Life and Death of Neurons

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Abstract

Zn^{2+} has emerged as a major regulator of neuronal physiology, as well as an important signaling agent in neural injury. The intracellular concentration of this metal is tightly regulated through the actions of Zn^{2+} transporters and the thiol-rich metal binding protein metallothionein, closely linking the redox status of the cell to cellular availability of Zn^{2+}. Accordingly, oxidative and nitrosative stress during ischemic injury leads to an accumulation of neuronal free Zn^{2+} and the activation of several downstream cell death processes. While this Zn^{2+} rise is an established signaling event in neuronal cell death, recent evidence suggests that a transient, sublethal accumulation of free Zn^{2+} can also play a critical role in neuroprotective pathways activated during ischemic preconditioning. Thus, redox-sensitive proteins, like metallothioneins, may play a critical role in determining neuronal cell fate by regulating the localization and concentration of intracellular free Zn^{2+}. *Antioxid. Redox Signal*. 15, 2249–2263.

Introduction

ZINC HAS BEEN REFERRED TO as the "calcium of the 21st century," reflecting its diverse roles in normal cell physiology and, in turn, its deregulation as a contributor to cellular pathological changes (29). Relatively abundant in the mammalian brain, Zn^{2+} plays a critical role as a structural component of numerous proteins and transcription factors, and has recently emerged as a neuromodulator and intracellular signaling factor (129). The concentration of intracellular free, or "chelatable" Zn^{2+}, is maintained in the picomolar range under normal conditions due to exquisite regulation by metal binding proteins, a family of Zn^{2+} efflux transporters, and compartmentalization into organelles (110). However, an accumulation of free Zn^{2+}, mediated by redox modulation of intracellular metal binding proteins (1) along with translocation from presynaptic vesicles into postsynaptic neurons (53), plays an important role in ischemic neurodegeneration.

Neuronal tolerance to lethal ischemic cell death can be conferred by sublethal preconditioning stimuli, which activate endogenous survival pathways that limit or resist subsequent lethal injury (51). Increasing evidence suggests that preconditioning stimuli induce the sublethal activation of cell death factors that trigger survival pathways, which, in turn, prevent subsequent lethal injurious signaling (32). Along these lines, a novel role for Zn^{2+} as an important, early signal in the initiation of survival pathways critical to neuronal tolerance has emerged (5, 65). The results from these studies offer insights into endogenous mechanisms that protect neurons in the face of lethal cellular injury. In this review, we discuss the roles for Zn^{2+} in cell physiology, neuronal cell death pathways, and neuronal tolerance, focusing on the intersection of Zn^{2+}-dependent pathways with redox-sensitive proteins and other damage-associated molecular patterns (DAMPs).

Physiological Roles of Zinc

Zinc is the second most prevalent trace element in the body and is very abundant in the brain (overall content of $\sim 150\,\mu M$) with highest concentrations in forebrain regions, including the hippocampus, amygdala, and cortex (127). Since its discovery in the brain almost 55 years ago, Zn^{2+} has been shown to play critical roles in protein structure, enzymatic activity, and gene regulation (29). Zn^{2+} can associate with over 300 enzymes, where it can interact strongly with electronegative sulfur, nitrogen, and oxygen moieties in multiple coordination geometries, serving catalytic and structural roles in maintaining active peptide conformations (23). In addition to metalloenzymes, Zn^{2+} is most known for its ability to bind and stabilize proteins involved in gene regulation in domains called zinc fingers, zinc clusters, and zinc twists (122). Because of the critical role Zn^{2+} plays in protein structure and function, it is not surprising that chronic dietary Zn^{2+} deficiency manifests in multisystemic dysfunction, including growth failure, skin changes, delayed wound healing, immune system impairment, neurosensory disorders, and lack of sexual development (101).

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Besides regulating the structure and function of proteins, brain Zn^{2+} may also be important in modulation of neuronal activity (29). Approximately 10% of neuronal Zn^{2+} is selectively stored in presynaptic vesicles of certain glutamatergic neuronal terminals by the neuronal-specific zinc transporter 3 (ZnT3). Because of this, much attention has been given to determining whether synaptic Zn^{2+} release is activity dependent (96). Activity-dependent Zn^{2+} release was elegantly and clearly demonstrated by Qian and Noebels, who used a membrane-impermeant form of the Zn^{2+}-selective indicator FluoZin-3 to show extracellular Zn^{2+} in mossy-fiber synapses of wild-type, but not ZnT3 knock-out mice after individual action potentials (102, 103). Zn^{2+} released from presynaptic neurons has several potential postsynaptic targets. Zn^{2+} inhibits N-methyl-D-aspartate (NMDA) and gamma-aminobutyric acid receptor subtypes, and potentiates glycine receptors (96, 110). In addition to modulating ionotropic pathways, synaptic Zn^{2+} has also been reported to transactivate receptor tyrosine kinase b (42) and regulate metabotropic activity through the activation of a recently described Zn^{2+} receptor, ZnR (10). Of interest, the molecular identity of the ZnR has been proposed to be the orphan G protein-linked receptor GPR39 (10). However, since the exact magnitude and time course of synaptic Zn^{2+} remain unclear (47), the full range of physiological roles for vesicular Zn^{2+} has yet to be fully described.

A critical new role of Zn^{2+} is its emergence as an intracellular signal. Yamasaki and colleagues recently found evidence for intracellular Zn^{2+} release in mast cells within minutes of extracellular stimulation of the high-affinity immunoglobulin E receptor 1 (FcεR1), a phenomenon they termed a "zinc wave" (129). Using confocal fluorescent imaging, the authors found that the Zn^{2+} wave originated near the endoplasmic reticulum and other perinuclear areas (129). Rapid Ca^{2+} influx and mitogen-activated protein/extracellular signal-regulated kinase (MEK) activity were required for the Zn^{2+} rise, although neither the Ca^{2+} ionophore ionomycin nor an MEK activator was sufficient to elicit a Zn^{2+} wave (129). Zn^{2+} chelation decreased FcεR1-induced interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) mRNA expression, while increasing free Zn^{2+} in mast cells with pyrithione/Zn^{2+} prolonged mRNA expression (129). Similarly, mitogen-activated protein kinase (MAPK) activation was decreased with Zn^{2+} chelation and prolonged with Zn^{2+} supplementation after FcεR1 stimulation (129). A likely role for such intracellular Zn^{2+} signals is the modulation of protein phosphorylation. While increased cellular Zn^{2+} can activate many kinase pathways, including extracellular signal-regulated kinase and p38 (135), these studies suggest a target upstream in the signaling cascade rather than direct effect of Zn^{2+} on these kinases. For the most part, however, activation of Zn^{2+}-dependent kinase cascades have been examined within the context of large exogenous Zn^{2+} exposure, sometimes leading to cellular toxicity. On the other hand, physiologically relevant nanomolar concentrations of Zn^{2+} have been shown to effectively inhibit protein tyrosine phosphatase (PTP) activity (38). These studies were performed with a truncated form of the phosphatase containing only the catalytic domain, suggesting that the inhibitory action of Zn^{2+} is through direct interaction with this highly conserved domain of PTPs (37). Thus, a major physiological role for Zn^{2+} may be the modulation of cell signaling cascades, especially those involving protein phosphorylation.

Regulation of cellular zinc

Zn^{2+} does not passively cross biological membranes and thus requires specialized mechanisms for its uptake and release. Dietary Zn^{2+} is absorbed from the gastrointestinal tract by a ZnT, ZIP4, located on the apical membrane of enterocytes, whereas another ZnT, ZnT1, is involved in zinc efflux from the enterocyte into the circulation (70). Mutations in the *ZIP4* gene cause Acrodermatits enteropathica, a rare, lethal autosomal recessive inherited human zinc deficiency (59, 125). Once in the circulation, Zn^{2+} is bound to albumin and other plasma proteins facilitating transport to tissues, including the brain. Despite relatively high Zn^{2+} content in the brain, extracellular Zn^{2+} concentrations are normally below 500 n*M* and the intracellular levels of free Zn^{2+} are generally 1 n*M* or less (127). Intracellular free Zn^{2+} is tightly regulated though the opposing actions of the solute-linked carrier 39 (SLC39) family of transporters (also known as Zrt- and Irt-like proteins or Zips) and the SLC30 transporter family (also known as ZnTs) (110). Zip and ZnT proteins appear to have opposing roles in Zn^{2+} homeostasis, as Zips increase cytoplasmic Zn^{2+}, whereas ZnTs promote Zn^{2+} efflux from the cytoplasm into intracellular compartments or across the plasma membrane (70). Detailed mechanisms and energetics of transport by these proteins are not well characterized, but are believed to be mediated by facilitated diffusion, secondary active transport, or symporters (70). Recent work investigating the activity of the ZnT5 transporter, which is localized in the Golgi apparatus and mediates Zn^{2+} transport into this compartment, found that Zn^{2+} transport is catalyzed *via* Zn^{2+}/H^{+} exchange and is powered by the vesicular H^{+} gradient (88). Further, using a bacterial Zn^{2+} transporter YiiP as a homology model, this study identified Asp 599 and His 451 as two residues on the C-terminal region of ZnT5 that are critically responsible for ZnT5-mediated Zn^{2+} transport and sequestration to the Golgi (88). This work represents an important step toward determining the transport mechanisms involved in Zn^{2+} homeostasis.

Metallothioneins (MTs) also play a major role in regulating intracellular Zn^{2+} homeostasis by binding, releasing, and distributing Zn^{2+} (Fig. 1). MTs are a family of nonenzymatic, cysteine-rich, metal-binding polypeptides (61–68 amino acids) found throughout mammalian tissues (6). In the mammalian adult central nervous system, three major MT isoforms are expressed. The precise cellular localization of MT I, II, and III is still a matter of debate due to the lack of isoform-specific antibodies leading to conflicting results between *in vivo* and *in vitro* studies and between mRNA and protein expression assays (41). The general consensus, however, is that MT I and MT II are the predominant astrocytic isoforms (where they are expressed over seven-fold higher than in neurons), whereas MT III is predominantly expressed in neurons (41). MT III was first identified during investigations of putative mechanisms underlying Alzheimer's disease neuropathology. Abundant MT III expression, which was then identified as growth inhibitory factor due to its ability to inhibit neurotrophic factors and neurite outgrowth, was found in normal brain astrocytes, but not in Alzheimer's disease brains (121). However, since then, the association between MT III and Alzheimer's disease has not fully developed (19). Instead, MTs are thought to play a major role in heavy metal detoxification and Zn^{2+} homeostasis. Expression of MT I and II is highly inducible in response to a range of stimuli in addition to Zn^{2+} and Cd^{2+},

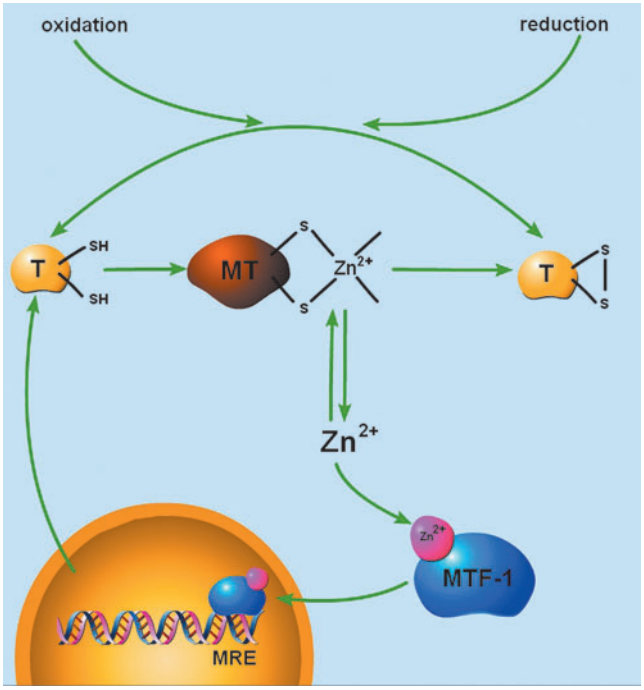

FIG. 1. Redox modulation of metallothionein (MT). The Zn^{2+}/sulfur coordination environments of metal-bound MT can be oxidized, causing the liberation of free Zn^{2+}. Intracellular free Zn^{2+} can activate the metal response element transcription factor-1 (MTF-1), resulting in the upregulation of the metal deficient thionein. Due to its Zn^{2+} chelating and reductive capacities, thionein can either bind-free Zn^{2+} or interact with other redox-active processes forming an oxidized thionein, which cannot bind Zn^{2+}. In this light, thionein/MT can effectively transduce redox signals to Zn^{2+}-mediated cellular signaling processes [adapted from Krezel and Maret (56)]. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

including hormones, cytokines, oxidative agents, inflammation, and ischemia (2). Because of this, MTs have been implicated in mediating a more general cellular response to injury (128). Exciting recent reports have shown that MT can be secreted by astrocytes and internalized by neurons after brain injury, promoting neuronal cell survival (25). Thus, MTs play diverse roles in regulating cellular Zn^{2+} homeostasis, heavy metal detoxification, and intercellular response to injury.

MTs are structurally composed of two globular metal-binding domains (α and β) containing a total of 20 sulfur-donating cysteine residues, allowing them to bind up to seven Zn^{2+} ions in Zn_3S_9 and Zn_4S_{11} configurations or up to seven Cd^{2+} ions or 12 Cu^{2+} ions (73). These cysteine residues in MTs occur in unique Cys-X-Cys, Cys-X-Y-Cys, and Cys-Cys motifs (45). For MTs to buffer cellular-free Zn^{2+}, these high-affinity ligands for Zn^{2+} must normally not be saturated with metal. Indeed, biochemical analysis of the association of Zn^{2+} with the apoprotein thionein revealed three classes of binding affinities that vary by several orders of magnitude. Four Zn^{2+} ions were bound tightly ($\log K_d = 11.8$), making the thionein a strong chelating agent; one Zn^{2+} was relatively weakly bound ($\log K_d = 7.7$), making MT a potential Zn^{2+} donor, and the remaining two Zn^{2+} ions were with intermediately bound ($\log K_d \sim 10$) (57). This suggests that MT molecules are not saturated with seven Zn^{2+} ions under normal physiological conditions and can actively participate in cellular Zn^{2+} buffering and distribution (57). In addition, differential fluorescent labeling of the MT cysteine clusters from rat kidney, liver, and brain tissue extracts showed that tissues contain almost as much of the apo-protein thionein as metal-bound MT (130). Thus, due to the presence of Zn^{2+}-lacking species along with apo-thionein, MT III serves a significant role in buffering neuronal free Zn^{2+}. Further, the unique Zn^{2+} coordination environment of MT allows the redox environment of the cell to ultimately dictate overall cellular Zn^{2+} availability.

Zinc-regulated gene expression

A major role for physiologic Zn^{2+} is the regulation of the proteins that control the intracellular concentration of the metal (Fig. 2). The inducible MT isoforms, MT I and MT II, are regulated in a coordinated manner through the activation of gene transcription (39). The promoter regions of MT I/II genes contain several *cis*-acting DNA elements, including metal response elements (MRE), glucocorticoid response elements (GREs), antioxidant response elements (ARE), and IL-6/signal transducers and activator of transcription responsive elements (IL-6 RE) that can bind *trans*-acting transcription factors involved in the regulation of both constitutive and inducible MT expression (39). MRE transcription factor 1 (MTF-1) is the predominant gene regulatory protein mediating constitutive as well as metal- and oxidative stress-induced MT I/II expression (2). MTF-1 is a 72.5-kDa, ubiquitous zinc-finger

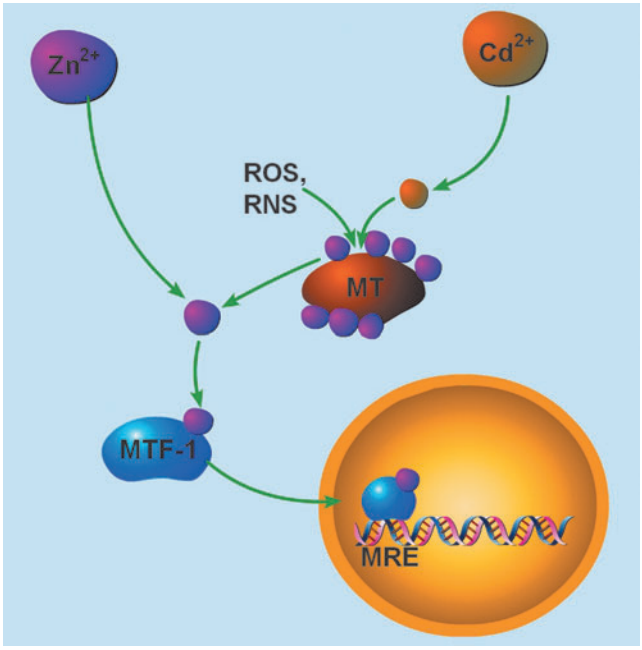

FIG. 2. Zn^{2+}-regulated gene expression. Free Zn^{2+} signals can be generated by (i) mobilization of the metal from MT *via* thiol modification by reactive oxygen or nitrogen species (ROS, RNS), or (ii) displacement of Zn^{2+} by Cd^{2+}, which binds to MTs with higher affinity. Once bound to Zn^{2+}, MTF-1 translocates to the nucleus and binds to metal response elements (MRE) on the promoter regions of Zn^{2+}-dependent genes. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

transcription factor in the Cys_2His_2 family that binds to DNA sequence motifs, known as MREs, at the consensus site TGCRCNC (3). MRE sequences are present in five, nonidentical copies (MREa-MREe) in the 5′ flanking region of the MT I/II gene (3). MTF-1-mediated MRE activation sufficiently upregulates MT I/II in response to Zn^{2+} or Cd^{2+}, oxidative stress, and hypoxia (2, 83). After Zn^{2+}, acute hypoxia, or oxidative stress induced by H_2O_2 or tert-butylhydroquinone, MTF-1 translocates from the cytoplasm to the nucleus and acquires tight DNA-binding ability (2). However, after exposure to other heavy metals, most notably Cd^{2+}, the DNA-binding ability of MTF-1 is inhibited in cell-free DNA binding assays, despite induction of MTF-1-dependent MT gene transcription *in vivo* (3). This paradox was solved by demonstrating that transcriptional activation after Cd^{2+} requires Zn^{2+}-saturated MT (134). Cd^{2+}, which binds to MTs with higher affinity than Zn^{2+}, can displace Zn^{2+} from the metal-binding protein, making Zn^{2+} available for MTF-1 activation (134). Activation of gene transcription also requires the occupancy of the Zn^{2+} fingers with Zn^{2+} and the interaction among three distinct transcriptional activation domains in the C-terminal region of MTF-1 (3). While most of the evidence characterizing Zn^{2+}-regulated gene expression has been performed studying MT gene activation, changes in ZnT1 transporter expression can also be induced by Zn^{2+} or Cd^{2+} (2). Constitutive and metal-induced activation of the ZnT1 gene also requires MTF-1 (62). While the organization of the promoter regions of all MT genes is conserved, the MT III gene was found to contain a "silencing element" in the 5′ flanking regions upstream from the first cluster of putative MREs (20), eliminating Zn^{2+}-regulation and making MT III a constitutively expressed protein in neurons. Close inspection of the silencing element of the rat MT III promoter reveals that it contains a quadruplicate CTG sequence ~400 base-pairs upstream of the translation start site (20). CTG-repeat elements have been shown to act as repressors on a number of heterologous promoters independent of its orientation and proximity to the gene, and thus may account for the silencing effect on the promoter region of the MT III gene (20). Besides MT I/II and ZnT1, many other MRE-containing MTF-1 target genes have been identified, including γ-glutamylcysteine synthetase (a key enzyme in glutathione synthesis), α-fetoprotein, and transforming growth factor β-1 (68).

Role of Zinc in Neuronal Cell Death

Translocation of vesicular zinc

Unregulated increases of free intracellular Zn^{2+} lead to cell death. Using TSQ (N-[6-methoxy-8-quinolyl]-P-toluenesulfonamide) and acid fuschin to simultaneously stain the presence of Zn^{2+} and degenerating neurons, respectively, Tonder *et al*. suggested a translocation of Zn^{2+} from mossy fibers terminals to degenerating dentate hilar neurons 2–24 h after 20 min of cerebral ischemia (118). This type of observation, coupled with reports suggesting that the amount of Zn^{2+} stored in synaptic vesicles ($\sim 300\,\mu M$) was sufficient to kill neurons (132), led to what is generally known as the Zn^{2+} translocation hypothesis. This hypothesis proposes that the extracellular release of vesicular Zn^{2+} and its uptake by postsynaptic neurons is responsible for the toxic accumulation of this metal in neurons after ischemia and other neurological disorders (28). In support of this model, Koh *et al*. demonstrated that intraventricular injection of the membrane-impermeant Zn^{2+} chelator, ethylenediaminetetraacetic acid saturated with Ca^{2+} (CaEDTA), blocked the accumulation of neuronal Zn^{2+} and prevented neuronal death after transient global cerebral ischemia (53). While Zn^{2+} was previously known to be toxic to neurons when applied exogenously (24), these reports were first to implicate endogenous accumulation of Zn^{2+} in ischemic neuronal death.

Extracellular Zn^{2+} can lead to toxic intracellular Zn^{2+} accumulations by entering through voltage-sensitive Ca^{2+} channels (VSCC), NMDA receptors, or Ca^{2+}-permeable α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)/kainate receptor channels (Ca^{2+}-A/K) (110). Of these, Ca^{2+}-A/K channels exhibit the highest Zn^{2+} permeability (111) and are thought to be the primary route of Zn^{2+} entry after ischemia (131). Indeed, in hippocampal slices, ischemia-induced Zn^{2+} accumulation and subsequent neurodegeneration can be attenuated with a Ca^{2+}-A/K channel inhibitor but not NMDA receptor or VSCC channel antagonists (131). Hippocampal pyramidal neurons, which are especially sensitive to ischemic injury, express Ca^{2+}-A/K channels on distal dendritic branches (87). Unlike all other heterotetrameric AMPA receptors, Ca^{2+}-A/K channels lack the GluR2 subunit (60). GluR2 subunits contain an arginine (R) in its pore-forming domain due to RNA editing of a genomically encrypted glutamine (Q) codon (60). The arginine is functionally dominant because it dictates gating kinetics, channel conductance, channel assembly, and, importantly, Ca^{2+} permeability (35). Edited GluR2(R) subunits form Ca^{2+}-impermeable channels, whereas unedited GluR2(Q) channels are Ca^{2+} permeable (60). Generation of mice with unedited GluR2(Q) exhibit fulminant seizures and death by 3 weeks of age (14). GluR2 expression can be modulated by ischemic injury, as ischemia has been shown to induce GluR2 downregulation (34) and disrupt Q/R editing (98). This modulation of GluR2 subunits render AMPA channels Ca^{2+} permeable, allowing entry of toxic Ca^{2+} and Zn^{2+} during ischemia (91).

Regulation of Ca^{2+}-A/K channel composition after ischemic injury may also involve Zn^{2+}-dependent signaling. In an *in vivo* model of stroke, application of the cell-impermeant Zn^{2+} chelator CaEDTA 30 min before global ischemia attenuated the ischemic downregulation of GluR2, the delayed rise in neuronal Zn^{2+}, and neuronal death (16). Ischemic downregulation of GluR2 occurs *via* the Zn^{2+}-dependent expression of the nine Zn^{2+}-finger transcription factor restrictive element-1 silencing transcription factor (REST-1), which suppresses neuronal-specific targets, including GluR2 (16, 17). Application of CaEDTA 48–60 h after global ischemia, presumably after GluR2-lacking A/K channels are already expressed, attenuated only the late rise in neuronal Zn^{2+} and cell death (16).

In spite of the aforementioned work, several key observations have shown that Zn^{2+} translocation cannot fully account for the accumulation of free Zn^{2+} in dying neurons after ischemia. For instance, Zn^{2+} accumulation in neurons after injury is observed in areas that are not highly innervated by glutamate- and Zn^{2+}-releasing fibers, such as thalamic neurons (53, 61). Intracellular sources of Zn^{2+} must play a major role in lethal Zn^{2+} accumulation in these brain regions. More importantly, if presynaptic Zn^{2+} were the only source of toxic Zn^{2+} contributing to ischemic injury, then animals without presynapic Zn^{2+} would not be susceptible to Zn^{2+}-dependent toxicity. However, mice lacking the ZnT3, which show no

histochemically reactive Zn^{2+} in presynaptic vesicles, still undergo significant Zn^{2+} accumulation in degenerating CA1 and CA3 neurons after kainate-induced seizures (64). This suggests that lethal neuronal injury can induce intracellular Zn^{2+} accumulation and neurodegeneration in the absence of presynaptic vesicular Zn^{2+}. In initial studies, CaEDTA nearly completely abolished Zn^{2+} accumulation and neuronal cell death, suggesting an extracellular source for Zn^{2+} (53). However, CaEDTA has since been shown to deplete intracellular Zn^{2+} compartments, including synaptic vesicles (30). CaEDTA may trap extracellular Zn^{2+}, giving rise to a steep Zn^{2+} gradient across the plasma membrane resulting in the removal of Zn^{2+} from the neuronal cytoplasm. As such, accumulation of intracellular Zn^{2+} after neuronal injury may not be solely dependent on translocation of the metal from presynaptic neurons. Intracellular sources of Zn^{2+} may therefore play a substantial and critical role in neurodegenerative processes.

Redox-dependent liberation of intracellular zinc

As Zn^{2+} accumulation can occur in degenerating neurons even in the absence of vesicular Zn^{2+}, liberation of Zn^{2+} from intracellular stores may be a significant mechanism of cell injury, especially in brain regions with little or no synaptic Zn^{2+}. Vallee and colleagues suggest that Zn^{2+} binding and release in cells are dynamic processes that are dependent on the intracellular redox status (Fig. 1) (44). Of the Zn^{2+} metalloproteins, only those with Zn^{2+}/sulfur coordination environments are susceptible to oxidative mobilization of Zn^{2+} during injury (72). These coordination environments allow for tight binding of Zn^{2+} and for the mobilization of redox-inert Zn^{2+} (9) by biological oxidants. Of the thiol-Zn^{2+} metalloproteins in neurons, MT III is a likely source of injury-induced mobilized Zn^{2+}. Despite binding Zn^{2+} with high thermodynamic stability ($K_d = 1.4\times10^{-13}$ *M* for human MT, pH 7.0), the redox potential for MT is very negative (−366 mV), causing even mild cellular oxidants to release Zn^{2+} from the metal-binding protein (73). In HT-29 cells, it was found that as the intracellular redox potential became more oxidizing, the buffering capacity of MT was lowered, leading to a pronounced elevation in free Zn^{2+} (55). Liberation of Zn^{2+} from MT has been demonstrated in a variety of systems, including cell-free assays (73), as well as in cortical neurons (1, 136), where it is an event critical to initiation of oxidant-induced neuronal apoptosis (1). In mouse hepatoma (Hepa) cells, oxidants activate MRE-binding by MTF-1 (2). Further, nitric oxide (NO) and subsequent peroxynitrite ($ONOO^-$) formation, two powerful cellular oxidants contributing to ischemic nitrosative stress, also liberate neuronal Zn^{2+} from intracellular stores triggering neurodegeneration (13, 136). NO-induced Zn^{2+} liberation in pulmonary artery endothelial cells was shown to effectively activate MTF-1 and transcription of MT (113). NO has been shown to more readily release Zn^{2+} from MT III than other MT isoforms due to the unique presence of consensus motifs for catalytic nitrosylation and the preferential reactivity of S-nitrosothiols with MT III through transnitrosylation, allowing direct transfer of NO between sulfhydryl groups that result in sequential release of Zn^{2+} ions (21). Other redox signals, including superoxide/peroxide, selenium compounds, and NADPH, have also been shown to induce the release Zn^{2+} from MT (71, 107). Thus, liberation of Zn^{2+} from intracellular stores, especially neuronal MT III, may be a significant source of the Zn^{2+} rise after ischemia. Depending on the availability of unoccupied Zn^{2+} binding sites as well as the intracellular redox environment, MT III can serve as an important sink, as well as a source, for neuronal-free Zn^{2+}.

Zinc-dependent signaling in neuronal death

Accumulation of neuronal Zn^{2+}, likely mediated by a combination of Zn^{2+} translocation from presynaptic vesicles and Zn^{2+} liberation from intracellular stores, can trigger subsequent neurodegenerative signaling after ischemia (Fig. 3). Zn^{2+}-induced cell death involves several serial and parallel signaling events, has features of both apoptosis and necrosis (50), and is likely mediated by oxidative and nitrosative stress (48). The diversity of cell death signaling attributed to Zn^{2+}

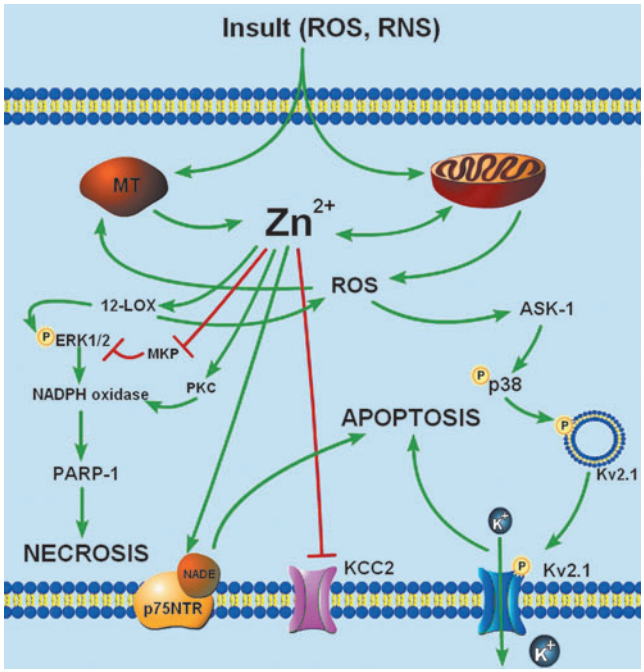

FIG. 3. Zn^{2+}-dependent signaling in neuronal cell death. Central to Zn^{2+}-dependent neuronal cell death are roles for mitochondria and oxidative stress. Free Zn^{2+} can be taken up by mitochondria leading to changes in mitochondrial potential ($\Delta\Psi_m$), release of ROS and pro-apoptotic proteins, and induction of mitochondrial swelling (110). These effects on mitochondria could also be produced after oxidant-induced liberation of intracellular Zn^{2+} (110). Several downstream neuronal cell death signaling pathways have been shown to be Zn^{2+}-dependent. Oxidant- or microglia-induced Zn^{2+} liberation triggers 12-lipoxygenase (12-LOX) activity, resulting in p38-dependent enhancement of K^+ efflux through newly inserted Kv2.1 channels. Alternatively, Zn^{2+}-coordinated activation of 12-LOX and protein kinase C (PKC) and inhibition of MAP kinase phosphatase can trigger a necrotic cell death pathway involving extracellular signal-regulated kinase (ERK) phosphorylation, NADPH-oxidase activation, and poly-ADP-ribose polymerase (PARP-1) cleavage. In addition, Zn^{2+} has also been shown to be critical for p75-NTR-mediated apoptosis and has recently shown to inhibit K^+/Cl^- co-transporter 2 (KCC2) after oxygen-glucose deprivation (OGD). (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

may depend on the intensity of Zn^{2+} exposure, as brief exposure to high concentrations of Zn^{2+} lead to signs of necrotic, caspase-independent cell death, whereas longer exposures to lower Zn^{2+} concentrations trigger apoptotic, caspase-dependent cascades (48). In addition, accumulation of Zn^{2+} after ischemia may trigger divergent signaling mechanisms along multiple temporal profiles. While a delayed rise in neuronal Zn^{2+} in degenerating neurons after ischemia has been well described (53), recent reports suggest that an early Zn^{2+} rise after ischemia onset also contributes to ischemic injury (79, 114). Mechanisms involved in Zn^{2+}-dependent ischemic neuronal death are incompletely understood, and may resemble the complexity in Ca^{2+}-mediated cell death pathways (22). Diverse Zn^{2+}-dependent cell death signaling events have been described, including regulation of Zn^{2+}-dependent transcription factors, induction of p75 neurotrophin receptor-mediated pathways, and activation of kinases and poly-ADP-ribose polymerase (PARP-1) (110). Recent work has also illuminated a previously unknown link between Zn^{2+} and the K^+/Cl^- cotransporter 2 (KCC2) after oxygen–glucose deprivation (40). Here, we highlight some recent examples of neuronal cell death pathways that critically involve an accumulation of free Zn^{2+}.

Role of Zn^{2+} in Kv2.1-facilitated apoptosis. A well-characterized caspase-dependent apoptotic pathway has been critically linked to oxidant-induced Zn^{2+} liberation. The enhancement of voltage-gated K^+ channel (Kv) activity, resulting in cellular K^+ efflux, is a critical step in many apoptotic programs (12). Apoptotic K^+ current enhancement leads to a decrease in the concentration of this cation in the cytoplasm (133), which serves as a permissive apoptotic signal (12), as pro-apoptotic factors are activated most efficiently at reduced K^+ concentrations (43). In our laboratory, Kv2.1 was identified as the critical mediator of K^+ efflux during neuronal apoptosis (92). Kv2.1 is the major component of delayed-rectifying K^+ current in cortical neurons (82), and exists in large, highly phosphorylated clusters on the somatic surface and proximal dendrites of cortical neurons (109). Stimulation of a caspase-dependent neuronal apoptotic cascade by oxidative injury triggers the liberation of intracellular Zn^{2+}, leading to p38 MAPK-dependent phosphorylation and insertion of new Kv2.1-encoded channels (Fig. 3) (77, 93, 105). Recent molecular studies have shown that, in addition to p38 phosphorylation, the apoptotic program requires Kv2.1 to be phosphorylated on a N-terminal tyrosine (Y124) residue (104), a target of Src kinase and cytoplasmic PTP-epsilon (PTPε). Zn^{2+} plays a critical role in Kv2.1-mediated apoptosis by coordinating the activation of p38 MAPK and Src kinase, along with the concomitant inhibition of cytoplasmic PTPε, thereby enabling the completion of the apoptotic program (104).

Role of Zn^{2+} in immune cell/DAMP-mediated neuronal death. Microglia are resident immune cells of the central nervous system that can serve several beneficial functions in neuronal cellular maintenance and innate immunity. Analogous to peripheral macrophages, microglia monitor the brain parenchyma through expression of diverse membrane receptors that can identify a wide range of signals (11). Integral to the innate immune response are constitutively expressed pattern recognition receptors that identify and bind DAMPs and pathogen-associated molecular patterns (PAMPs). Indeed, microglia have been shown to express many of the well-recognized pattern recognition receptors, including toll-like receptors 1–9 (90), scavenger receptors, and the receptor for advanced glycation endproducts (11). However, the microglial response to signals in innate immunity can differ from that elicited by neuronal damage.

When stimulated during brain injury, the overactivation and dysregulation of microglia can compound neuronal damage by releasing neurotoxic cytokines, matrix metalloproteinases, and NO and superoxide (O^-_2), leading to the formation of the powerful cellular oxidant, peroxynitrite ($ONOO^-$) (66). Since $ONOO^-$ can traverse membrane lipids faster than its decomposition pathways (74), it is possible that activated microglia may trigger oxidant-induced Zn^{2+}-dependent neuronal cell death. Indeed, in a coculture model, lipopolysaccharide (LPS)-activated microglia triggered an apocyanin- and 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron(III) chloride-dependent accumulation of neuronal free Zn^{2+} (52). Further, microglia-derived reactive oxygen and nitrogen species (ROS and RNS) triggered the well-characterized apoptotic signal-regulating kinase-1 (ASK1)/p38-dependent, Kv2.1-mediated K^+ current surge (52). When these studies were repeated in neurons overexpressing MT III, thereby chelating free Zn^{2+}, the K^+ current surge and neuronal cell death were markedly reduced (52). Thus, microglial activation can lead to a neuronal free Zn^{2+} rise and Zn^{2+}-dependent neuronal cell death. Of note, peroxynitrite is also responsible for the LPS-induced degeneration of developing oligodendrocytes in coculture experiments (66). However, when astrocytes were present along with oligodendrocytes and microglia, the LPS-induced microglia-dependent toxicity to developing oligodendrocytes was mediated by a mechanism involving TNF-α signaling (67). Thus, in addition to redox-triggered signaling, microglia-mediated toxicity is also dependent on a number of immunologically relevant cytokines that can activate conserved DAMPs and PAMPs.

Microglia can stimulate an accumulation of free Zn^{2+} in target neurons, and the metal itself can trigger microglial activation. After exogenous Zn^{2+} exposure, cultured mouse microglia transformed into the activated morphology, increased NO production and F4/80 expression, and altered cytokine release (46). Zn^{2+}-induced microglial activation was dependent on NADPH oxidase and PARP-1 activation (46). Importantly, intracerebroventricular injection of Ca^{2+}-EDTA prevented microglial activation in an *in vivo* model of cerebral ischemia (46). Thus, a positive feedback model emerges in which ischemic injury leads to microglial activation causing the release of cellular oxidants and other DAMPs, which lead to the liberation of neuronal free Zn^{2+} (Fig. 4). In addition to activating cell death signaling pathways, the accumulation of free Zn^{2+} may overwhelm homeostatic mechanisms and lead to Zn^{2+} release, further activating microglia. Other DAMPs may also contribute to microglia-mediated neurodegeneration after stroke. For example, the prototypical DAMP, high-mobility group box 1 (HMGB1) is elevated in the serum of stroke patients (81), may be secreted from neurons triggering microglial activation (49), and contributes significantly to neurodegeneration after ischemia (69, 81). Thus, further studies are needed to better understand the precise mechanisms by which immune cells and inflammatory signaling contributes to neurodegeneration after acute neuronal injury, as well as the relative roles of Zn^{2+} in these processes.

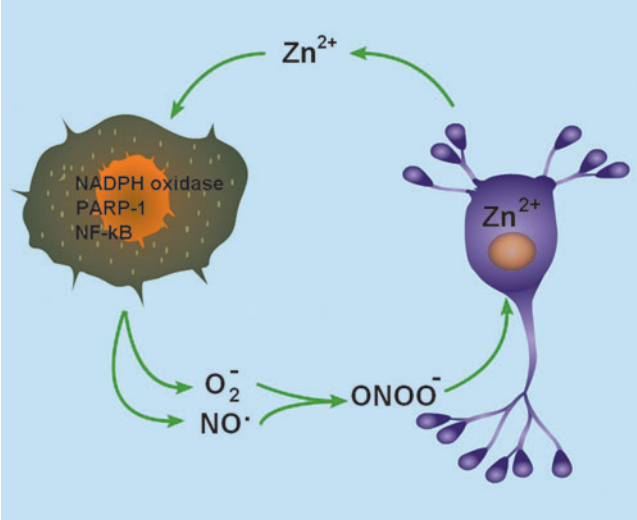

FIG. 4. Zn^{2+} and microglial activation. After brain injury, overactivated microglia release nitric oxide (NO) and superoxide (O^{-}_2), forming the neurotoxic oxidant, peroxynitrite ($ONOO^{-}$). This oxidative injury results in the accumulation of neuronal free Zn^{2+}, triggering a p38-dependent K^{+} efflux surge and neuronal cell death. Sustained Zn^{2+} accumulation in injured neurons may lead to release of Zn^{2+} to the extracellular space, which can sustain microglial activation, fueling a positive feedback loop in microglia-mediated neurodegeneration. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

Zinc in Neuronal Preconditioning

Preconditioning refers to the activation of endogenous adaptive processes by sublethal stimuli, which in turn can increase cellular tolerance to subsequent, lethal injury (86). Tolerance to lethal ischemic injury was first described by preconditioning cardiac myocytes with brief sublethal ischemic episodes (84). This phenomenon was later shown to also be present in the brain (51). A variety of stress stimuli known to cause ischemic brain injury when utilized at sublethal levels have been reported to trigger neuronal tolerance, including ischemia, hypoxia, metabolic inhibitors, excitotoxins, and inflammatory cytokines (32). Thus, the ultimate fate of neurons may not depend on which molecules are activated after a particular insult, but rather the extent and duration of their activity.

Cell death pathways trigger tolerance

Recent evidence suggests that pro-survival mechanisms conferring neuroprotection in ischemic preconditioning may involve sublethal activation of cell death pathways. For example, antioxidants and protease inhibitors, which limit cell death after lethal stimuli, can paradoxically increase vulnerability to subsequent lethal injury when administered during the preconditioning period (76). Similarly, autophagy, a lysosomal degradation process of the cell's own components, has been shown to be protective after a preconditioning stimulus, but may contribute to cell death after lethal insults (97). Caspase-3, an apoptosis executioner cysteine protease, has also been implicated in neuronal tolerance (Fig. 5). Our group first demonstrated that preconditioning led to caspase-3 cleavage *in vivo* and that caspase-3 activity was required for neuronal tolerance *in vitro* (76). Pro-caspase cleavage and caspase enzymatic activity reach maximum levels 6 h after a preconditioning stimulus and are dependent on ROS generation and ATP-sensitive potassium channel (K_{ATP}) channel opening (31, 76). Although widespread caspase-3 activation characterizes many cell death processes, the relatively modest preconditioning-induced caspase-3 activation is held in check by sequestration with caspase-binding proteins, including the constitutively active heat shock protein 70 (HSP70) homolog HSC70 (76) and the pro-survival inhibitor-of-apoptosis (IAP) family member cIAP (116). The depletion of the free pool of HSC70 leads to increased synthesis of HSP70, which is observed 24 h after preconditioning. HSP70 is then able to buffer lethal cellular signaling processes, including caspase-3 generation (76). Importantly, inhibiting the activation of caspase-3 dramatically attenuates the neuroprotective effect of preconditioning (76).

Aside from upregulating survival proteins in preconditioned neurons, caspase-3 has also been shown to target PARP-1 (31). PARP-1, which accounts for >80% of nuclear PARP activity, facilitates DNA repair by mediating the enzymatic transfer of ADP-ribose groups from NAD^{+} to form branched ADP-ribose polymers on acceptor proteins in the

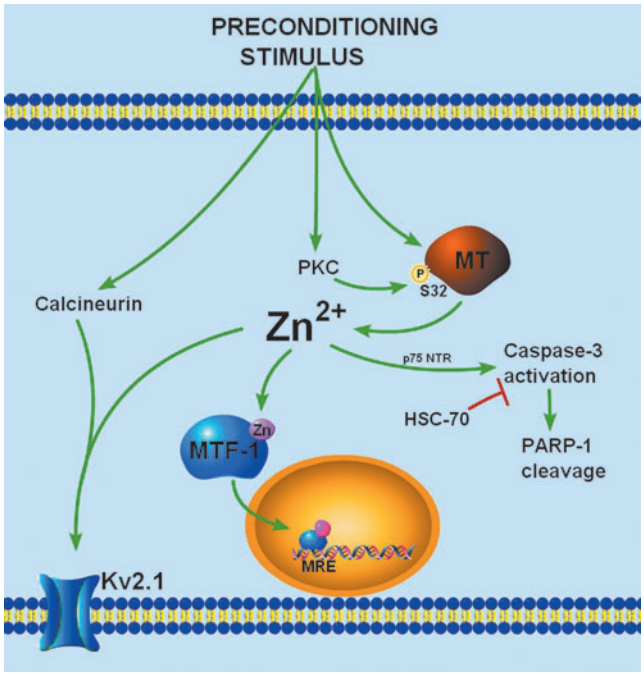

FIG. 5. Zn^{2+}-dependent signaling in neuronal tolerance. A sublethal preconditioning stimulus can cause a transient accumulation of neuronal free Zn^{2+}. The Zn^{2+} rise originates mainly from intracellular sources, like neuronal MT-3, and is facilitated by PKC-dependent phosphorylation at serine 32 of MT. Neuronal free Zn^{2+} can affect several downstream targets. In mediating long-term tolerance, Zn^{2+} can activate the transcription of Zn^{2+}-regulated genes or can activate a caspase-3 mediated pathway leading to the upregulation of heat shock proteins. In addition, Zn^{2+} can contribute to immediate homeostatic processes in regulating neuronal excitability by modulating the activity and localization of Kv2.1 channels. Together, Zn^{2+} plays a central role in several neuroprotective strategies after sublethal ischemia. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

vicinity of DNA strand breaks or kinks (26). However, extensive PARP-1 activation, which occurs in ischemia (124), depletes NAD and ATP, leading to cellular energy failure and cell death (36). Caspase-3 can irreversibly cleave the catalytic site of PARP-1 from its DNA binding domain, effectively inactivating the polymerase (63). Accordingly, preconditioning stimulates caspase-dependent PARP-1 cleavage, attenuating PARP-1 activity, and protecting neurons from subsequent PARP-1-mediated cell death (31). Caspase inhibition during preconditioning blocked PARP-1 cleavage and severely diminished the neuronal tolerance (31). Thus, the killer protease caspase-3, when activated to sublethal levels, is a critical mediator of neuroprotection in preconditioned neurons.

Zinc accumulation in preconditioned neurons

Although preconditioning-induced caspase activation and some of its downstream targets have been characterized, little is known about proximal signaling events that contribute to sublethal caspase activation. Until recently, the intracellular accumulation of free Zn^{2+} has been largely identified as a characteristic of degenerating neurons. Using *in vivo* and *in vitro* systems, two recent reports have shown that a sublethal stimulus led to an accumulation of free Zn^{2+} in preconditioned neurons (5, 65). The free Zn^{2+} rise occurred immediately after the preconditioning stimulus, subsided 24 h after the sublethal stimulus, and was required for neuronal tolerance (5, 65). The increase in neuronal free Zn^{2+} activated caspase-3-dependent PARP-1 cleavage, HSP70 upregulation, and Zn^{2+}-regulated gene transcription conferring delayed neuronal tolerance (Fig. 5) (5, 65). In addition, the rise in free Zn^{2+} led to modulation of Kv2.1 channel activity and localization (4), which may be an important immediate response to ischemic injury (80). Together, these studies have described a critical, novel role for an accumulation of neuronal free Zn^{2+} in the activation of cell survival signaling pathways.

Source of zinc rise in preconditioning

While oxidative- and nitrosative-stress mediated liberation of neuronal Zn^{2+} has been previously described as a characteristic of cell death (1, 13), a similar mechanism may account for the Zn^{2+} signal in preconditioned neurons. Indeed, sublethal oxidative signaling has been shown to be required for tolerance in models of preconditioning (76). In preconditioned neurons, sublethal ROS generation can modify redox-sensitive protein kinases, phosphatases, or redox-sensitive transcription factors, such as nuclear factor-κB and activator protein-1, modulating gene expression and conferring neuronal tolerance (100). Similarly, NO generation, triggered by the influx of Ca^{2+} through NMDA receptors, has been shown to play a central role in preconditioning signal transduction by activating downstream kinases that ultimately modulate gene transcription (33). Thus, multiple redox signals known to trigger Zn^{2+} release from MT are activated in preconditioned neurons. The predominant source of the preconditioning-induced Zn^{2+} rise is indeed intracellular, as the low-affinity extracellular Zn^{2+} chelator tricine and the AMPA receptor blocker 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) had little effect on preconditioning-induced increase in Zn^{2+} fluorescence (5). Our laboratory found evidence for a highly conserved, putative protein kinase C (PKC) phosphorylation site on MT at serine 32 (S32) that is critically involved in Zn^{2+} regulated gene expression and in conferring neuronal tolerance (5), providing a novel role for PKC in modulating the MT/Zn^{2+} interaction to facilitate Zn^{2+} release from the metal-binding protein. Thus, it is likely that the preconditioning-induced Zn^{2+} signal is mediated by the redox modulation of the MT/Zn^{2+} interaction. Further, signaling kinases can serve as regulating factors in neuronal Zn^{2+} homeostasis.

Preconditioning triggers zinc-regulated gene expression

One of the hallmark characteristics of delayed neuronal tolerance is its dependence on *de novo* protein synthesis elicited by the preconditioning stimulus (8). However, the genomic response to preconditioning may not simply involve an immediate activation of quiescent survival genes. Instead, DNA oligonucleotide microarray analysis has shown that a large number of genes may be repressed after preconditioning (112). Nevertheless, protein synthesis inhibition by cycloheximide before, or during preconditioning blocks neuronal tolerance *in vivo* and *in vitro* (76), suggesting a major role for gene activation and protein synthesis in neuroprotection. Changes in gene expression/repression may occur along several temporal profiles. Some genes are altered within minutes or hours (*e.g.,* adenosine A_{2a} receptor and vascular endothelial growth factor), whereas others are affected days after the preconditioning stimulus (*e.g.,* calbindin and serine/threonine protein kinases) (117). Thus, the highly regulated modulation of gene expression in preconditioned neurons is an important cellular response that may ultimately confer neuronal tolerance.

We found evidence suggesting that a preconditioning-induced Zn^{2+} rise triggers the activation of processes that can prevent subsequent toxic Zn^{2+} accumulations (5). Matsushita and colleagues (75) reported that exogenous $ZnCl_2$ pretreatment reduced neuronal death after *in vivo* global ischemia when Zn^{2+} was administered 24 and 48 h, but not 1 h, before ischemia. In light of the requirement of new protein synthesis in neuronal tolerance, and that proteins involved in buffering-free Zn^{2+} are regulated at the level of transcription, it is likely that preconditioning triggers the upregulation of proteins involved in maintaining cellular Zn^{2+} homeostasis. To this end, our group found that preconditioning activates MRE-dependent transcription in cortical neurons, suggesting that Zn^{2+}-regulated gene expression can be stimulated in preconditioned cells. Thus, the Zn^{2+}-induced upregulation of Zn^{2+}-regulated proteins, such as MT I/II and ZnT1, may play a role in neuronal tolerance. Indeed, upregulation of MT I and II have been shown in multiple models of preconditioning (18, 27, 117). A two-fold induction of MT I/II mRNA can be detected as early as 3 h after preconditioning, peaking at 12 h with a 6-fold change in expression, and returning to baseline levels 24 h after preconditioning (18, 27). The induction of MT by application of transition metals protects the heart against oxidative damage (108) and cortical cells against irradiation damage (15). Moreover, mice overexpressing MT I have reduced lesion volume and sensorimotor deficits after *in vivo* ischemia compared with wild-type mice (123). On the other hand, mice lacking MT I/II suffer worse outcomes than wild-type mice after a range of CNS injuries, including focal cryolesion, experimental autoimmune encephalomyelitis (an experimental model of multiple sclerosis), motor neuron dis-

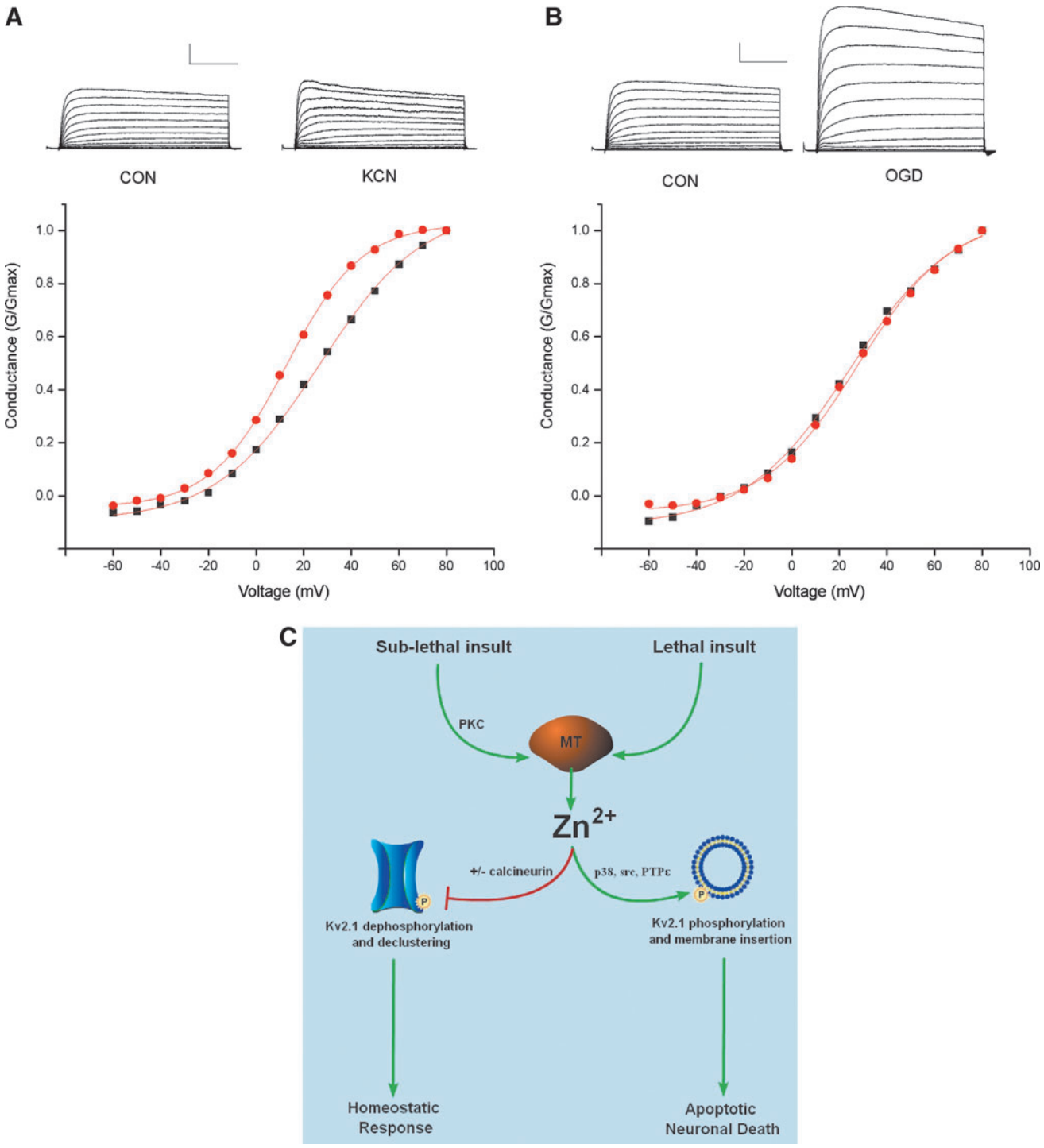

FIG. 6. Divergence of Zn^{2+}-dependent modulation of Kv2.1 channels. (A) Representative whole cell K^+ currents in cortical neurons measured immediately after either control (CON) or sublethal potassium cyanide (KCN; 3 m*M*, 90 min) evoked with a series of 200-ms depolarizing steps from −60 to +80 mV recorded under whole-cell voltage clamp. Plot shows the conductance-voltage (G-V) relationship of peak K^+ current recorded from a representative neuron from each treatment group. Note the leftward, hyperpolarizing shift in voltage-dependency in neurons exposed to sublethal ischemia, but no significant change in the absolute K^+ current. Calibration 2 nA, 50 ms. **(B)** In contrast, when whole cell K^+ current was measured 4 h after 75 min of lethal OGD, an enhancement of absolute K^+ current was evident without alteration of voltage-dependency. **(C)** Thus, after a sublethal stimulus, the liberation of Zn^{2+} leads to the modulation of Kv2.1 channel kinetics, which is accompanied by dephosphorylation and declustering of Kv2.1 channels. These immediate modifications of Kv2.1 are thought to limit neuronal excitability after ischemia. After a lethal stimulus, free Zn^{2+} coordinates the phosphorylation of new Kv2.1 channels, which lead to plasma membrane insertion, a delayed K^+ current surge, and neuronal apoptosis. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

ease, and stroke (99). Finally, MT I/II double-knockout mice had approximately three-fold larger infarcts and significantly worse neurological outcome than wild-type mice after transient ischemia (119). Thus, MT plays an important neuroprotective role in response to cellular injury.

Despite the abundance of data describing the upregulation of MTs in limiting neuronal injury, the inducible isoforms MT I and II are predominantly expressed in astrocytes (54). Thus, if MT I/II buffer lethal neuronal free Zn^{2+} in models of neuronal tolerance, then brain astrocytes may respond to preconditioning by upregulating MT I/II and secreting it into the extracellular space. In fact, accumulating evidence has supported the injury-induced secretion of astrocytic MT, the presence of MT in the extracellular space, and a potential receptor that may mediate MT internalization in neurons (25). In response to brain injury, extracellular MT has been shown to promote axonal regeneration and neurite outgrowth (25). It is also conceivable that, in addition to its effects on neuronal proliferation, secreted MT could also chelate Zn^{2+} released into the extracellular space from injured neurons. By chelating extracellular Zn^{2+}, MT may be able to indirectly reduce intracellular Zn^{2+}, analogous to the previously reported effects of CaEDTA (30). In addition, the internalization of astrocytic MT by neurons (25) may buffer lethal intracellular Zn^{2+} accumulations. These various hypotheses, however, require additional experimental studies to be validated.

Another likely target of preconditioning-induced Zn^{2+}-regulated gene expression is the plasma membrane Zn^{2+} efflux transporter, ZnT1. ZnT1 is expressed by neurons (120) and can be induced by Zn^{2+} in an MTF-1-dependent pathway (62). ZnT1 facilitates Zn^{2+} efflux (95, but also see 89), and may actually be more efficient than MTs in rapidly regulating intracellular accumulation of Zn^{2+} (94). Using *in situ* hybridization, ZnT1 mRNA expression has been shown to be upregulated in gerbil CA1 pyramidal neurons 12 h after transient forebrain ischemia (120). However, not all changes in ZnT1 mRNA have been realized as proportional changes in protein levels (78, 120), indicating possible post-translational regulation of ZnT1 protein expression. It is also unclear whether blocking ZnT1-mediated Zn^{2+} efflux after ischemia exacerbates neuronal injury. Unlike MT, changes in ZnT1 mRNA and protein expression in preconditioned neurons have not been extensively investigated. However, a conceptually similar investigation has been performed in astrocytes (85). In these cells, sublethal exogenous Zn^{2+} exposure induced ZnT1 protein expression and conferred resistance to subsequent lethal Zn^{2+} toxicity (85). Further, in non-preconditioned astrocytes, heterologous expression of ZnT1 reduced toxic intracellular Zn^{2+} accumulations and induced Zn^{2+}-tolerance (85).

Zinc mediates Kv2.1-dependent homeostatic response after sublethal ischemia

Ischemia leads to profound changes in neuronal excitability, manifested as an early phase of cellular hyperpolarization and depression of neural activity, followed by a second phase of dramatic enhancement of excitability (58). Changes in metabolic state or intracellular Ca^{2+} concentration after ischemia can modulate a variety of K^+ channels, including K_{ATP} channels, Ca^{2+}-activated large conductance K^+ channels, and delayed rectifier voltage-dependent K^+ channels (7, 80, 106). Recent evidence has shown that sublethal ischemic injury is associated with a protein-phosphatase 2B (PP2B or calcineurin)-dependent dephosphorylation of existing Kv2.1 channels, which is accompanied by a dispersal of somatodendritic Kv2.1 clusters and hyperpolarizing shifts in voltage-dependency (80). The latter has been proposed to limit neuronal excitability and thus prevent widespread excitotoxic cell death (115). We have observed that chelation of Zn^{2+} during sublethal ischemia blocks the hyperpolarizing shifts in the activation kinetics and prevents Kv2.1 de-clustering (Fig. 6) (4). As ischemia-induced Zn^{2+} accumulation is not blocked by a calcineurin inhibitor, nor is calcineurin activity altered by Zn^{2+}, the Zn^{2+} rise and calcineurin activation may act in concert to modulate Kv2.1 channels (4).

Concluding Remarks

Currently, the most effective therapy for ischemic stroke is timely reperfusion. Intravenous administration of human recombinant tissue plasminogen activator within 3 h of ischemic stroke onset can restore blood flow before major brain damage occurs (126). However, thrombolytic drugs must be used with caution, as lethal intracranial bleeding may result. In light of the serious side effects and limited time window of efficacy, it is imperative that alternative therapeutic strategies for ischemic stroke be developed. Over a decade, experimental evidence has described a central role for a toxic accumulation of intracellular Zn^{2+} in ischemic neuronal death. In several animal models of cerebral ischemia, Zn^{2+} chelation has consistently been shown to reduce neuronal death (16, 53). However, emerging *in vivo* and *in vitro* studies suggest that a sublethal increase in neuronal free Zn^{2+} may also trigger pathways that limit cell injury after injury and confer long-term tolerance (5, 65). The mechanisms accounting for the free Zn^{2+} accumulation and downstream signaling pathways mediating neuroprotection may provide novel therapeutic targets for regulating intracellular Zn^{2+} signals, and ultimately cell survival. This new, neuroprotective role for increases in free Zn^{2+} could aid in the development of future therapies for ischemic stroke.

Acknowledgments

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Abbreviations Used

AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ASK1 = apoptotic signal-regulating kinase-1
CNQX = 6-cyano-7-nitroquinoxaline-2,3-dione
DAMP = damage-associated molecular patterns
EDTA = ethylenediaminetetraacetic acid
ERK = extracellular signal-regulated kinase
HMGB1 = high-mobility group box 1
IAP = inhibitor-of-apoptosis
IL-6 = interleukin-6
K_{ATP} = ATP-sensitive potassium channel
KCC2 = K^+/Cl^- co-transporter 2
Kv = voltage-gated potassium channel
LPS = lipopolysaccharide
MAPK = mitogen-activated protein kinase
MEK = mitogen-activated protein/extracellular signal-regulated kinase
MRE = metal response element
MT = metallothionein
MTF-1 = metal response element transcription factor 1
NMDA = N-methyl-D-aspartate
NO = nitric oxide
OGD = oxygen–glucose deprivation
PAMPs = pathogen-associated molecular patterns
PARP-1 = poly-ADP-ribose polymerase
PKC = protein kinase C
PTP = protein tyrosine phosphatase
RNS = reactive nitrogen species
ROS = reactive oxygen species
TNF-α = tumor necrosis factor-alpha
VSCC = voltage-sensitive Ca^{2+} channels
ZnT = zinc transporter