# Chapter 7
# Zinc Overload in Stroke

**Yang V. Li**

**Abstract** Although zinc (Zn or $Zn^{2+}$) signals are necessary for cell communication and survival, abnormal cellular zinc load can be a precursor for cell death and subsequent brain degeneration following a stroke. Zinc mediates cell death by causing mitochondrial dysfunction, by reinforcing apoptotic signaling cascades, and by inhibiting neuronal metabolism. The mechanisms leading to the sustained zinc dysregulation in excitotoxic conditions are only in part elucidated. This chapter examines the cytotoxicity and sources of zinc overload, and its interaction with neurotransmitter receptors/ion channels. Recent studies show that zinc can be released through the mobilization of zinc from intracellular stores such as mitochondria and endoplasmic reticulum. Changes in the expression of zinc transporters may also be responsible for zinc dyshomeostasis. The question of possible interplays between zinc and calcium overloads in ischemic-hypoxia and oxidative stress are also discussed. Although zinc-induced damage seem to follow the similar trajectory of cell death that has been recognized for calcium overload, neurons appear to be more sensitive to zinc-mediated toxic effect. Therefore, an effective therapeutic strategy for stroke may be a combined treatment that also targets the multiple events involved in zinc dyshomeostasis.

**Keywords** Stroke • Ischemia • Endoplasmic reticulum • Zinc • Calcium • Mitochondria • Cytotoxicity • Neurotoxicity • Dyshomeostasis • Zinc transporter

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## Introduction

The accumulation of endogenous zinc (Zn) in neurons following ischemic stroke is now well recognized (Frederickson et al. 2005; Sensi et al. 2009; Shuttleworth and Weiss 2011). Staining of affected brain regions with zinc indicator and cell viability probe revealed a striking correlation or colocalization between zinc accumulation in neurons and cell death (Frederickson et al. 1988; Stork and Li 2006a, 2009). While Zn is found abundant in a great number of proteins and organelles (such as synaptic vesicles), Zn homeostasis is tightly controlled since intracellular and extracellular free Zn concentration is considerably low comparing to other divalent. Therefore, an excess of Zn accumulation is a detrimental factor that is critical to the neuron viability after stroke. The treatment of rodent models of cerebral ischemia with Zn chelator reduces both infarct volume or neuronal death and Zn accumulation in affected neurons (Koh et al. 1996; Calderone et al. 2004; Diener et al. 2008; Rosenberg et al. 2011). Additionally, the idea of endogenous Zn toxicity as a contributing mechanism has been investigated and shown to be valid in other injury models, including epilepsy, brain trauma, and Alzheimer's diseases (Frederickson et al. 2005; Sensi et al. 2009). The term "overload" has been used to describe an excessive intracellular free metal ion such as calcium (Ca)-mediated neurotoxicity. There is an intracellular Zn overload that contributes significantly to the brain injury after stroke (Fig. 7.1).

## Cytotoxicity of Zinc Overload

Neurons undergo changes in ion homeostasis as a result of normal physiologic stimuli. When these changes become uncontrolled or overloaded, as in stroke and excitotoxicity, they cause pathologic dysfunction (Stork and Li 2009). The level of labile or free Zn ions in the cytosol is normally low (in ρM) because of tight regulation of Zn homeostasis by cellular mechanisms (Frederickson and Bush 2001; Outten and O'Halloran 2001). It reaches the lethal concentrations in the range of high nanomolar (Li and Maret 2009; Sensi et al. 2009) or micromolar as shown by detections with the low-affinity indicator Newport Green ($K_{D\ zinc} = 1–3\ \mu M$) (Stork and Li 2006b, 2009). Growing evidence indicates that Zn accumulated in cytoplasm can be liberated from intracellular stores after oxidative stress and translocated into cells from extracellular space (see Sources of Zn overload) (Frederickson and Bush 2001). Zn-overload-initiated cell death pathways appear complex and multiple, involving oxidative stress, mitochondria dysfunction, and multiple signaling pathways (Galasso and Dyck 2007; Zhang et al. 2007).

Zn overload appears to cause the dysfunction of mitochondria. Under normal conditions, Zn is sequestered in mitochondria through the activation of a cation-permeable channel, the mitochondrial Ca uniporter, or other unidentified independent pathways (Jiang et al. 2001; Csordas and Hajnoczky 2003; Gazaryan et al. 2007). Mitochondrial Zn uptake functions as an organelle storage and may provide clearance of cytosolic accumulation, especially in neurons undergoing excitotoxicity

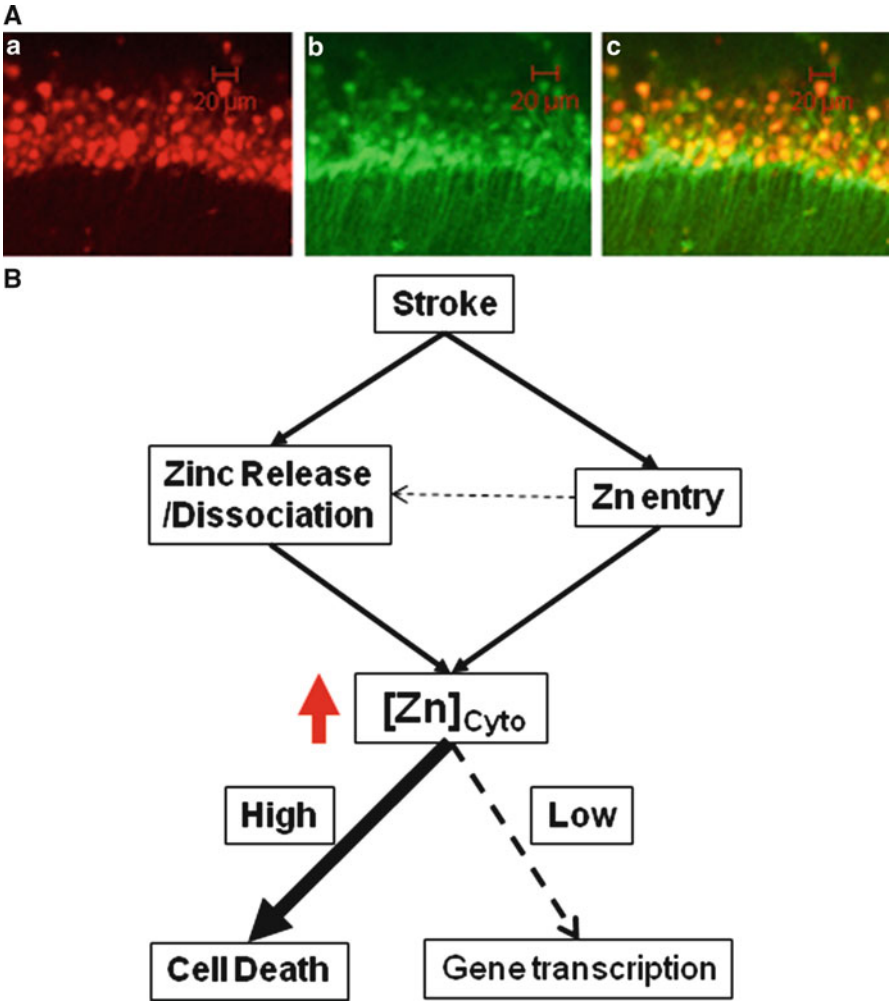


**Fig. 7.1** Zn overload in stroke. (**A**) Effects of ischemic conditions (oxygen–glucose deprivation and reperfusion, OGD/R) on free cytosolic Zn levels and cell death demonstrated in hippocampal CA1 neurons by loading with the fluorescent Zn indicator Newport Green and the cell death indicator propidium iodide (Stork and Li 2006b). (**a**) Image of pyramidal neurons labeled with propidium iodide. (**b**) Image of the same neurons labeled with Newport Green. (**c**) Colocalized fluorescence of propidium iodide and Newport Green, indicating that rising $[Zn]_{cyto}$ induced by OGD/R occurs coincident with the development of OGD/R induced neuronal damage. (**B**) The working hypothesis of cytosolic Zn overload. The accumulation of cytosolic Zn is a detrimental factor triggering cell death after stroke

(Frederickson et al. 2004a; Medvedeva et al. 2009). Excessive and prolonged intramitochondrial Zn overload can trigger ROS production by inhibiting the activity of complex III of the electron transport chain or by interfering with complex I and α-ketoglutarate dehydrogenase (KGDHG) (Sensi et al. 2009), and also inducing

multiconductance cation channel activity in the inner mitochondrial membrane that is consistent with the activation of the mitochondrial permeability transition pore (mPTP) (Shahbaz et al. 2011; Bossy-Wetzel et al. 2004). A consequence of mPTP opening is the release of pro-apoptotic mitochondrial proteins such as cytochrome C and apoptosis-inducing factor (AIF). Several studies support contributions of endogenous Zn to mitochondrial dysfunction in models of ischemia (Capasso et al. 2005; Bonanni et al. 2006; Gazaryan et al. 2007). Zn chelation at the onset of ischemia attenuated the mitochondrial release of cytochrome C and downstream caspase 3 activation. Therefore, mitochondria may become the first victim of cytosolic Zn overload. In return, injured mitochondria release ROS and Zn (worsen Zn dyshomeostasis), triggers the pro-apoptotic signaling pathways, and results in neuronal death.

Zn overload also triggers mitochondrial independent apoptotic signaling pathways, by activating NADPH oxidase through protein kinase C (PKC) activation (Noh and Koh 2000), and by increasing the activity of neuronal nitric oxide synthase (nNOS) (Kim and Koh 2002). The latter is a key enzyme that together with superoxide produces peroxynitrite ($ONOO^-$), a highly neurotoxic free radical species. The linkage of Zn and NO has been directly demonstrated by studies in vitro and in vivo showing the ability of NO or NO-derived molecules to increase free Zn levels in cells and from oxidation and nitrosylation of metalloproteins such as metallothionein and cytochrome oxidase (Cuajungco and Lees 1998; Bossy-Wetzel et al. 2004). The intracellular NO-induced accumulation of Zn has been related to neuronal apoptosis (Zhang et al. 2007). Zn also interferes with the glycolytic metabolism of neurons and inhibits glyceraldehyde-3-phosphate dehydrogenase (GAPDH) through a reduction of cytosolic $NAD^+$, leading to ATP depletion and neuronal death (Cai et al. 2006). Zn-induced cell death seems to occur through poly-ADP-ribose polymerase (PARP) activation, which has been shown in other cases of predominantly necrotic cell death (Kim and Koh 2002; Kunzmann et al. 2008). Furthermore, in rat models of ischemia neurotrophin receptor p75 (p75NTR) and NADE, a p75NTR-associated cell death executor, are co-induced in neurons that undergo cell death. Zn increases the expression and secretion of nerve growth factor (NGF), the agonist of p75NTR, and by modulating p75NTR and NADE, which is a combination that can triggers caspase activation and apoptosis (Park et al. 2000).

Zn may promote the expression of Zn-dependent metalloproteases. Among them, matrix metalloprotease-9 (MMP-9) and tumor necrosis factor alpha converting enzyme (TACE) have been repeatedly shown to be toxically active during ischemic neuronal injury (Romera et al. 2004; Amantea et al. 2007; Burggraf et al. 2007; Machado et al. 2009). Inhibitors of their activity provided significant neuroprotection from experimental cerebral ischemia. TACE and MMP-9 activities both depend on a single catalytic Zn ion in the active site, thus falling into the category of mononuclear Zn enzymes. A unifying theme for such mononuclear Zn metalloproteases is that they are activated by divalent cations and inactivated by chelators such as EDTA (Hurtado et al. 2001; Loy et al. 2002; Amantea et al. 2007; Burggraf et al. 2007; Machado et al. 2009). It was shown that elevated Zn in ischemia deleteriously activates MMP-9 and TACE (Hwang et al. 2005; Stork and Li 2010a). Zn also promotes the expression of early growth response factor 1 (EGR1), an immediate-early gene transcription factor that is induced after cerebral ischemia (Park and Koh 1999).

Zn rather than Ca influx selectively promotes extracellular signal-regulated kinase 1 (ERK1) and ERK2 activation, an event upstream of EGR1 induction as inhibition of ERK1 and ERK2 activation blocks both EGR1 activity and Zn-dependent neurotoxicity (Klein et al. 2006). Taken together, there is no shortage of Zn-dependent factors that might promote cell death in ischemic neurons and thereby might be inactivated by chelator application; leading to a protective effect achieved by Zn removal.

The Zn buffering capacity determines whether intracellular free Zn is physiological (cytoprotective) or cytotoxic (Maret 2008), with equilibrium constants in the $10^{-8}$ to $10^{-9}$ range would maintain pZn in the optimal range ($9 > pZn > 8$), thereby preventing excess Zn damage while avoiding a harmful degree of Zn deficiency(Frederickson et al. 2006). Although excessive Zn exerts direct neurotoxic action, this metal is also essential for the activity of numerous biological systems. Zn is an intrinsic factor for neuron survival, and in low amounts, is an active neuroprotectant against neurotoxic cell death. For example, it is involved in ischemic preconditioning, a neuroprotective phenomenon by which a brief sublethal ischemic attach is able to protect from a subsequent stronger ischemic insult. A recent study shows that sublethal ischemia triggers neuroprotective mild intracellular Zn rises in neurons undergoing ischemic preconditioning (Aras et al. 2009). Furthermore, since many radical scavengers such as Cu–Zn-SOD are Zn containing proteins, the moderate increase in cytosolic Zn may be protective to neurons. In this way, initial moderate Zn increase in ischemic condition or the sublethal addition of Zn may promote the expressions of these protein (increasing Zn buffering capacity) and serves as an antioxidant and cytoprotectant.

Cell membranes act as protective walls to exclude molecules that are not actively transported by viable cell in normal condition. When Zn is applied briefly outside of cells, the actual free Zn signal produced inside of the cells is very low (except in prolonged presence). However, under favorable conditions such as increased membrane permeability to Zn (also see the section "Intracellular Sources of Zinc Overload"), significant amounts of Zn can enter into the cell. If Zn increase exceeds buffering capacity, the resulting elevated free intracellular Zn concentrations induce oxidative stress. When a few hundred micromolar Zn was added in the presence of KCl (to open voltage dependent channels) or Na-pyrithione (a Zn ionophore to increase membrane permeability), cytotoxic amount of Zn was achieved with intracellular concentration estimated to be about 10–200 nM (Sensi et al. 1997; Li and Maret 2009). Although these are less than 0.1% of total Zn applied extracellularly, they are lethal and kill most neurons in a short time (Weiss et al. 1993; Frederickson et al. 2005). In general, it is widely accepted that excessive increases of this metal ion in ischemic stroke is detrimental (Fig. 7.2).

## Intracellular Sources of Zinc Overload

It is now well recognized that significant amount of Zn can be released through the mobilization of Zn from intracellular storages such as organelles, mitochondria and metallothioneins within postsynaptic neurons (Frederickson et al. 2005;

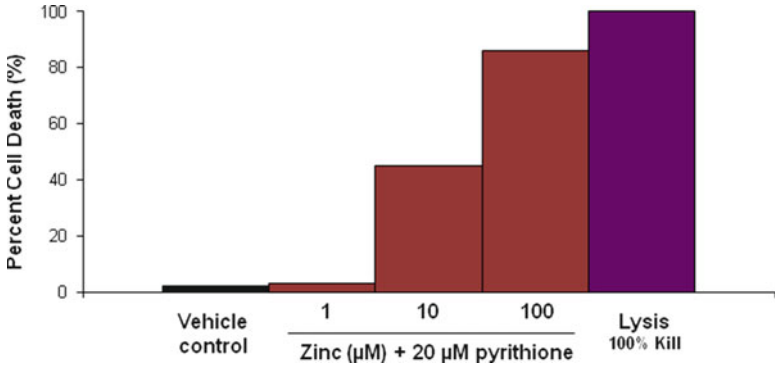


**Fig. 7.2** Zn concentration dependent cell death measured by MTT assay following 30 min Zn treatments (cell lysis with triton X-100 as positive control)

Stork and Li 2010b). Zn is an important structural and functional component in many cellular proteins and enzymes. As such, Zn levels are normally tightly regulated, limiting the extent of cytosolic labile (or free) Zn concentrations (Vallee and Falchuk 1993; Outten and O'Halloran 2001). For example, levels of free Zn are several orders of magnitude less than that of Ca (Finney and O'Halloran 2003) (cytosolic free Zn is in the picomolar range). While the mechanisms responsible for regulating Zn homeostasis are not well established, available data support that, like Ca, intracellular Zn levels are determined by the interaction of membrane Zn transporters and cytoplasmic Zn buffers (Cousins et al. 2006; Eide 2006). Intracellular Zn rises become lethal and neuronal loss is induced when the peak concentrations in the range of high nanomolar to low micromolar are reached (Sensi et al. 2009).

Most recent work demonstrates that Zn is released from thapsigargin-sensitive and $IP_3R$-mediated stores (Stork and Li 2010b), and raises the possibility that neuronal cells maintain a substantial concentration of Zn in ER-like storage and that Zn could be released alongside of Ca upon a stimulation. ER dysfunction and its association with protein folding have been implicated in ischemic stroke pathology (Mattson 1997; DeGracia and Montie 2004; Roberts et al. 2007). Thapsigargin, a plant derived compound that specifically inhibits SERCA activity (Young and Stokes 2004). By blocking the ability of the cell to pump Ca into the ER, thapsigargin causes these stores to become depleted and thereby raise the cytosolic Ca concentration. Apparently, Ca is not the only metal ion that is sequestered in the ER. The source of thapsigargin-induced elevation in intracellular Zn is of intracellular origin, since the elevation of intracellular Zn is independent from either extracellular Ca or extracellular Zn (Stork and Li 2010b). The applications of caged $IP_3$ or $IP_3$-3K inhibitor, which both elevate intracellular levels of $IP_3$ (Chang et al. 2002; Akhkha et al. 2004; Antigny et al. 2008; Watanabe et al. 2009), resulted in a rapid increase in intracellular Zn (Stork and Li 2010b). These results indicate that Zn homeostasis, like Ca homeostasis, is controlled by $IP_3Rs$ that may also gate Zn into the cytosol, and by thapsigargin sensitive ATPase activity that pumps Zn from the cytosol into the ER (Fig. 7.3).

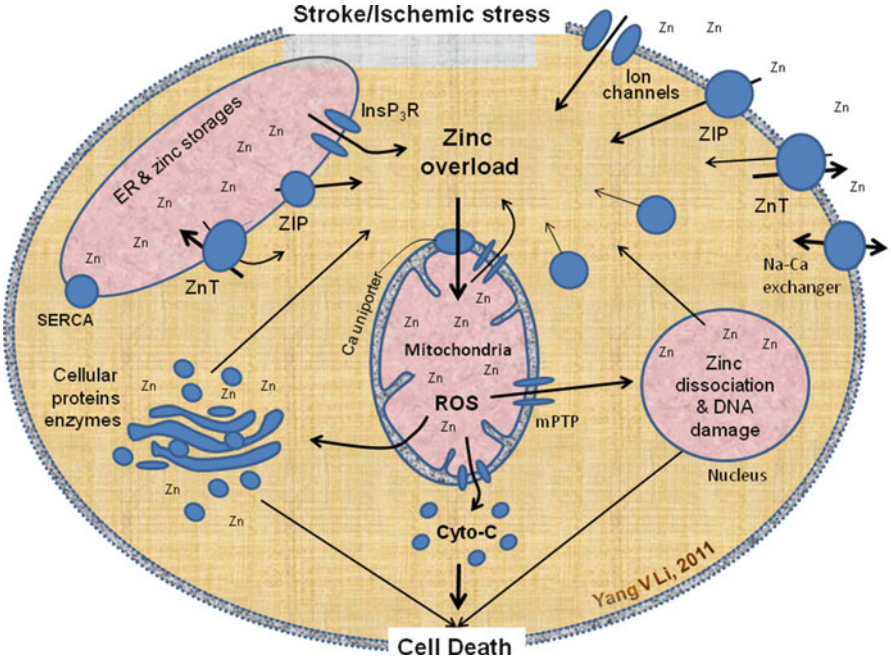


**Fig. 7.3** Schematic depiction of mechanisms leading to cell death in response to Zn overload after stroke. During hypoxic-ischemic stress, Zn may be released from ER and other intracellular storage. Zn is also dissociated from Zn-binding proteins/enzymes. Zn enters neurons through various ion channels including voltage-gated Ca channels, Ca permeable AMPA receptors, NMDA receptors. Zn can also be transported in or out by Na–Ca exchanger and Zn transporters (ZIP, ZnT). Mitochondria can actively sequester Zn via Ca-uniporters. Zn accumulation in mitochondria can trigger ROS production and are responsible for the activation of the mitochondria permeability transition pore (mPTP) and the release of pro-apoptotic factors such as cytochrome C (Cyto C). ROS productions can further deteriorate Zn dyshomeostasis by facilitating Zn dissociation in cytosol and nucleus, which deposit more free Zn, and consequent cell death

Zn transporters (ZnT) ZnT5/ZnT6 hetero-oligomeric complexes are involved in ER homeostasis by transporting Zn under stress condition (Suzuki et al. 2005a; Ishihara et al. 2006), and may function as bidirectional transporters (Ellis et al. 2004, 2005; Eide 2006; Qiao et al. 2009). Studies in *Saccharomyces cerevisiae* by Eide and his colleagues suggest Zn and ZnTs are required for ER function (Ellis et al. 2004, 2005; Eide 2006; Qiao et al. 2009). Zn deficiency (or depletion) induced the UPR and ATF6 activities as well as ZnT upregulations. Specifically, *ZnT5* mRNA was up-regulated by the ER stress in various cell lines (Suzuki et al. 2005a; Ishihara et al. 2006), suggesting a compensation mechanism to uptake Zn into ER. Taken together, these studies suggest that Zn is required for correct ER functions. Within the ER, it is known that Ca is buffered by the abundant luminal resident chaperone protein calreticulin which binds Ca. Although calreticulin was first identified as a Ca binding protein (Ostwald and MacLennan 1974), this protein is multifunctional (Corbett et al. 2000) and binds other ions including Zn with multiple

binding sites (Khanna et al. 1986; Baksh et al. 1995; Guo et al. 2003; Tan et al. 2006). Zn also binds with several other resident luminal proteins (Abdelwahab et al. 2011; Suzuki et al. 2005b; Urbaniak et al. 2005; Qiao et al. 2009). There are reports suggesting that thapsigargin/$IP_3$ regulate mitochondrial Ca signaling and function (Csordas and Hajnoczky 2003). It remains to be studied how thapsigargin and $IP_3$ induced Zn release affect mitochondrial function.

There has been considerable focus on mitochondria taking up cytosolic Zn in maintaining intracellular Zn concentration in stroke. The mitochondrial Zn uptake and depolarization is associated with early Zn accumulation following ischemic assaults (Shuttleworth and Weiss 2011). Like Ca, Zn can accumulate in the mitochondrial matrix through the activation of the mitochondrial Ca uniporter (Saris and Niva 1994; Jiang et al. 2001; Gazaryan et al. 2007). While the Zn uptake provides clearance of cytosolic Zn, especially in neurons undergoing excitotoxicity (Sensi et al. 2009), excessive Zn overload in mitochondrial lumen alter or, consequently, impair its function, leading to a loss of mitochondrial membrane potential, as well as opening of the mPTP (Saris and Niva 1994; Lee et al. 2009; Medvedeva et al. 2009). A consequence of mPTP opening is the efflux of Zn from mitochondria (contributing to cytosolic Zn overload) and increased generation of reactive oxygen species (ROS) and eventually cell death (Bossy-Wetzel et al. 2004; Medvedeva et al. 2009). Hence, the biphasic control of cytosolic Zn by mitochondria in response to the rising Zn: early uptake to remove cytosolic Zn and late release to promote neurotoxicity, which provides novel basis for complex pathological patterns of intracellular Zn signaling (Fig. 7.3).

Zn is also sequestered into lysosomes that serve as the main degradative factory in cells (Hwang et al. 2008). Like in mitochondria, following oxidative stress and cytosolic Zn overload, free Zn levels in lysosomes rises rapidly along with Zn dependent accumulation of the toxic 4-hydroxy-2-nonenal (HNE) adduct (Hwang et al. 2008; Kim et al. 2009). HNE is a key endogenous neurotoxin and causes lysosomal membrane permeabilization. The Zn overload and HNE elicit the lysosomal stress, leading to the release of Zn and the lysosomal protease cathepsin, and triggering neuronal loss (Hwang et al. 2008).

Another critical source of this Zn is the metallothioneins (MTs), from which Zn can be released rapidly by nitrosylation or oxidation of the thiol ligands (Maret 2008). The sequestration and storage of Zn in metallothiones have been extensively investigated (Colvin et al. 2010; Vallee and Galdes 1984). The metallothionein 3 (MT3) isoform is found only in the brain and testis, whereas other isoforms are more widespread (Cole et al. 2000; Lee et al. 2003). They function physiologically by accepting Zn from other Zn-binding ligands, including Zn binding proteins. Oxidation or nitrosylation of cysteine residues in the Zn cluster results in the release of Zn (Aizenman et al. 2000; Maret 2008), so these proteins can function as Zn donors to other Zn-binding proteins. Oxidative stress and acidosis, both of which occur prominently in ischemia, can induce Zn release from MT proteins, resulting in substantial increases intracellular Zn (Frederickson et al. 2005). Zn has been shown to activate a number of protein kinases such as protein kinase C, CaMKII, TrkB, Ras and MAP kinase (Hubbard et al. 1991; Quest et al. 1992), CaMKII (Brewer et al. 1979; Weinberger and Rostas 1991; Park and Koh 1999; Lengyel et al. 2000).

While the mechanism(s) that govern Zn trafficking remain elusive, there is little doubt that the intracellular free Zn level must be maintained within a physiological limit. On the other hand, abnormal levels of Zn may lead to either Zn-induced toxicity or apoptosis (Frederickson et al. 2005). Therefore, the mechanism such as thapsigargin/IP3 sensitive Zn storage may function as a source of intracellular free Zn in response to stimuli, and is likely to play an important role in the regulation of intracellular levels of Zn. Determining the relative contributions of MT proteins, mitochondria, ER and possibly other intracellular stores to Zn homeostasis is an interesting area for future work that may yield new interventions to limit pathologic Zn elevations in the post-ischemic period.

## Extracellular Sources of Zinc Overload

Free or labile Zn in extracellular space is extremely low (<1 nM) (Frederickson et al. 2004b), with total serum Zn concentration 10–15 μmol/L. Unlike Ca, Mg (magnesium) or K (potassium), Zn is found to be selectively stored in, and released from, the presynaptic vesicles of a specific subset of glutamatergic nerve terminals heterogeneously throughout the mammalian cortex and limbic region with highest densities in cortical supragranular and infragranular as well as hippocampal hilus/CA3 regions (Frederickson et al. 2000). Similar to glutamate, Zn concentration outside the cells of the nervous system is normally kept low and appears to be sensitive to neuronal activity. Evidence demonstrated that Zn release was highly correlated with glutamate release and depended on vesicular exocytosis (Howell et al. 1984; Li et al. 2001a, b; Ueno et al. 2002; Qian and Noebels 2005), supporting that Zn is co-localized and co-released with glutamate. Like glutamate, in ischemic and excitotoxic conditions, the concentration of extracellular Zn is rapid increased (Wei et al. 2004) while disappeared from presynaptic terminals (Koh et al. 1996; Sorensen et al. 1998; Suh et al. 2001), which occurs via mechanisms expectedly similar to those previously suggested for extracellular glutamate accumulation after stroke.

Is extracellular Zn accumulation released from nerve terminals during stroke a source of cytotoxicity? Ischemic stroke occurs because of a loss of blood supply to part of the brain, as oxygen or glucose becomes depleted in ischemic brain tissue, leading to depolarization and massive glutamate and Zn accumulation in the extracellular space. Neurons store up ~1 mM of free Zn in their terminals (Frederickson et al. 1983, 2005). The peak concentrations of synaptically released Zn in extracellular pace is estimated to be about tens μM under moderate stimulation (Assaf and Chung 1984; Vogt et al. 2000; Li et al. 2001b; Bastian and Li 2007), and may approach several hundred micromolar (μM) in ischemic or other extreme conditions (Frederickson et al. 1983, 1989). The exposure of cells to such high concentrations of free ionic Zn is proven to be detrimental (Yokoyama et al. 1986; Frederickson et al. 2005; Stork and Li 2009) (Fig. 7.2). In animal stroke model in vivo, treatment with Zn increased infarct volume and brain edema, and also worsened neurological deficits (Shabanzadeh et al. 2004). The striking cytotoxic

actions of Zn were observed when the application of Zn were accompanied with membrane depolarization or when Zn were co-applied with its ionophore to allow free ionic Zn influx into neurons (Lee et al. 2002; Sensi et al. 2009; Stork and Li 2009).

The translocation of Zn into neurons is critical in Zn-induced cell injury in excitotoxicity (Frederickson et al. 2005). Both excessive glutamate accumulation and massive depolarization in stroke provide a favorable condition for opening the Zn-permeable channels; therefore, maximum Zn translocation would be expected in stroke. Zn could enter neurons through various voltage- and glutamate-gated channels (Sensi et al. 2009). However, presynaptically-released Zn is not the only source of toxic Zn that contributed to the degeneration of postsynaptic neurons (see Intracellular sources of Zn overload). Brain regions with low or little presynaptic Zn are also be susceptible to such Zn toxicity in the condition of excitotoxic brain injury. The best example is that the CA1, the most sensitive region to ischemic injury in hippocampus, shows the highest intracellular Zn accumulation following ischemia although it contains lowest Zn in nerve terminals among hippocampus regions (Frederickson et al. 2000; Wei et al. 2004; Stork and Li 2006a, b).

## Zinc Transporters and Zinc Overload

Zn is one of the most abundant transition metals in the brain and is in particularly high concentrations in the mammalian brain (Frederickson et al. 2000). The total Zn concentration in the brain was estimated to be approximately 150–200 μmol/L or 10 μg/g wet brain tissue (Markesbery et al. 1984). Zn homeostasis is regulated with Zn transporters, Na–Zn exchangers, ion channels and other mechanisms, but the transporters may play a dominant role in maintaining Zn equilibrium. Zn homoeostasis is regulated by two large metal-transporter families; the Zip family that mediates Zn influx into the cytosol and the ZnT family that reduce cytosolic zinc level with facilitating Zn efflux from the cytosol, either into intracellular cell compartments or out of the cell (Ishihara et al. 2006).

There are at least ten members of the ZnT family, most of which are ubiquitously expressed, although ZnT3 and ZnT8 are found only in the brain and pancreas, respectively. ZnT1 is expressed on the plasma membrane of neurons and glia, which has been implicated in neuroprotection against toxic Zn surges (Nolte et al. 2004; Palmiter 2004). ZnT2 is expressed at low levels in the brain and it has been associated with the vesicular Zn uptake (Palmiter et al. 1996a). The best studied ZnT in the brain, ZnT3, is mainly localized at Zn-containing synaptic glutamatergic vesicles in the cortex, hippocampus and other limbic regions (Palmiter et al. 1996b). ZnT3-knockout mice are deficient in synaptic Zn and are an invaluable tool for studying its role in physiological and neuropathological conditions (Cole et al. 1999). Other ZnT family members, ZnT2, ZnT4, ZnT5 and ZnT6, are also expressed in several brain regions. Like ZnT1, ZnT4 are located in the plasmatic membrane acting as Zn exporters to avoid an excessive rise of its

intracellular concentration (McMahon and Cousins 1998). ZnT7, ZnT8, and ZnT9 have not been detected in the brain (Lichten and Cousins 2009) though they have been found in other tissues (Kambe et al. 2004). ZnT5, ZnT6 and ZnT7 are present on plasma membrane of intracellular organelles such as the ER, Golgi and the secretory vesicles in the biosynthetic-secretory pathway (Ellis et al. 2004, 2005; Suzuki et al. 2005b; Ishihara et al. 2006). The exact function of these transporters in transporting Zn is elusive. ZnT5 and ZnT6 are expressed abundantly in the brain and are responsible for the accumulation of Zn in ER-Golgi network of mammalian cells (Abdelwahab et al. 2011; Huang et al. 2002; Ellis et al. 2004; Suzuki et al. 2005b).

One of the brain responses to stroke is to up-regulate proteins that may alleviate brain injury. The upregulation of ZnTs has been associated with resistance to Zn toxicity (Cousins et al. 2003; Aguilar-Alonso et al. 2008). The harmful effect of Zn overload is possibly caused by the inability of ZnTs to efficiently remove it. There are several studies showing the up-regulation of the mRNA for ZnT1, ZnT2, and ZnT4 after transient forebrain ischemia, which may be responsible to decrease to cytosolic Zn levels (Tsuda et al. 1997; Cousins et al. 2003; Nolte et al. 2004; Aguilar-Alonso et al. 2008). On the other hand, ZnT5 and ZnT6 appeared to be up-regulated under stressful conditions such as Zn deficiency (or depletion) in various cell lines (Suzuki et al. 2005a; Ishihara et al. 2006; Jackson et al. 2008), to maintain Zn equilibrium. These studies suggest that ZnTs expressions are actively regulated to avoid raising levels that trigger a pathological condition.

Another family of Zn transporters is ZIP (ZRT1-IRT1-related protein) superfamily. The ZIPs has a fundamental role in mediating Zn influx at the plasma membrane and in intracellular organelles, through a process that may be facilitated by or coupled to $HCO3-$ or H+ gradients (Gaither and Eide 2000). Thus far, more than ten members of ZIPs have been identified, which are ubiquitously expressed in brain and peripheral tissues and, notably, the membrane distribution of these proteins is regulated by Zn levels (Gaither and Eide 2001; Dufner-Beattie et al. 2006). ZIP1 and ZIP3 was highly enriched in brain regions with high densities of neuronal cell bodies, including the hippocampus, thalamus, and perifontal cortex, and zip4 mRNA was identified in the brain but was restricted to choroid plexus and brain capillaries (Dufner-Beattie et al. 2006; Belloni-Olivi et al. 2009). ZIP5, ZIP6 and ZIP10 show also strong hippocampal mRNA transcript expression and presumably contribute to the remaining passive influx. Expression of ZIP6 was also found in hippocampal and cortical neurons (Chowanadisai et al. 2008; Qian et al. 2011). ZIP7 is a functional Zn transporter that acts by transporting Zn from the Golgi apparatus to the cytoplasmic matrix of the cell (Huang et al. 2005). However, the precise function of ZIPs in cellular Zn homeostasis is still not clear. These proteins appear to be more sensitive to the effect of dietary or immediate Zn supplies (Dufner-Beattie et al. 2006; Belloni-Olivi et al. 2009), and excessive Zn levels may down-regulate the expression of ZIPs-mediated neuronal Zn uptake (Chowanadisai et al. 2008). Although we can hypothesize these transporters are up/down regulated in response to Zn overload, how ZIPs regulate Zn homeostasis in stroke is not known.

## Zinc and Receptors/Channels

There is a growing body of evidence indicating a neurotransmitter or neuronal signaling role for Zn by its interactions with membrane receptors, ionic channels, and intracellular signaling pathways. Zn interacts with various neurotransmitter receptors and ion channels (Li et al. 2003; Frederickson et al. 2005; Sensi et al. 2009). The effect of Zn on ischemia should take a perceptive of its action to particular receptor or channel, as well as the action of Zn overload to other receptors/channels as a whole in stroke. The striking reality of this "whole" picture is that Zn appears to inhibit both excitatory and inhibitory receptors. Application of Zn exhibits profound modulatory effects on a variety of ligand- and voltage-gated mammalian ion channels, including attenuation of current through NMDA and GABA receptor-gated channels, potentiation of AMPA, glycine, and ATP receptor-mediated currents and modulation of the transient outward $K^+$ current $I_A$ (Li et al. 2003). Although it has not been accurately measured, the estimated amount of synaptically released Zn to be about 10s μM (Assaf and Chung 1984; Vogt et al. 2000; Li et al. 2001b; Bastian and Li 2007) or >100 μM in extreme conditions (seizure or brain trauma), as neurons store up ~1 mM of free Zn in their terminals (Frederickson 1989; Frederickson et al. 1989).

The co-localization of Zn and glutamate implies that Zn is largely involved in the function of the glutamatergic synapses (Frederickson 1989). Electrophysiological studies on cultured neurons and the hippocampus have shown the amount of released Zn is more than enough to decrease the NMDA receptor (NMDAR) mediated responses (Chen et al. 1997; Choi and Lipton 1999; Vogt et al. 2000; Chen and Liao 2003) also see (Molnar and Nadler 2001; Timofeeva and Nadler 2006). Zn directly inhibits NMDA-sensitive glutamate-gated channels by two separate mechanisms: a high-affinity (IC50 < 5 nM) voltage-independent inhibition, as well as a low affinity (IC50 < 20 μM) voltage dependent inhibition of NMDAR function (Williams 1996; Paoletti et al. 1997). Although NMDAR blockers have been shown to ameliorate ischemia in in vitro models, Zn overload under ischemic condition may override any possible protective action of Zn inhibition of NMDAR. There is evidence that Zn and GABA are also co-localized in nerve terminals (Sandler and Smith 1991; Ruiz et al. 2004). Micromolar concentration of Zn antagonizes GABAA receptor mediated responses (Westbrook and Mayer 1987) and the application of Zn chelators reveal the effects of endogenous Zn signals on GABA receptors (Xie et al. 1994; Ruiz et al. 2004), suggesting that the modulation of GABA receptors by Zn is probably a vital factor in normal brain function. Changes in the Zn modulation of GABA receptors have been implicated in the etiology of epilepsy (Buhl et al. 1996; Coulter 2000; Nadler 2003). Both pilocarpine-induced status epilepticus (Gibbs et al. 1997) and kindling (Buhl et al. 1996) markedly increase the sensitivity (to Zn) of GABAergic receptor, thereby reducing GABAA receptor-mediated inhibition and enhancing excitotoxic susceptibility.

Zn has been proposed to affect aminergic, purinergic and cholinergic receptors, but the physiological importance of such putative effects remains uncertain

(Frederickson et al. 2005). Glycine and Zn co-localize in presynaptic terminals in the brain stem and spinal cord (Birinyi et al. 2001). Zn appears to exhibit biphasic modulation of glycine receptors: inhibiting at high concentrations (>10 μM) and facilitating at lower concentrations (<10 μM) (Trombley et al. 2011). At μM concentrations, Zn markedly potentiated transient potassium current ($I_A$) (Huang et al. 1993). Recent study shows that Zn regulates neuronal apoptosis via an exocytotic membrane insertion of Kv2.1-encoded ion channels, resulting in an enhancement of voltage-gated potassium currents and a loss of intracellular potassium during neuronal cell death (Redman et al. 2009). Zn inhibits the Ca current through acid-sensing ion channels (ASICs) that is activated by lowering extracellular pH (Chu et al. 2004). However, the ASIC activation could also be important in Zn mediated cytotoxicity, especially in ischemic stroke, in which both Zn overload and falling pH are likely to occur. As Zn current is enhanced by extracellular acidity (Kerchner et al. 2000), the ASICs could be one of Zn influx routs in the ischemic condition. Zn could induce the release of $[Ca]_i$ by acting on G-protein coupled putative Zn-sensing receptors (Hershfinkel et al. 2001), which has been described on epithelial cells, initiates Ca mobilization through Ca/calmodulin dependent protein kinase activation. Finally, evidence shows that Zn inhibited both glutamate uptake as well as release by acting on glutamate transporters in retinal glial cells (Spiridon et al. 1998). If similar Zn action is present in other brain regions, the inhibition of glutamate uptake into glia by Zn overload could have a significant detrimental effect in stroke. Taken together, Zn interacts with almost all categories of receptor/channels with distinct sensitivity. The Zn overload in stroke will likely affect all these interactions. The outcome of these interactions is probably determined not only by amount of Zn but also the expression of particular receptor/channels in stroke.

## Conclusion and Therapeutic Perceptive

The role of Ca overload in ischemic-hypoxia and oxidative stress has come under greater scrutiny recently, resulting in part from the sensitivity of fluorescent Ca indicators to Zn (Stork and Li 2006b). As reviewed in this work, Zn induced damage seem follow the similar trajectory of cell death that has been recognized for Ca signaling or Ca overload. A major issue that is still animating the field is the question of possible interplays between these two ions (Stork and Li 2006b; Medvedeva et al. 2009). While the rising $[Ca]_i$ has been conventionally detected with fluorescent Ca-indicators, it is recognized that the most commonly used Ca indicators such as Fura-2, and Calcium Green-1 are not selective solely for Ca but rather show a higher affinity to Zn (Grynkiewicz et al. 1985; Thompson et al. 2002; Martin et al. 2006). Most of these fluorescent indicators are derivatives of the Ca chelators BAPTA, EGTA and APTRA, which bind Zn with significantly higher affinity (Basolo and Pearson 1967; Martell and Smith 1974; Grynkiewicz et al. 1985; Hering and Morel 1988; Csermely et al. 1989; Bers et al. 1994; Davis et al. 1999;

**Table 7.1** Fluorescent Ca indicators are derivatives of "Ca" chelators, which bind Zn with higher affinity

| Chelators | $K_{D\,Zn}$ | $K_{D\,Ca}$ |
|---|---|---|
| BAPTA | 8 nM | 160 nM |
| EGTA | 7.8 pM | 0.38 nM |
| APTRA | 19 nM | 12 μM |

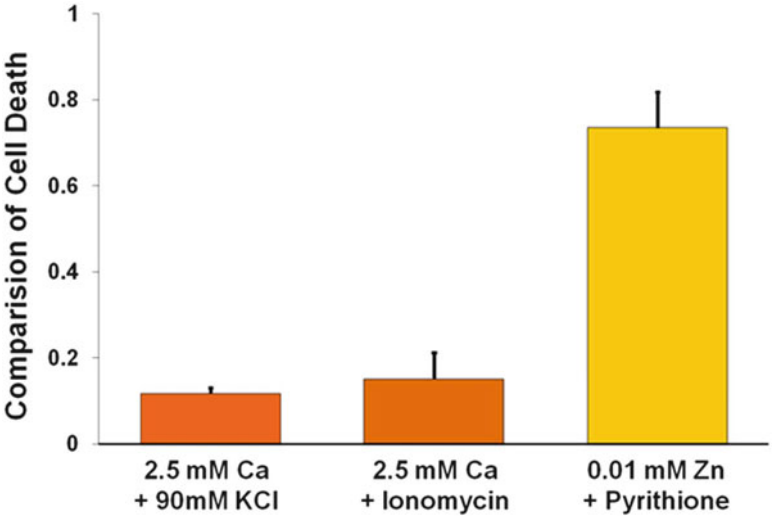


**Fig. 7.4** The comparison of cell death caused by Ca and Zn (Stork and Li 2009). The cell death induced by the treatment of 10 μM Zn/20 μM pyrithione, a Zn ionophore, was significantly higher than that induced by Ca. The Ca influx was facilitated with 90 mM KCl or ionomycin

Haugland 2005) (Table 7.1). For example, the affinity of the classical Ca-sensitive fluorescent probe, Fura-2, for Zn is 100-fold higher than its affinity for Ca (Tsien and Pozzan 1989; Haugland 2005; Sensi et al. 2009).

Induced Zn elevation shows potently greater toxicity than induced Ca elevation (Stork and Li 2009). At the center of the Ca-overload hypothesis is the notion that the influx of extracellular Ca triggers a rise in $[Ca]_i$ and subsequently causes cellular injury/death. As shown in Fig. 7.4, also see (Stork and Li 2009), a comparatively low concentration of exogenous Zn (10 μM) produces the greatest magnitude of cell death compared to that measured following the 2.5 mM application of exogenous Ca, indicating that Ca influx result in significantly less injury as compared to Zn influx. These results are in agreement with the assessment that neurons appear to be more sensitive to Zn mediated toxic effect when comparing the damage in the similar condition (Frederickson et al. 2005; Sensi et al. 2009). Moreover, the neuronal injury mediated by Ca influx is largely prevented by Zn chelation (Stork and Li 2009), suggesting that a link between Ca influx and Zn dyshomeostasis exists. One explanation is that the influx Ca may trigger the release of Zn from its storages. Another explanation is that

the influx of Ca initiates upstream processes which lead to the liberation of intracellular $[Zn]_i$ as has been demonstrated with nitric oxide synthase activation (Frederickson et al. 2002; Bossy-Wetzel et al. 2004; Stork and Li 2006b).

Alternatively, recent work simultaneously tracking changes in Ca and Zn suggests that Zn overload can serve as an upstream contributor to deregulation of Ca to toxic levels within neurons (Vander Jagt et al. 2008b; Medvedeva et al. 2009). They observed that Zn increases were observed before toxic Ca overload, and prevented Zn accumulation delayed the onset of irrecoverable Ca increases. Intracellular Zn accumulation appeared to contribute significantly to triggering the degenerative cascades and initiates Ca overload by decreasing ATP availability (Vander Jagt et al. 2008a, b). Collectively, these results indicate that although Ca mobilization may serve a key role for physiological intracellular signaling, most of the death signaling associated with neurological conditions such as cerebral ischemia might be mediated not only by Ca but also by Zn (Sensi et al. 2009). Since several other metal ions such as copper and iron share the similar properties with Ca or Zn, their potential interplays need to be further investigated. Specific tests of the involvement of these interactions in global and focal ischemia models will probably reveal new targets for effective therapeutic intervention.

The application of a Zn chelator to prevent damages caused by Zn overload is under active investigation as a treatment for stroke. Among chelators, the moderate or weak chelators such as DP-b99 and Clioquinol have been tested and already produced promising results (Wang et al. 2010; Diener et al. 2008; Barkalifa et al. 2009). The application of another Zn chelator DEDTC (diethyldithiocarbamate) improved the survival and tolerance under the hypoxic-ischemic treatment in a newly developed in vivo zebrafish model (Yu and Li 2011a, b). As Zn is required for normal biological process, the advantage for using these relative weak chelators is to maintain Zn in the optimal range, thereby preventing excess Zn damage while avoiding an unwanted Zn deficiency (Frederickson et al. 2005). As Zn toxicity is largely mediated by oxidative and nitrosative stress, antioxidants, NOS inhibitors and anti-apoptosis measures caspase inhibition might be useful. NADPH oxidase might be a target, because Zn overload induces and activates NADPH oxidase (Noh and Koh 2000). At present, despite clear demonstration of numerous agents that can prevent the cascade of events leading to ischemic neuronal death in animal models, it is not known whether any particular neuroprotectant is more effective than others to conclusively improve stroke outcome in humans (White et al. 1996; O'Collins et al. 2006; Marler 2007; Saver et al. 2009) (also see Report of the Stroke Progress Review Group, NIH, 2002 & 2006). As discussed in this review, diverse serial and parallel events contribute to Zn-induced cell death. Therefore, the effective strategy may be a combined treatment that targets the multiple events involved in Zn dyshomeostasis.

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