Studies of the Change of Intracellular Zinc Triggered by Exogenous NO and the Induction of Tolerance to OGD by Exogenous NO and Lipophilic Metal Chelators in Cultured Cortical Neurons

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This dissertation titled

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Cultured Cortical Neurons

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ABSTRACT

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Studies of the Change of Intracellular Zinc Triggered by Exogenous NO and the Induction of Tolerance to OGD by Exogenous NO and Lipophilic Metal Chelators in Cultured Cortical Neurons. (146 pp.)

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Intracellular Zn\(^{2+}\) release and concomitant cell death after prolonged exposure to NO was demonstrated by different studies. In this study, cortical neurons were loaded with the Zn\(^{2+}\) selective fluorophore FluoZin-3 and treated with NO generator, spermine NONOate. Microfluorimetry was used to detect intracellular Zn\(^{2+}\) change. N,N,N’,N’-Tetrakis-(2-pyridylmethyl)-Ethylenediamine (TPEN) was applied to eliminate Zn\(^{2+}\) signals. A significant increase of intracellular fluorescence was detected during a 5 min perfusion with NO. The increased intracellular Zn\(^{2+}\) appeared to peak at 1 µM. The NO scavenger hemoglobin blocked the effects and the inactive analog of the spermine NONOate, spermine, was without effect. These data indicate that \textit{in vivo} release of NO may elevate intracellular Zn\(^{2+}\) in neurons that may have physiological significance.

Neurons exposed to oxygen glucose deprivation (OGD) were used as an \textit{in vitro} model to study ischemic injury. After a 5 min pretreatment with spermine NONOate 24 hrs prior to OGD, neurons exhibited tolerance. Coincubation of NO with TPEN prevented the development of tolerance. The effect of TPEN could be blocked by coadministration with Zn\(^{2+}\) but not Cu\(^{2+}\) or Fe\(^{2+}\). Increased intracellular Zn\(^{2+}\) alone was not sufficient to induce tolerance. Cycloheximide (CHX) blocked the induction of
tolerance suggesting that de novo protein synthesis is necessary for development of tolerance. Using S\textsuperscript{35} incorporation assay, TPEN was shown to inhibit new protein synthesis and addition of Zn\textsuperscript{2+} reversed the effect by TPEN. ERK1/2 and RSK inhibitors blocked the induction of tolerance indicating that ERK1/2-RSK pathway was involved.

Clioquinol (Cq), a metal chelator with neuroprotective effects in Alzheimer’s disease, was shown to induced tolerance. TPEN prevented the induction of tolerance by Cq. DP-b99 is a lipophilic metal chelator shown to be effective in the treatment of stroke. A 5 min pretreatment with DP-b99 induced a robust tolerance. TPEN or CHX blocked tolerance indicating that DP-b99 shares similar mechanisms with NO. ERK1/2 and RSK inhibitors blocked the induction of tolerance by DP-b99 indicating that ERK1/2-RSK pathway is also involved. Incubation with DP-b99 caused an increase in cellular Ca\textsuperscript{2+} content detected by ICP-OES. The presence of extracellular Ca\textsuperscript{2+} was shown to be necessary for the induction of tolerance. The NOS inhibitor, L-NAME was able to prevent the induction of tolerance by DP-b99 suggesting that the production of NO is the downstream effector.

This study suggests that Zn\textsuperscript{2+} action is involved in the neuroprotection mechanism against ischemic cell death.

Approved: _____________________________________________________________

Robert A. Colvin
Professor of Biological Sciences
Dedicated to my parents, Xicai Lin and Lijuan Dai
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INTRODUCTION

I. Introduction zinc in the brain

A. Zinc overview

Zinc is shown to have structural, catalytic and regulatory functions. The number of genes coding for proteins with zinc-binding domains is conservatively estimated to be more than 3% of the human genome but is reasonably guessed to be as much as 10% (Andreini et al., 2006). Zinc is an essential micronutrient and is required for the normal development and function of the nervous system (Takeda, 2000). As an essential component of many enzymes, DNA binding proteins and structural proteins, zinc contributes to important biological processes, including DNA synthesis, gene expression, hormone control, enzymatic reactions, and cell proliferation (Beyersmann & Haase, 2001). Zinc is critical for many physiological functions (Yanagisawa 2008, Beyersmann & Haase, 2001) including growth and development, wound healing, skin metabolism, spermatogenesis and oogenesis (Falchuk & Montorzi, 2001), normal thyroid, pancreatic and parathyroid function (Neve, 1992), normal immune function (Rink & Gabriel, 2001), bone mineralization, sense of taste and olfaction (Tomita, 2002), function of central nervous system (Prasad, 1997), retinal function, synthesis and action of insulin, and lipid metabolism. Zinc deficiency has been shown to cause a series of syndromes which include growth retardation, hair loss, skin problems, immune dysfunction, cognitive impairment, and testicular atrophy (Brown et al., 2002). In humans, about 1% of the total
body zinc content is replenished daily by the diet. The replenished zinc is delivered by tight control of two systems, absorption from the intestine and endogenous loss via pancreatic and other intestinal secretions (Cousins et al., 2006).

B. The role of zinc in brain

The brain has one of the highest zinc content of any organ and is estimated to be approximately 150 µmol/L (Mocchegiani et al., 2005). In the brain, approximately 90% of the total zinc is bound to zinc-binding proteins (Frederickson 1989). Zinc levels were shown to be highest in the hippocampus and caudate nucleus and lowest in the middle frontal lobe and globus pallidus (Markesbery et al., 1984). However, the free zinc concentration estimated in the cytosol of cultured neurons is from picomolar to nanomolar range (Colvin, 2003; Colvin et al., 2008; Frederickson et al., 2007). Neurons that contain zinc ions in the vesicles of their presynaptic boutons are present also in other brain areas and are generally termed zinc-enriched (ZEN) neurons (Frederickson 1989; Danscher et al., 1994; Frederickson et al., 2000). As ZEN neurons are not uniformly distributed, the distribution of zinc itself in the brain is not uniform. Higher zinc concentrations are present in grey than white matter, and the highest regions are found in certain forebrain regions, including the hippocampus, amygdala and neocortex (Frederickson et al., 2000).

The transport of zinc into the brain parenchyma occurs via the blood brain barrier system (Takeda, 2001). After the uptake, zinc can be transferred freely through the cerebrospinal and the brain extracellular fluid compartments, as shown by autoradiographic studies (Takeda, 2001). The entry routes, which regulate zinc uptake
from extracellular fluids into neurons and glial cells, are still not completely described, but transporters of the Zip family seem involved in this process (Law et al., 2003). Whereas specific gated zinc-permeable membrane-spanning channels such as voltage L-type Ca$^{2+}$ channels (Kim et al., 2000; Segal et al., 2004), Na$^+$/Zn$^{2+}$ exchangers (Cheng & Reynolds, 1998), NMDA receptors-gated channels (Koh & Choi, 1994) and Ca$^{2+}$ permeable AMPA/kainite channels (Jie et al., 2002) can also mediate the neuronal uptake of zinc. IN contrast, zinc transporter proteins belonging to ZnT family regulate the efflux of Zn$^{2+}$ from neurons as well as the vesicular Zn$^{2+}$ uptake (Qin et al., 2008; Seve et al., 2004). Another possible mechanism consists of the modulation of cellular Zn$^{2+}$ homeostasis by neuronal mitochondria uptake of Zn$^{2+}$ (Sensi & Jeng, 2004). A putative Zn$^{2+}$ transporter could be implicated in this uptake, which might involve a direct exchange of Zn$^{2+}$ from intracellular zinc-ligands to the transporter (Guan et al., 2003).

It has been suggested that Zn$^{2+}$ acts like a neurotransmitter during synaptic transmission (Frederickson et al., 2005). Numerous studies have shown evidence of Zn$^{2+}$ release after stimulus. A calcium-dependent release of Zn$^{2+}$ was induced by electrical stimulation in hippocampal granule cells (Howell et al., 1984). Stimulation with high K$^+$ also demonstrated a calcium-dependent release of Zn$^{2+}$ from hippocampal slices (Assaf & Chung, 1984). Kainate was also shown to induce Zn$^{2+}$ release from hippocampal mossy fibers (Frederickson et al., 1988). Moreover, Zn$^{2+}$ release was also found in the amygdala stimulation with high K$^+$ (Takeda et al., 1999). It was also demonstrated that presynaptic stimulation of hippocampal mossy fibers caused Zn$^{2+}$ release from terminal vesicles into the surrounding milieu (Li et al., 2001; Ueno et al., 2002). A recent study showed direct
observation of decreasing vesicular $\text{Zn}^{2+}$ from presynaptic axonal boutons in the stratum lucidum of CA3 when induced by depolarization (Ketterman & Li, 2008). The $\text{Zn}^{2+}$ released by excitation is thought to be coreleased with glutamate (Howell et al., 1984). Additionally, exogenous $\text{Zn}^{2+}$ was shown to be taken into mossy fiber neuropil by electrical stimulation (Howell et al., 1984). Recent data provide further evidence that synaptically released $\text{Zn}^{2+}$ can be taken up into presynaptic terminals (Ketterman & Li, 2008).

The physiological role of synaptic $\text{Zn}^{2+}$ is poorly understood. Recent studies suggest that it is mainly involved in the modulation and release of glutamate and $\gamma$-aminobutyric acid (GABA) (Xie et al., 1994; Frederickson & Moncrieff, 1994; Colvin et al., 2000; Li et al., 2001; Takeda et al., 2003). Glutamate is the major excitatory transmitter employed in the central nervous system and exerts rapid effects by interacting with ionotropic (ligand-gated ion channels) and metabotropic (G-protein linked glutamate receptors) receptors (mGluRs). Recently, by combining $\text{Zn}^{2+}$ imaging and electrophysiological recordings, direct evidence for a quantal co-release of $\text{Zn}^{2+}$ and glutamate has been provided (Vogt et al., 2000; Li et al., 2001; Molnar & Nadler, 2001; Ueno et al., 2002; Qian and Noebel, 2006). The most convincing findings were pbtaomed bu using FluoZin-3. In acute hippocampal slices, $\text{Zn}^{2+}$ exocytosis can be reliably detected after individual action potentials, not only at the $\text{Zn}^{2+}$-enriched mossy fiber synapses, but also at CA3-CA1 synapses, despite their lower $\text{Zn}^{2+}$ content (Qian & Noebels, 2005). The lack of $\text{Zn}^{2+}$ signals in slices from ZnT3 KO mice confirmed the origin of the released $\text{Zn}^{2+}$ (Qian & Noebels, 2006). Both high affinity and low affinity zinc-binding sites have
been found in NMDA receptors. The corelease of Zn\(^{2+}\) with glutamate from synaptic vesicles of gluzinergic (neurons that sequester both zinc and glutamate) and other ZEN neurons (Frederickson et al., 2000) may be directly involved in the modulation of receptor-mediated excitatory post-synaptic currents. Electrophysiological studies showed the release of Zn\(^{2+}\) with glutamate reduces the ability of glutamate to activate post-synaptic NMDA receptors and favors synaptic non-NMDA receptor activation (Mocchegiani et al., 2005). Zn\(^{2+}\) was also shown to inhibit NMDA receptor-mediated channels in cultured neurons (Christine & Choi, 1990; Legendre & Westbrook, 1990). A most recent paper showed that Zn\(^{2+}\) could activate the receptor tyrosine kinase TrkB and potentiate hippocampal mossy fiber-CA3 pyramid synapses (Huang et al., 2008). Therefore, Zn\(^{2+}\) functions are similar to neurotransmitters that are stored in membrane-enclosed synaptic vesicles and released by exocytosis. Zn\(^{2+}\) then binds to gated ion channels and activates postsynaptic cells (Colvin et al., 2003).

**C. The role of zinc transporters and zinc-binding proteins**

Two protein families have been shown to be involved in Zn\(^{2+}\) transport. The ZnT (solute-linked carrier 30 (SLC30A)) proteins lower intracellular Zn\(^{2+}\) by mediating Zn\(^{2+}\) efflux from cells or influx into intracellular vesicles. The Zip (Zrt- and Irt-like proteins (SLC39A)) proteins promote Zn\(^{2+}\) transport from the extracellular fluid or from intracellular vesicles into the cytoplasm (Cousins et al., 2006). The mammalian ZnT family consists of 10 members (ZnT1-10). Zinc transporter activity for most ZnT proteins has been confirmed indirectly by survival of cells in medium of high Zn\(^{2+}\) content or directly through measuring Zn\(^{2+}\) uptake/efflux or Zn\(^{2+}\) accumulation in different types of
cells (Cousins et al., 2006). There is considerable sequence homology among human ZnT proteins. The sequences vary in size, and most of them are predicted to have six transmembrane domains. They have both their N and C terminals on the cytoplasmic side of the membrane. Most ZnT proteins also have a long intracellular loop with a variable number of histidine residues (Cousins et al., 2006). Most ZnT proteins have been found in intracellular compartments, usually associated with endosomes, Golgi or endoplasmic reticulum. ZnT1 seems to be the only ZnT transporter located at the plasma membrane, consistent with its role as the primary regulator of cellular Zn\(^{2+}\) efflux.

The mammalian Zip family consists of 14 members. Most Zip proteins are predicted to have eight transmembrane domains. Zip proteins are predicted to have their N and C terminals on the cytoplasmic side of the membrane and a long intracellular loop with a histidine-rich repeat. The transport activity has been measured by \(^{65}\)Zn uptake or by using fluorescent probes binding intracellular labile Zn\(^{2+}\). The mechanism of Zip-mediated transport is not well understood. The concentration gradient could drive Zn\(^{2+}\) uptake as a facilitative process. There is no evidence showing that hZip1 and hZip2 transporters require ATP. Most Zip proteins are located at the plasma membrane except for Zip7, which is located in the Golgi apparatus.

In addition to the Zn\(^{2+}\) that is released from presynaptic terminals into extracellular fluid, there is a pool of releasable zinc in perikarya. One source of this Zn\(^{2+}\) is the metallothioneins (MTs), from which Zn\(^{2+}\) can be released rapidly by nitrosylation or oxidation of the thiol ligands (Krezel et al., 2007). In humans, MT is involved in translocations of Zn\(^{2+}\) from the cytoplasm to either the nucleus undergoing proliferation
or to the intermembrane space of mitochondria, where it modulates respiration (Tsujikawa et al., 1991; Ye et al., 2001). MT is also found in the extracellular space and taken up by cells through an endocytotic pathway, in which the metals, but not protein, translocate to the cytoplasm (Hao et al., 2007). Critical to its protein structure and function are twenty cysteine residues (Maret, 2008). Thioneins are small proteins (~3,000Da) that contain several cysteine residues that allow them to bind metals, including Zn\(^{2+}\). They function physiologically by accepting Zn\(^{2+}\) from other zinc-binding ligands. Thionein can bind seven zinc atoms through twenty cysteine residues in zinc clusters. Oxidation or nitrosylation of cysteine residues in the zinc cluster results in the release of Zn\(^{2+}\), so these proteins can function as zinc donors to other zinc-binding proteins. Metallated thionein is in equilibrium with the unmetallated thionein. Using the fluorescent chelating agents FluoZin-3 and RhodZin-3, it has been revealed that at least three classes of sites with affinities that differ by 4 orders of magnitude (Krezel and Maret, 2007). The rate of thiol oxidation increases in the presence of Zn\(^{2+}\) acceptors but decreases if more Zn\(^{2+}\) becomes available.

The metallothionein III (MT-III) isoform is found only in the brain and testes, not like other isoforms, which are widespread in different parts. Three of four known isoforms are identified in the nervous system (Aschner et al. 1997). The expression of MT-I and MT-II are mainly localized in glial cells (Aschner et al. 1997), whereas, MT-III is mostly present in neurons (Masters et al. 1994). In response to oxidized glutathione (GSSG), Zn\(^{2+}\) can be released from MTs (Maret & Vallee, 1998). MTs seem to act as a cytosolic Zn\(^{2+}\) buffer but the physiological conditions of Zn\(^{2+}\) release are mostly unknown.
Experimental evidence has shown that long duration exposure to millimolar levels of exogenous NO results in increased levels of intracellular free Zn$^{2+}$ (Berendji et al. 1997). Research has suggested that NO induces Zn$^{2+}$ release from MT by S-nitrosylation and disulfide formation both in vitro and in vivo (Kroncke et al. 1994, Zhang et al. 2004).

Changes in MTs have been implicated in the control of cellular Zn$^{2+}$ fluctuations. Upon homocysteinylination, MT no longer scavenges superoxide ions and the released Zn$^{2+}$ activates the zinc finger transcription factor Egr-1 (Barbato et al., 2007). Such an impairment of Zn$^{2+}$ and redox homeostasis is believed to be a mechanism of how hyperhomocysteinemia, a major risk factor for heart disease and stroke, increases production of reactive species and may cause chronic inflammation, and atherothrombotic disease (Colgan & Austin, 2007). MT also can be nitrosylated (Kroncke et al., 1994). In this case, the released Zn$^{2+}$ activates MTF-1, which controls the expression of genes involved in the antioxidant defense (Stitt et al., 2005).

II. The role of Zinc in signaling pathways

Zinc is known to be a critical structural constituent of a great number of proteins, including enzymes from cellular signaling pathways and transcription factors. In addition, zinc may also function as a signaling molecule. Microfluorescence imaging of Zn$^{2+}$ dynamics following presynaptic stimulation of hippocampal mossy fibers shows Zn$^{2+}$ release from terminal vesicles into the surrounding milieu (Murakami & Hirano, 2008). Zn$^{2+}$ is then taken up into the cytoplasm of neighboring cells through gated Zn$^{2+}$ channels. Rapid Zn$^{2+}$ influx through Ca$^{2+}$ permeable AMPA/kainite (Ca-A/K) channels
triggers the generation of reactive oxygen species, which are potently neurotoxic (Weiss & Sensi, 2000). Zn\textsuperscript{2+} is also known to inhibit NMDA receptor activity via two mechanisms: voltage-dependent channel blockade and voltage-independent reduction in the probability of channel opening (Christine et al., 1990; Legendre & Wesbrook, 1990). Thus, it is likely that Zn\textsuperscript{2+} acts as a neurotransmitter to carry information between neuronal cells.

There is increasing evidence for a direct signaling function for Zn\textsuperscript{2+} at different levels of signal transduction pathways through regulatory mechanisms that are still largely unknown (Beyersmann & Haase, 2001; Maret, 2001). Zn\textsuperscript{2+} seems to be functioning as an intracellular secondary messenger from these resources: (i) extracellular stimuli; (ii) an intracellular compartment or organelles; and (iii) free Zn\textsuperscript{2+} at a level similar to that observed in the Zn\textsuperscript{2+} wave, a release of Zn\textsuperscript{2+} from perinuclear area, can affect intracellular signaling molecules to modulate the final output triggered by extracellular stimuli. When investigated with a microarray experiment, increased intracellular Zn\textsuperscript{2+} has been shown to affect the expression of more than 1,000 genes in the monocytic cell line THP-1, a considerable number of which were involved in signal transduction, immune function, and cytokine production (Cousins et al., 2003). Zn\textsuperscript{2+} was found to stimulate lipopolysaccharides (LPS), tyrosine phosphorylation and protein kinase C in monocytes (Haase & Rink, 2007). Zn\textsuperscript{2+} was also found to inhibit cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) in monocytic cells. The incubation of PC12 rat pheochromocytoma cells with Zn\textsuperscript{2+} led to an increase in the cellular cGMP concentration which was explained by a Zn\textsuperscript{2+}-mediated inhibition of
cGMP hydrolysis (Watjen et al., 2001). Moreover, not only does Zn\(^{2+}\) modulate cGMP signaling, but also cGMP modulates the uptake of Zn\(^{2+}\) (Hasse, 2001). These data indicate that elevation of intracellular Zn\(^{2+}\) results in a rise in cGMP, which inhibits further Zn\(^{2+}\) import. In T cells, it has been shown by an \textit{in vitro} activity assay that IL-1 receptor associated kinase (IRAK-1) activity is reduced after incubation of T-cells with Zn\(^{2+}\) (Wellinghausen et al., 1997). A direct interaction between Zn\(^{2+}\) and NF-κB signaling pathways was also found. IκB Kinase (IKK) immunoprecipitated from LPS-stimulated murine RAW 264.7 macrophages is directly inhibited by Zn\(^{2+}\) \textit{in vitro} (Jeon et al., 2000). Several laboratories have investigated the activation of mitogenic signaling pathways by Zn\(^{2+}\) (Zago et al., 2005; Kohda et al. 2006; Uzzo et al. 2006). Zn\(^{2+}\) increased the affinity of both IGF-1 and IGF-2 to the type 1 IGF receptor on murine myoblasts with a half-maximal concentration of about 50μM (McCusker et al., 1998). In other researches, Zn\(^{2+}\) chelation by Diethylene triamine pentaacetic acid (DTPA) partially abolished the stimulation of MAPK by IGF-1 in rat fibroblasts and this inhibition was reversed by addition of equimolar concentration of Zn\(^{2+}\) sulfate (Lefebvre et al., 1999). In human bronchial epithelial cells, Zn\(^{2+}\) induced EGF receptor phosphorylation and MAPK activation (Wu et al., 1999). The stimulation effect of Zn\(^{2+}\) on tyrosine phosphorylation may be caused at least partially by interference with tyrosine dephosphorylation, because Zn\(^{2+}\) inhibited various protein tyrosine phosphatases in human airway epithelial cells (Samet et al., 1999). The isolated recombinant protein tyrosine phosphatase was inhibited by Zn\(^{2+}\) with an IC\(_{50}\) of 200 nM (Maret et al., 1999). The incubation of mouse cortical cells with toxic Zn\(^{2+}\) concentrations caused an activation of the MAP kinase ERK,
leading to increased expression of the immediate early gene egr-1 (Park & Koh, 1999). Treatment of human bronchial epithelial cells with subtoxic concentration of Zn\(^{2+}\) activated the MAP kinases ERK, JNK and p38, and evoked increased phosphorylation of the transcription factors Jun and ATF-2, which are substrates of MAP kinases (Samet et al., 1998). In Swiss 3T3 cells, phosphorylation and activation of P70S6 kinase was observed after treatment with Zn\(^{2+}\), and experiments with kinase inhibitors indicated activation through the phosphatidylinositol 3-kinase (PI3K) signaling pathway (Kim et al., 2000). For protein kinase C (PKC), a regulatory function of Zn\(^{2+}\) is inferred from the observation that nanomolar concentration of Zn\(^{2+}\) can activate PKC and cause a translocation of PKC (Csermely et al., 1988). Zn\(^{2+}\) also seems to regulate the translocation of PKC to the cytoskeleton (Zalewski et al., 1990). Moreover, it was shown that a chelatable pool of intracellular Zn\(^{2+}\) increases the binding of the PKC activator phorbol dibutyrate (Forbes et al., 1999). In addition, Zn\(^{2+}\) affects the regulation of transcription factors. Zn\(^{2+}\) can induce the expression of some genes, including those coding for molecules involved in Zn\(^{2+}\) homeostasis, like Zn\(^{2+}\) transporters and metallothioneins (Palmiter, 2004). The gene expression of metallothioneins by Zn\(^{2+}\) is regulated by metal response element-binding transcription factor-1 (Lichtlen & Schaffner, 2001). Zn\(^{2+}\) may also inhibit transcription factor activities directly. The activation of nuclear factor-κB in bovine cerebral cells is suppressed if cellular Zn\(^{2+}\) is elevated by application of the Zn\(^{2+}\) ionphore (Kim et al., 1999). Also, the binding of steroids to the murine glucocorticoid receptor is reversibly inhibited by Zn\(^{2+}\) (Telford & Fraker, 1997).

Since most of the experimental evidence comes from \textit{in vitro} experiments, it is not yet
generally accepted that $\text{Zn}^{2+}$ is indeed a signaling molecule \textit{in vivo}. In particular, the mechanism by which intracellular $\text{Zn}^{2+}$ activates various signaling pathways is still unknown.

\section*{III. The role of zinc in ischemia}

\subsection*{A. The neurotoxic role of zinc in ischemia}

Tonder and colleagues conducted the first study showing indirect evidence for the toxic translocation of $\text{Zn}^{2+}$ from presynaptic neurons into selective postsynaptic neurons during the experimental paradigm of global ischemia (Tonder et al., 1990). Johansen and colleagues also found that intra-ischemic hypothermia prevented ischemic-induced intracellular $\text{Zn}^{2+}$ accumulation and subsequent cellular demise, likely by inhibiting $\text{Zn}^{2+}$ translocation (Johansen et al., 1993). More direct evidence for the translocation of $\text{Zn}^{2+}$ was shown during a brief period of global ischemia, intracellular $\text{Zn}^{2+}$ accumulation in vulnerable CA1 pyramidal hippocampal neurons preceded degeneration, which could be prevented with the intracerebroventricular administration of Ca-EDTA (Koh et al., 1996). These researchers concluded that selective neuronal death of CA1 neurons during global ischemia was mediated by the release of synaptic vesicle $\text{Zn}^{2+}$ from a subset of excitatory terminals and its subsequent translocation into vulnerable post-synaptic neurons. Tsuda and colleagues examined the induction of ZnT-1 mRNA expression in the CA1 subfield of the hippocampus following global ischemia (Tsuda et al., 1997). The $\text{Zn}^{2+}$-mediated neuronal death following ischemia was achieved through the specific induction of $p75_{\text{NTR}}$ and its associated death executor, NADE (Park et al., 2000). Studies in cultured cells have demonstrated that $\text{Zn}^{2+}$ neurotoxicity may promote the disruption of different stages
of cellular respiration through depletion of ATP and the oxidized form of the coenzyme, NAD+ (Galasso & Dyck, 2007). Different studies have shown the administration of pyruvate can achieve neuroprotection by antagonizing Zn$^{2+}$ neurotoxicity following global ischemia (Galasso & Dyck, 2007). The intracerebroventricular administration of Ca-EDTA prior to mild focal ischemia achieved early neuroprotection against Zn$^{2+}$ accumulation (Lee et al., 2002). It is also demonstrated that hypothermia (29°C) reduces interneuronal Zn$^{2+}$ movement and subsequent death (Johansen et al., 1993). The above studies demonstrated that elevated intracellular Zn$^{2+}$ levels during ischemia serve as critical mediator of neuronal death; therefore Zn$^{2+}$ inhibition through either early or late chelation may be effective in alleviating Zn$^{2+}$ induced neurotoxicity.

**B. The neuroprotective role of zinc in ischemia**

In contrast to chelation-based therapeutic intervention, some studies have also shown neuroprotective benefits following the administration of various Zn$^{2+}$ compounds. The therapeutic effect of zinc protoporphyrin (ZnPP) was found to reduce ischemic brain edema significantly by blocking IL1 activity (Yamasaki et al., 1992). Kadoya and colleagues demonstrated that ZnPP was limited in its level of neuroprotection by the temporal parameters of its administration and whether or not reperfusion followed the onset of ischemia (Kadoya et al., 1995). In a follow-up study, Zhao and colleagues set out to determine whether the Zn$^{2+}$ or protoporphyrin complement of ZnPP possessed neuroprotective capabilities. Equimolar doses of zinc chloride, PP and ZnPP were all found to reduce the lesion size, but only ZnPP and PP were found to ameliorate ischemic brain edema (Zhao et al., 1996). As such, this study suggested that Zn$^{2+}$ ions, in
comparison to protoporphyrin, provide neuroprotection by mechanism other than reducing brain edema. Matsushita and colleagues also demonstrated that Zn\(^{2+}\) supplementation could provide neuroprotection to the CA1 hippocampal subfield during global ischemia in the gerbil (Matsushita et al., 1996). Recently, it was found that reduction in Zn\(^{2+}\) levels following intracerebroventricular injection of Ca-EDTA prior to focal ischemia accelerated the early development of the infarct, suggesting the need for a minimum complement of Zn\(^{2+}\) to maintain cellular viability, even during cerebral ischemia (Kitamura et al., 2006).

C. The change of zinc level during ischemia

There are some studies focusing on monitoring dynamic changes in zinc levels during cerebral ischemia in the absence of therapeutic interventions. Relying on the neo-Timm sulfide-silver staining procedure, it was shown that staining at zinc positive-terminals was decreased within the ischemic region 7 min after ischemic onset (Galasso & Dyck, 2007) and was relatively absent for all remaining times examined, up to seven days after ischemia. The reason was attributed to the release of Zn\(^{2+}\) from synaptic vesicles. Kitamura and colleagues tested the temporal release profile of extracellular Zn\(^{2+}\) using micro-dialysis and also examined subsequent intracellular Zn\(^{2+}\) accumulation in the CA1 subfield of the hippocampus during global ischemia (Kitamura et al., 2006). Within 15 min post-ischemia, extracellular Zn\(^{2+}\) levels reached a peak (~600nM) that was double the basal level. Subsequently, extracellular Zn\(^{2+}\) levels decreased and returned to baseline 15 min following reperfusion. Frederickson and colleagues also used micro-dialysis to examine extracellular Zn\(^{2+}\) levels during global ischemia and reperfusion (Frederickson et
al., 2006). Extracellular Zn\(^{2+}\) levels increased and were synchronous with glutamate release. However, the reperfusion-induced Zn\(^{2+}\) release was more pronounced (>100 nM, in some cases), in both intensity and duration than the initial ischemic-induced Zn\(^{2+}\) release. The delayed reperfusion-induced Zn\(^{2+}\) release was unaccompanied by glutamate release, possibly reflecting a release of Zn\(^{2+}\) from intracellular stores (Galasso & Dyck, 2007).

The liberation of Zn\(^{2+}\) from intracellular stores contributes to neuronal injury. Previous studies demonstrated that glutamate-induced changes in intracellular Zn\(^{2+}\) were completely dependent on Ca\(^{2+}\) entry in cultured rat forebrain neurons (Dineley et al., 2008). The entry of Ca\(^{2+}\) could induce reactive oxygen species that arises from both cytosolic and mitochondrial sources. The reactive oxygen species also caused intracellular Zn\(^{2+}\) changes. This interaction of Zn\(^{2+}\) and Ca\(^{2+}\) might give a possible mechanism in neuronal death resulting from ischemia.

**IV. An introduction to ischemic tolerance**

**A. An overview of ischemia**

Worldwide, stroke is an important cause of long-term disability and the most common life-threatening neurological disorder (Rothwell, 2001). The most common symptom for stroke is the disruption of blood flow. The brain is one of the most sensitive organs to ischemic injury in the entire body. A constant flow of blood to the brain is essential in delivering oxygen and glucose to neurons. If this flow is disrupted, the result is cell damage or death. Neurons are rarely replaced once they have died, therefore the
damage to affected regions may be permanent. Stroke is the result of blood flow disruption to the brain with many different causes. Although stroke is a heterogeneous condition that includes a variety of etiologies, strokes mainly result from the obstruction of an intra-cranial artery by a thrombus. Unfortunately, there are few therapies available to lyse clots and restore blood flow to the compromised brain.

Although calcium was involved in ischemia, more and more evidences suggest that calcium may serve as an accomplice to zinc, which is a possibly a more potent ionic mediator of ischemic injury (Galasso & Dyck, 2007). More and more studies suggested that the increase of intracellular Zn$^{2+}$ during and after ischemia played an important role in concomitant ischemic injury. But how the increase of intracellular Zn$^{2+}$ led to damage to the neurons is still largely unknown. Better understanding of the exact role of intracellular Zn$^{2+}$ in the ischemic damage would give us more hints to develop therapies for stroke.

**B. An overview of ischemic tolerance**

Ischemic preconditioning (IPC) is a phenomenon in which brief episodes of a subtoxic insult induce a robust protection against the deleterious effects of subsequent, prolonged, lethal ischemia. The subtoxic stimuli that constitute the preconditioning event are quite diverse, ranging from brief ischemic episodes, spreading depression or potassium depolarization, chemical inhibition of oxidative phosphorylation, or exposure to excitotoxins and cytokines. The beneficial effect of preconditioning was first demonstrated in the heart (Murry et al., 1986), but was subsequently found to occur in the
brain as well (Kitagawa et al., 1991). It is now clear that preconditioning can induce ischemic tolerance in a variety of organ systems including brain, heart, liver, small intestine, skeletal muscle, kidney and lung. A common method to precondition neural tissues is hypoxia, or exposure to reduced atmospheric oxygen concentration (Miller et al., 2001). Reduced atmospheric pressure has also been used in combination with reduced oxygen concentration (Romanovskii et al., 2001). A variety of chemical agents have been used to initiate protection, often by interfering in the action of major proteins involved in neuronal damage, such as inhibitors of succinic dehydrogenase, which an important enzyme involved in citric acid cycle and electron transport chain (Kuroiwa et al., 2000), glutamate receptor agonists (Lam et al., 1998) and hormone analogs, which was shown to enhance cerebral metabolism (Urayama et al., 2002). Tolerance to ischemic insults can also be produced by cortical spreading depression (Yanamoto et al., 1998), sleep deprivation (Hsu et al., 2003), dietary restriction (Yu & Mattson, 1999), and both hyperthermia and hypothermia (Dirnagl et al., 2003). Exposure of cortical cell cultures to low levels of glutamate or NMDA to induce NMDA receptor activation has also been found to cause tolerance (Grabb & Choi, 1999). Moreover, tolerance to oxygen glucose deprivation (OGD) was blocked if an NMDA antagonist was applied (Lees, 1997). NMDA receptor activation also results in the production of NO (Nandagopal et al., 2001). The activity or nitric oxide synthase (NOS) and the total amount of NO present in the brain are increased following exposure to hypoxia (Lu & Liu, 2001).

Molecules and proteins that are important for the development of ischemic tolerance in the brain are potential targets for the development of new treatments for ischemia and
stroke. Often, potential targets are first identified by the observation of changes in gene expression level, protein level or protein activity following ischemia or the preconditioning treatment. Microarrays are one way to accomplish such broad screening. Changes in gene expression must then be confirmed to cause a corresponding change in protein expression level. Although the mechanisms behind the formation of protection are still largely unknown, once the processes involved in preconditioning are more fully understood, the potential benefits for prevention and treatment of brain damage due to ischemia could be enormous.

V. NO and ischemia

A. An overview of NO

NO is a unique diffusible molecular messenger in the vascular and nervous system. NO is produced by a family of enzymes called NOS. NOSs enzymatically oxidize the guanidine group of L-arginine to produce NO (Marletta et al., 1998). The synthesis of endogenous NO occurs in two sequential monooxygenase reactions, during which NADPH and molecular oxygen are also needed (Marletta et al., 1998). The three NOSs are constitutive calcium dependent neuronal NOS (nNOS), constitutive endothelial NOS (eNOS) and inducible calcium independent NOS (iNOS). They are located in the cytosol or associated with the cell membrane. Recently, NOSs activity or proteins bound to NOS-specific antibodies have also been found in isolated mitochondria (Ghafourifar & Richter, 1997). One possibility is that one of the three known NOSs can bind to or be transported into mitochondria. Another explanation is that there is a new mitochondrial NOS
Glutamate, depolarization with KCl and the calcium ionophore ionomycin stimulated NOS activity by increase intracellular Ca$^{2+}$.

The physiological concentration of NO in cells is considered less than 10µM. While under pathological conditions, the concentration of NO in cells is higher than 100µM. The effect of NO in cells remains controversial because both cytotoxic and cytoprotective effects of NO were reported. Different results were related to different variables used in the assay systems (Virag et al, 2002). In the cases where NO was found to be cytotoxic, it is questioned whether NO directly or indirectly, through the formation or more reactive oxidative species such as peroxynitrite, exerts its cytotoxic effects (Beckman & Koppenol, 1996).

NO can play a role as a signal molecule in neuronal systems to trigger downstream pathways to regulate physiological functions. During the formation of the brain, neuronal cell migration and neurite extension are controlled by extracellular guidance cues. NO was found to be an additional positive regulator of cell motility. In nerve cells, NO is generated in an activity-dependent process by Ca$^{2+}$/calmodulin-stimulated NO synthase. The major function of NO appears to be as an activator of the heme protein soluble guanylyl cyclase (sGC), which leads to the formation of cGMP in neuronal cells. Synthesis of cGMP may directly gate ion channels, stimulate protein kinase G (PKG) and cGMP-dependent phosphodiesterases, and regulate other downstream signal transduction cascades. Experimental manipulations of NO signal transduction during the formation of the vertebrate nervous system indicates that NO mediates the refinement of retinotectal...
projections and the activity-dependent synaptic suppression by guanylyl cyclase pathway in developing neuromuscular synapses (Bicker 2005).

NO was also shown to be involved in the activation of MAPK/ERK pathway in different experiments. Endothelin (ET-1), an important molecule in diabetic vascular complications, was found to be required for NOS activation. The addition of NOS inhibitor suppressed the contraction induced by ET-1. MEK/ERK-pathway inhibitor PD98056 and U0126 were able to reduce contraction (Matsumoto et al., 2009). In another study, NO was found to induce interleukin (IL)-8 production. The NO donor was found to increase phosphorylation of MAPK and ERK. The application of MAPK and ERK inhibitors blocked IL-8 secretion (Min et al., 2008). The activation of p21Ras protein induced by NO plays a critical role in THP-1 monocyte/macrophage apoptosis (Tsujita et al., 2008). Only ERK1/2 in the three major MAP kinase pathways was activated by NO donor. The inhibitor of ERK1/2 markedly attenuated apoptosis in THP-1 cells, but had a marginal effect on THP-1 cells expressing NO-insensitive p21Ras. Ras was found to be activated by endogenous NO. The mechanism of Ras activation by NO is possibly due to S-nitrosylation of a critical cysteine residue which stimulates guanine nucleotide exchange (Lander et al., 1995).

**B. The neurotoxic effect of NO in ischemia**

NO contributes to a wide variety of pathological processes in the central nervous system, including stroke and neurodegenerative disorders such as Parkinson’s disease, Alzheimer’s disease, multiple sclerosis, epilepsy, and HIV-associated dementia (Bossy-
Wetzel et al., 2004). Excessive stimulation of the NMDA receptor, which in turn leads to Ca\(^{2+}\)-mediated activation of nNOS, results in pathological levels of NO (Bredt et al., 1990). NO reacts rapidly with endogenous superoxide anion to form peroxynitrite, a neurotoxic molecule (Beckman et al., 1990). Peroxynitrite-induced neurotoxicity is thought to be an important mechanism of neuronal injury in hypoxic-ischemic brain and spinal cord injury (Szabo, 1996) and a variety of neurodegenerative diseases (Chabrier et al., 1999). The mode of cell death induced by peroxynitrite can be necrosis, apoptosis, or mixed types of cell death, depending on the concentration of peroxynitrite, the duration of peroxynitrite exposure and intracellular ATP levels (Zhang et al., 2004).

Peroxynitrite is formed in biological systems when superoxide and nitric oxide are produced at near equimolar ratio. Although not a free radical by chemical nature (as it has no unpaired electron), peroxynitrite is a powerful oxidant exhibiting a wide array of tissue damaging effects, ranging from lipid peroxidation, inactivation of enzymes and ion channels via protein oxidation and nitration to inhibition of mitochondrial respiration (Beckman & Koppenol, 1996). Low concentrations of peroxynitrite trigger apoptotic death, whereas higher concentrations induce necrosis. Cellular energetics (ATP and NAD) is proposed to serve as a switch between the two modes of cell death (Leist et al., 1997).

Research efforts have focused on understanding the relationship between increased NO levels and Zn\(^{2+}\) release from MT. When cultured neurons were treated with high concentrations of NO, peroxynitrite is produced, which leads to the release of Zn\(^{2+}\) from MT. The increase of free Zn\(^{2+}\) can result in more peroxynitrite production (Bossy-Wetzel
et al., 2004). The accumulated peroxynitrite and ROS can oxidize and activate the phosphorylation of p38 MAPK to trigger downstream signaling pathways. All these events lead to the activation of caspase to cause cell death (Bossy-Wetzel et al. 2004). Another pathway that can be induced by increased Zn\(^{2+}\) is the 12-lipoxygenase (12-LOX) pathway. The activation of 12-LOX can mediate arachidonic acid (AA) metabolism to increase ROS production (Zhang et al., 2004). In these experiments, high concentrations of NO were used to cause Zn\(^{2+}\) release. Under physiological conditions, endogenous NO is much lower, thus whether Zn\(^{2+}\) release occurs under physiological conditions has not been demonstrated.

C. The neuroprotective effect of NO in ischemia

NO has been shown to activate protective intracellular signaling during ischemic preconditioning in the brain using in vitro and in vivo models. Several studies suggested that the induction of iNOS is necessary for the development of ischemic preconditioning. Preconditioning stimuli such as ischemia, LPS or isoflurane anesthesia upregulate iNOS expression, and pharmacological inhibition or genetic inactivation of iNOS prevents the induction of tolerance to ischemia in rat brain (Kapinya et al., 2002; Cho et al., 2005; Kawano et al., 2007). Genetic deletion of epithelial NO synthase (eNOS) abrogated the neuroprotection induced by preconditioning. The eNOS played an obligate role in ischemic preconditioning by activating the PKC epsilon-MEK-1/2-p44-42 MAPK pathway to upregulate the STAT-dependent genes (Xuan et al., 2007). Using cultured cortical neurons exposed to sublethal OGD it was shown that an increase of neuronal
NOS and NO production during sublethal OGD resulted in neuroprotection against subsequent more severe OGD (Gonzalez-Zulueta et al., 2000, Scorziello et al., 2007).

The mechanism underlying induction of tolerance to OGD by NO still remains to be resolved. Previous studies using cultured cortical neurons have shown that Ras activation by NO is necessary and sufficient for the induction of tolerance to OGD. Guanylyl cyclase (GC) inhibitors showed no effect suggesting that the GC/cyclic GMP pathway is not involved in NO actions (Gonzalez-Zulueta et al., 2000). By using recombinant adenoviruses and pharmacological inhibitors, the ERK cascade was shown to be required as the downstream modulator of Ras activation. The activation of ERK was observed within 10 min after exposure to OGD. One downstream target for NO-induced tolerance to OGD is suggested to be the stimulation of $K_{ATP}$ channel (Lin et al., 2004). On the other hand, the activation and expression of Mn superoxide dismutase (Mn-SOD), a nuclear encoded mitochondrial enzyme that plays a role in scavenging excessive superoxide radicals, was also found to be regulated in a NO–dependent and Ras/ERK1/2 involved pathway during ischemic preconditioning (Scorziello et al., 2007).

D. An introduction of calcium

Under normal conditions, cytosolic $Ca^{2+}$ combines with calcium-binding proteins, such as calmodulin, or exists in storage in cellular organelles, such as the endoplasmic reticulum and the mitochondria (Corbett & Michalak, 2000; Duchen, 2000). Free $Ca^{2+}$ released into the intracellular space is actively excreted by the $Na^+/Ca^{2+}$ exchange system, which is maintained by ATPase much less than outside the cell (Ogawa et al., 2007). $Ca^{2+}$ plays a central role in signal transduction in the cell, which in turn controls the main
cellular activities, including differentiation and proliferation. But in different neurodegenerative disease such as epilepsy, hypoxia-ischemia, hypoglycemia, Alzheimer’s disease and schizophrenia, sustained Ca$^{2+}$ influx through glutamate receptor channels is thought to represent a final common pathway of neuronal cell death that is associated with these diseases (Choi, 1995; Lipton, 1999; Freidman, 2006). Glutamate, the major excitatory neurotransmitter of the central nervous system, is released in excess in all of these diseases and consequently stimulates Ca$^{2+}$ from neuronal stores by exerting its actions on receptor membranes from three structurally related fast acting iontropic gene families: NMDA, AMPA and KA (kainic acid) (Freidman, 2006). NMDA receptors are Ca$^{2+}$-preferred glutamate-gated ion channels that are expressed throughout most central neurons. NMDA-type glutamate receptors were initially held responsible for neuronal injury, because of their high Ca$^{2+}$ permeability conductance properties (Rothstein, 1996). AMPA-type glutamate receptors, however, have also been implicated in excitotoxicity because these receptors assemblies are highly permeable to Ca$^{2+}$ (Geiger et al., 1995) and also contribute to the delayed neuronal cell death processes by Ca$^{2+}$ overload (Pellegrini-Giampietro et al., 1997). High signal efficacy can be achieved because excess levels of glutamate in the central nervous system can result in elevated intracellular Ca$^{2+}$ concentration that exist for several hours even after glutamate has been removed from the synaptic cleft (Stout et al., 1998). This increase, in turn, causes a rise in Ca$^{2+}$ in sensitive organelles, such as the mitochondria and endoplasmic reticulum (Orrenius et al., 1992; Pelletier et al., 1999). The molecular events linking glutamate receptor activation and elevated cytosolic free Ca$^{2+}$ to eventual cell death include
downstream activation of proteases, endonucleases and the generation of nitric oxide and free radicals, which eventually destroy cells by lipid peroxidation, apoptosis or both (Choi, 1988; Lipton, 1999; Kristian & Siesjo, 1998).

The influx of Ca\(^{2+}\) through the NMDA-type glutamate receptor is further accelerated by delayed depolarization. This impairment is much enhanced by energy starvation under ischemia. Furthermore, energy-metabolism impairment also paralyzes the intracellular storage of calcium, which results in an unregulated increase of intracellular Ca\(^{2+}\). The influence of the disengagement of Ca\(^{2+}\) regulation on the cellular organelle includes the activations of endonuclease, which will initiate apoptosis pathway; acceleration of lipid degradation, which will increase intracellular free fatty acid to generate reactive oxygen intermediate to cause DNA damage; the activation of Ca\(^{2+}\) dependent protease, which leads to autolysis of cytoskeleton proteins; the activation of Ca\(^{2+}\) dependent protein kinase C, which will increase unregulated protein phosphorylation to cause the dysfunction of cellular signal transduction system; the dissociation of microtubulins, which will disturb axon transport; the activation of NOS, which will produce large amount of NO to activate apoptotic pathways (Ogawa et al., 2006).

However, hypoxic or ischemic preconditioning models demonstrate that certain levels of intracellular Ca\(^{2+}\) can protect against subsequent insults. In support of this notion, antagonizing the NMDA receptor with MK-801 during ischemic preconditioning in the gerbil hippocampus in vivo abolishes subsequent tolerance to lethal ischemia, presumably by blocking glutamate-triggered Ca\(^{2+}\) influx (Kato et al., 1992). In another study, cortical spreading depression induces transient Ca\(^{2+}\) influxes and results in delayed
adaptation to ischemia (Matsushama et al., 1996). Sublethal hypoxia or glucose deprivation in hippocampal cultures produce modest elevation of intracellular Ca\(^{2+}\) that can uncouple subsequent increase in intracellular Ca\(^{2+}\) to promote cell survival (Bickle & Fajlman, 2004). Ca\(^{2+}\) influx during preconditioning is required for the induction of tolerance during anoxic preconditioning (Perez-Pinzon et al., 1999). Although Ca\(^{2+}\) is generally viewed as an agent of neuronal death, particularly within an excitotoxic setting of cerebral ischemia, Ca\(^{2+}\) is now regarded as a key mediator of IPC. The incubation of an NMDA receptor antagonist during IPC in vivo (Mabuchi et al., 2001) and during OGD-preconditioning in vitro (Raval et al., 2003) blocks development of ischemic or OGD tolerance. Ca\(^{2+}\) influx through this receptor is required, since decreasing extracellular Ca\(^{2+}\) during preconditioning prevents acute electrical recovery in brain slices (Perez-Pinzon et al., 1999) and delayed OGD tolerance in cortical cell cultures (Gonzalez-Zulueta et al., 2000) or organotypic hippocampal brain slices (Raval et al., 2003). NMDA-preconditioning induces rapid uptake of mitochondrial Ca\(^{2+}\), but prevention of this process by collapsing the mitochondrial membrane potential with FCCP or dinitrophenol had minimal effect on preventing tolerance to OGD. Cerebral IPC may induce ER stress, possibly in a Ca\(^{2+}\)-dependent manner. A Ca\(^{2+}\)-dependent role in ischemic tolerance is suggested by findings that overexpression of ORP150, a 150-kDa oxygen-regulated protein, suppresses a glutamate-induced increase in intracellular Ca\(^{2+}\) and neurotoxicity (Mizayaki et al., 2002). CREB and NF-κB are associated with broad neuroprotective properties and may be activated by Ca\(^{2+}\) influx through NMDA receptor and L-type Ca\(^{2+}\) channels and other stimuli (Tauskela & Morley, 2004).
Since majority of the current studies about the roles of Ca\(^{2+}\) elevation are based on its interaction with fluorescent indicators. The interference by Zn\(^{2+}\) during monitoring intracellular Ca\(^{2+}\) change was omitted based on the assumption of intracellular free Zn\(^{2+}\) levels are very low. But it is now well established that several classical Ca\(^{2+}\) fluorescent indicators are also sensitive to Zn\(^{2+}\) (Thompson et al., 2002; Devinney et al., 2005; Stork & Li, 2006). It is necessary to carefully distinguish the changes observe by Ca\(^{2+}\) changes in either Ca\(^{2+}\), Zn\(^{2+}\) or both ions.

VI. An introduction to DP-b99

Different metal ion chelators are being developed as a way of modulating metal dependent functions. Clioquinol, a Zn\(^{2+}\) and Cu\(^{2+}\) chelator, affected the stable markers Tau and GAP43 in patients with Alzheimer’s disease and clinical ratings after 3 weeks of treatment showed slight improvements (Gouras & Beal, 2001; Cherny et al., 2001; Regland et al., 2001). BAPTA-AM, a cell permeable Ca\(^{2+}\) chelator was highly neuroprotective in cerebral ischemic models (Tymianski et al., 1993). DP-b99 is a membrane-active lipophilic chelator of divalent metal ions derived from the metal ion chelator BAPTA. It is designed to chelate these ions only in the vicinity of membranes and when their concentrations exceed physiologic levels, thus affecting the function of proteins that require these ions for their activation (Angel et al., 2002). The affinity of DP-b99 to Zn\(^{2+}\) and Cu\(^{2+}\) is different. Ca\(^{2+}\) is 4 mM, while the K\(d\) to Zn\(^{2+}\) is 100 nM. DP-b99 was shown to mediate the transfer of Ca\(^{2+}\) from aqueous phase to octanol, leading to the transfer and eventual accumulation of these ions in the organic phase. The redistribution of Ca\(^{2+}\) by DP-b99 within cell membranes affected Ca\(^{2+}\)-dependent
functions at the vicinity of the membrane. In vitro, DP-b99 protected primary neurons from cell death induced by glucose-oxygen deprivation, H₂O₂ and S-nitroso N-acetyl penicillamine, a NO donor. DP-b99 was also shown to block basal activation of MMP-9 as well as reducing TNF-α-induced expression of MMP-9 in primary cultured glial cells (Angel et al., 2002). It has demonstrated potent efficacy in several animal models of cerebral ischemia, with a large therapeutic window, and is effective for up to 8 hrs after ischemia (Angel et al., 2002). In rodent models of cerebral ischemia, DP-b99 therapy reduced infarct volume and neuron-specific enolase release and increased the survival rate (Krakovsky et al., 2001). In Phase-I studies using single and repeated escalating doses, DP-b99 were found to be safe and well-tolerated in young or elderly volunteers in the dose range of 0.003-1.0 mg/kg/day, when administered for up to 4 consecutive days. In a clinical trial phase-II study for stroke, the recovery rate was significantly improved under the treatment with DP-b99 when compared with placebo in a clinical trial phase-II study for stroke (Diner et al., 2008). Above experiments demonstrated that DP-b99 is effective in acute ischemic injury, but whether the pretreatment of DP-b99 could induce ischemic preconditioning effect is unknown.

**VII. Overview of completed studies**

As previous experiments have used long term exposures to NO to increase intracellular Zn²⁺, little is known about what will happen to intracellular free Zn²⁺ levels after brief physiological exposure to NO. Therefore, I studied short term, low
concentration exposure to extracellular NO, that were not neurotoxic, to determine if the release of intracellular Zn\(^{2+}\) was still a feature of such experimental manipulations.

Recent studies have shown that increased intracellular Zn\(^{2+}\) improved myocardial recovery after ischemia/reperfusion (Karagulova et al. 2007). The exogenous NO exposure was shown to increase intracellular Zn\(^{2+}\) in cultured primary cortical neurons. Previous studies have also shown that the activation of the production of endogenous NO induced a robust tolerance to OGD. It is interesting to investigate that whether the changes of intracellular Zn\(^{2+}\) play some roles in NO-induced tolerance.

Previous studies have also shown that lipophilic metal chelators, such as DP-b99 or Cq produced protection during acute ischemic injury, but the mechanism of protection is not clear. Whether the pretreatment of these metal chelators induced tolerance to OGD is investigated in this study.
MATERIALS AND METHODS

1. **Primary cortical neuron culture**

Pregnant rat (E17) was euthanized with CO$_2$. The fetuses were removed from the uterine horns of the pregnant rat. The fetal heads were then separated from the body and placed in a Petri dish containing cold 70% ethanol, then moved to 0.15 M ice-cold saline solution containing 50 µg/ml gentamycin. The heads were then transferred to Hank’s Balanced Saline Solution (HBSS) (without Ca$^{2+}$ and Mg$^{2+}$) under the dissecting microscope. Using forceps, the two cortical lobes were separated from the cerebellum, olfactory bulb, hypothalamus and meninges. The cortex pieces were then placed into a sterile tube containing three milliliters of 0.1% Trypsin in HBSS and gently inverted and allowed to stand for 15 minutes. The trypsin solution was removed and the cells were washed with 6-7 ml of fresh HBSS (Appendix A). The HBSS wash is removed and 3 ml of 0.1% soybean trypsin inhibitor added to the cells. The cells were triturated by pipetting up and down through a series of pipettes with fire polished increasingly narrow openings. Cell density was then checked with a 1:10 dilution of the sample in a hemocytometer.

About 10 µl to 50 µl of the cell suspension was added to each well of cell culture plates coated with polyethyleneimine coating solution (Appendix A). In 24-well plate, 0.5 ml MEM$^+$ (Appendix A) was already added to each well; in 6-well plate, 2 ml was already added to each well so that the cell density was kept low or high. For rectangular coverslips (22 x 40 mm, Warner Instruments, No.1 Cover Glass, CT), vacuum grease (Dow Corning, MI) was put on the four sides of the coverslip to keep the cells in the
grease dam. 0.5 ml MEM+ was added to each coverslip to maintain cell metabolism. Cells were then allowed to attach to the bottom of the plate for 3-6 hours at 37 °C, and then the media is changed to 0.5 ml of Neurobasal media (Appendix A) and incubated overnight. After one day, neurons were treated for 48 hours with 1 µM Ara-C to inhibit the proliferation of non-neuronal cells. Neurons were maintained in Neurobasal media (Gibco BRL) with B27 supplement (Gibco BRL) and L-glutamine.

2. Measurement of intracellular Zn$^{2+}$ changes in cultured cortical neurons

Microfluorometry was used so that changes in intracellular free Zn$^{2+}$ could be tracked in individual neurons. Intracellular free Zn$^{2+}$ concentration was monitored using a Zn$^{2+}$ specific fluorophore, FluoZin3 (Kd$_{Zn^{2+}}$~15nM, Invitrogen). Cultured neurons were preloaded with 10 µM FluoZin3 and 1% pluronic acid F127 in Locke’s buffer (Appendix A) for 30 minutes. The neurons grown on rectangle coverslips were then placed in a perfusion chamber (Warner Inst., model RC-30HV), so that the media could be changed and the effects could be observed in real time (Lin et al., 2007). The rate of perfusion was held at approximately 0.5ml per minute for all buffers. Neurons were examined with a 60X oil objective (Plan Apo/1.40 oil DIC, Nikon) using an inverted epifluorescence microscope (Nikon, Diaphot 300) and FITC filter (Chroma 41001). Fluorescent images were captured with a CCD camera (Spot, RT ES, model 9.1 Monochrome w/IR -6). Neutral density filters were applied to decrease photobleaching. For each coverslip, three different regions were observed. Each region contained three to ten neurons. After
perfusion with each treatment, the same three regions were observed in sequence and the fluorescence images captured every minute. Each experiment was replicated several times usually using different preparations of cortical neurons from different rats (n equals the number of neurons used for data analysis). Changes in intracellular FluoZin3 fluorescence are quantified using standard image analysis software tools (MetaMorph). On each coverslip, the soma of each cell was traced and the average intensity of the fluorescence within the trace was determined. The trace outline was then copied onto all subsequent images of the experiment and the average intensity of each cell was determined similarly. The control was obtained by perfusing the cells with low calcium Locke’s buffer containing 100 µM EDTA for the entire length of the experiments. The low calcium concentration minimizes the potential effects of calcium influx on FluoZin3 fluorescence (Colvin et al., 2008). The perfusion with EDTA resulted in a decrease in fluorescent intensity, this most likely due to Zn\(^{2+}\) efflux from the neurons and photobleaching. After 5 and 10 min perfusion with 100 µM EDTA alone, the fluorescence intensity decreased to 90.69±0.50% (mean±SEM) and 84.05±0.56% respectively compared to the beginning fluorescence level. For this reason, data obtained from co-perfusion with EDTA were corrected by the formula: 

\[ \text{F}_{\text{corrected}} = \frac{\text{F}_{\text{observed}}}{\text{Ratio}_{\text{EDTA}}} \]

(Ratio\(_{\text{EDTA}}\) was the ratio of fluorescent intensity changes before and after the EDTA perfusion. The value equals 0.91 and 0.84 for 5 min and 10 min EDTA perfusions respectively), to eliminate EDTA effects.

Fluorescent images were captured and saved as 16 bit TIFF uncompressed files with no post processing. Using MetaMorph (Version 4.6) software, the cell body of each
neuron was outlined (region of interest) and the average pixel intensity was obtained. The background average pixel intensity of the same size on the same image was also measured. The background average pixel intensity was subtracted from the cellular average pixel intensity.

To compare fluorescent changes across different coverslips, fluorescence intensity was normalized by transforming the data to \( F/F_0 \). \( F_0 \) was the average pixel intensity of all the cells after perfusion with Locke’s buffer in the three different areas observed on each coverslip minus the average intensity of the same numbers of regions without cells. \( F \) was the background corrected, which is the basal fluorescence intensity on the coverslip without cells, cellular pixel average intensity at various times after different experimental treatments. Also, to compare the effect of different concentrations of exogenous NO, a normalized \( \Delta F_{\text{max}} \) was calculated. To determine the maximum response to each concentration of NO, the largest increase of the three measurements during perfusion with spermine NONOate was selected (\( \Delta F_{\text{max}} \)). The average of \( \Delta F_{\text{max}} \) was converted into percentage increase over \( F_0 \), and then was plotted.

3. **MTT (methyl-thiazolyl-diphenyl-tetrazolium) assay for cell viability**

High-density cortical neuronal cultures grown in 24-well plates were used for this experiment. During the experiment, they were treated with various concentrations of spermine NONOate or ZnCl\(_2\) in Locke’s buffer (pH 7.4) for the same time period as cells on coverslips exposed to the perfusion protocol. After the treatments, Neurobasal medium was added to replace the solutions. 24 h later, the medium was removed and 500 µl of
MTT (Sigma) solution (1:10 diluted in Locke’s buffer from 5mg/ml stock solution) was added and incubated for 1 h at 37 °C. Then the solution was removed and 1 ml DMSO was added and incubated for 5 min at 37 °C. The whole medium was transferred to a cuvette and read the absorbance in the spectrophotometer (Cary 50 Probe, Varian) at 550 nm as test wavelength. The value was subtracted by the reading from a reference wavelength at 650 nm to correct for nonspecific background values.

4. **OGD (oxygen glucose deprivation) treatment**

During experimental treatments, NB was replaced with Locke’s buffer. All the preconditioning treatments were performed at 37°C for 5 min. Where indicated various compounds were included in the Locke’s buffer, or neurons were treated similarly with Locke’s buffer alone (control). Finally, the buffer was removed and the neurons were returned to the incubator in fresh NB media. After 24 hrs, the neurons were exposed to OGD conditions. 1 µM spermine NONOate was made in Locke’s buffer. 125 mM DP-b99 (D-pharm, Israel) was made in ethanol and kept at -20°C for stock. Before usage, the stock DP-b99 was diluted to 5 mM in 10% BSA in 50 mM chelex treated HEPES buffer (pH7.4). Then, DP-b99 was further diluted to different concentrations in Locke’s buffer for preconditioning treatments. 1 mM clioquinol (Cq) was fresh made in methanol. Then it was further diluted to 1 µM in Locke’s buffer for preconditioning treatment. The MAPK inhibitors PD98059 and U0126 (Calbiochem, CA), and the inactive form U0124 (Calbiochem, CA) were made in DMSO for a stock concentration of 10 mM. Then, they were further diluted to 10 µM in Locke’s buffer. The p90
ribosomal S6 kinase (RSK) inhibitor, SL0101-1 (Tocris, MO) was dissolved in DMSO for a stock concentration of 20 mM. Then, it was further diluted to 30 µM in Locke’s buffer. All these inhibitors were added 30 min prior to spermine NONOate or DP-b99 treatments. The inhibitors were present during the 5 min preconditioning treatment and in the media after the preconditioning stimulus until OGD treatment. The NOS inhibitor L-NAME (Cayman chemical, MI) was made as a 10 mM stock solution in water. 50 µM L-NAME in Locke’s buffer was added 30 min prior to DP-b99 treatment. L-NAME was present during 5 min DP-b99 incubation and in the media after incubation until OGD treatment.
To create OGD conditions, neurons were incubated in Locke’s buffer without glucose added and bubbled with 95% N₂/5% CO₂ in a sealed chamber filled with 95% N₂/5% CO₂ at 37°C for 90 min. After OGD treatment, Locke’s buffer was replaced with fresh NB media and neurons returned to the CO₂ incubator as before. Cell viability was evaluated 24 hrs later (Fig. 2). Controls were treated for 90 min Locke’s buffer with the same number of wash and buffer replacements.
Figure 2: The time frame of preconditioning and OGD treatments. On day one, neuronal cells were exposed to different preconditioning stimuli in Locke’s buffer for 5 min. After the pretreatment, cells were returned to fresh NB media. On day two, cells were kept in OGD conditions for 90 min or in Locke’s buffer only for 90 min. The cells were returned to fresh NB media after treatments. On day three, cellular viability was measured by alamar blue.

5. Alamar blue assay for detecting cell survival rate

Twenty-four hrs after OGD treatment, NB media was removed, 500 µl Alamar Blue
(1:10 dilution in NB, AbD, CA) was added to each well and incubated for 6 hrs at 37°C. After 6 hrs, 200 µl of each solution was transferred to a 96-well plate. Fluorescent intensity of the media was determined using a fluorescence spectrometer (FluoMax-3) at 560 nm excitation and 580 nm emission. The excitation and emission wavelength was optimized by scanning the whole region of wavelength for best response. NB with alamar blue incubated at 37°C for 6 hrs was used as background reading. The 100% reduced form of alamar blue was obtained by autoclaving alamar blue in NB media for 15 min. The fluorescent signal of this solution was used as a maximal reading. The percent reduction of alamar blue in each experimental well was obtained using the following formula: Percent reduction of alamar blue = (Fluorescent Intensity (FI) of the media obtained from cells–FI of the background) / (FI 100% reduced alamar blue-FI of the background) X 100. A typical control was obtained from cells incubated with Locke’s buffer (pH7.4) only for 5 min on day 1. On day 2 they were treated with Locke’s buffer (pH7.4) for 90 min. 24 hours later, the cell viability was measured by alamar blue. This reading was used as 100% cell viability to compare with all other experimental runs treated similarly and expressed as percent cell survival rate in each figure. The cellular morphological changes before and after different preconditioning treatments and OGD treatment were checked by light microscope and images collected. Neurons were examined with a 20X objective using an inverted microscope (Olympus, EX-71) and a Hoffman modulation condenser. Images were captured with a CCD camera (Spot, RT ES, model 9.1 Monochrome w/IR -6). The Hoffman modulation condenser was used to show the cellular morphology. Typical views of neuronal morphology before and after various
treatments were taken and saved as 12 bit TIFF uncompressed files.

6. **Analysis of translational activity in cultured neurons**

   Different treatments were applied to the neuronal cells to investigate their effects on the translational activity of the cells. First, neurons were incubated in methionine/cysteine-free minimal essential medium (Invitrogen) for 20 min for starvation. Then the neurons were labeled with Redivue Pro Mix \[^{35}\text{S}]\text{Met/Cys}\ (100 \mu\text{Ci/ml}; 1,000\text{Ci/mmol}; \text{Amersham Biosciences}) for 30 min. After washing with phosphate-buffered saline, cell extracts were prepared by lysing the cells in Nonidet P-40 lysis buffer (Appendix A). The \(^{35}\text{S}\) incorporation was analyzed by SDS-PAGE. For electrophoresis equal amounts of protein from cell lysates were resolved on a 12% SDS-PAGE. The gel was stained with Coomassie Blue (R-250) for 20 min, then prepared for fluorography by treatment with En3Hance (PerkinElmer Life Sciences). The gel was washed in 10% acetic acid/40% methanol overnight. The gel was scanned by the scanner before getting dried. The scanned image was saved. The gel was dried thoroughly then exposed to film in an autoradiography cassette for 1 to 7 days to detect the radiolabeled protein content. The exposed film was scanned and the intensity of the bands were quantified by ImageJ (Version 1.41) software.

7. **BioRad protein assay for measuring protein concentration**

   After different treatments, cells were scraped off in NP-40 lysis buffer or 50 mM HEPES for ICP-OES experiment. Then the cells were homogenized with a micro
homogenizer. Then 10 µl of the cell suspension was added to 790 µl of dH₂O in a tube. Two replications were made for each treatment. 200 µl of BioRad reagent was added and vortexed well. CaryWin UV Simple Reads program and the Spectrophotometer (Cary 50 Probe UV visible, Varian) at wavelength 595.0 nm were used. A blank (200 µl of BioRad reagent added to 800 µl dH₂O) was read and the program zeroed, then the standards 5 µl, 10 µl, 15 µl, 20 µl and 25 µl made from 1 mg/ml Bovine Serum Albumin were read. Again a blank was read, and then the samples were read in duplicate. Using Graphpad Prism 4.0 program, linear regression was performed to obtain the protein concentration.

8. Measurement of total trace elements in neurons by ICP-OES

Neurons were treated with 50 µM DP-b99 or Locke’s buffer for 5 min then frozen in -80 freezer. The neurons were scraped in 50 mM HEPES chelex-treated buffer and two wells were placed into one tube. 10 µl protein samples were taken to measure protein concentration by BioRad assay. The rest samples were desiccated in a vacuum desiccator for overnight. Then 75 µl concentrated HNO₃ was added to each tube overnight. The dissolved solution was heated for 20 minutes. The volume was then brought to 1 ml with 1% HNO₃. Measurements were taken using an UltraMass 700 (Varian Inc.) ICP-OES according to the instrument manuals. A total of seven metals were analyzed using the ICP-OES including aluminum, calcium, copper, iron, magnesium, manganese and zinc. Metal ion concentration in nmol/mg was calculated by dividing ion amount by the protein concentrations.
9. Data analysis

All data plots and appropriate statistical analyses were generated using Graph Pad Prism 4.0 (Graph Pad Software). The data are presented as mean ± SEM. Statistical analysis was performed using one-way ANOVA and post hoc multiple comparison tests. There are some variations between different treatments in OGD experiments. For the results from OGD experiments, the statistical significance of differences was set as P < 0.01. For other results, the statistical significance of differences was set as P < 0.05.
RESULTS

The changes of intracellular Zn\(^{2+}\) induced by low brief exogenous NO exposure

Visualization of intracellular Zn\(^{2+}\) changes in primary cortical neuronal cells with FluoZin-3 AM

Intracellular Zn\(^{2+}\) levels were visualized by the high affinity Zn\(^{2+}\) indicator FluoZin-3 AM. To make sure this indicator is capable of detecting intracellular Zn\(^{2+}\) changes, 25 μM pyrithione, a Zn\(^{2+}\) ionophore, and 100 μM Zn\(^{2+}\) was used as positive control conditions to load cells with large amounts of Zn\(^{2+}\). Images were captured before and after Zn\(^{2+}\) and pyrithione perfusion followed by perfusion with 100 μM TPEN. As expected, the observed intracellular fluorescence intensity increased rapidly and significantly after perfusion with Zn\(^{2+}\) and pyrithione. The intracellular fluorescence levels obtained in Fig.3B would presumably reflect fluorophore saturation. Fluorescence decreased to a level below that of the resting FluoZin-3 fluorescence (Fig.3A) after perfusion with 100 μM TPEN for 5 min (Fig.3C). TPEN is a cell-permeable metal chelator, which should reduce free intracellular Zn\(^{2+}\) to very low levels below the detection limit of FluoZin-3. Presumably then, the fluorescence levels obtained after TPEN perfusion reflect cellular autofluorescence. The decrease in fluorescence observed after TPEN addition confirmed that the increase in fluorescence caused by Zn\(^{2+}\) and pyrithione perfusion was most likely due to increased cellular uptake of Zn\(^{2+}\). The cellular fluorescence showed a uniform distribution in all neurons. There was no evidence
of compartmentalization of the fluorophore in subcellular organelles because the fluorescent in the cells are uniformly distributed.

Figure 3: Represented images of the effects of perfusion with Locke's Buffer, 100 μM Zn$^{2+}$ and 25 μM Pyrithione, and 100 μM TPEN sequentially on FluoZin-3 fluorescence intensity: Coverslips containing cortical neurons were incubated with 10 μM FluoZin-3 and 0.01% pluronic acid (1:1 mixture) for 30 min and mounted in a perfusion chamber. Different treatments were applied to the neuronal cells sequentially. A. 5 min after perfusion with Locke's buffer. B. 5 min after perfusion with Zn$^{2+}$ and Pyrithione. C. 5 min after perfusion with TPEN. Legend bar = 10 μm.

**Addition of extracellular spermine NONOate causes release of intracellular Zn$^{2+}$**

To investigate the effects of extracellular NO on intracellular free Zn$^{2+}$ levels, the NO generator spermine NONOate was used. The NO generator, spermine NONOate has a 1:2 stoichiometry and a $t_{1/2}$ of 39 min for NO production at 37 °C. There is as much as 1 μM contaminating Zn$^{2+}$ present in the Locke’s buffer (154 mM NaCl, 5.6 mM KCl, 2.5 mM CaCl$_2$, 1 mM MgCl$_2$, 5 mM HEPES, 10 mM Glucose; pH 7.4) (determined by ICP-MS, data not shown). This extracellular Zn$^{2+}$ could cross the cellular membrane to cause an intracellular fluorescence increase. To avoid this, 100 μM EDTA, an extracellular metal
chelator, was included in the perfusion solutions to eliminate any possible Zn\(^{2+}\) influx, without changing extracellular Ca\(^{2+}\) or Mg\(^{2+}\) concentrations. Figs. 2 A-C show representative images, while Figs 4D-F show average fluorescence changes observed. The cellular fluorescence was increased during perfusion with 1 µM spermine NONOate (Fig.4B) compared to the perfusion with Locke’s buffer only (Fig.4A). After perfusion with 100 µM TPEN for 5 min (Fig.4C), the cellular fluorescence decreased. Fig.4D illustrates the effect of perfusion with 100 µM EDTA; followed by 1 µM spermine NONOate and finally 100 µM TPEN, averaged over 20 neurons. The effect of spermine NONOate was significant (P<0.001) when compared to cells prior to spermine NONOate perfusion. Since these effects were obtained in the presence of extracellular 100 µM EDTA (Zn\(^{2+}\) influx blocked), the observed increase in intracellular Zn\(^{2+}\) must represent the release of Zn\(^{2+}\) from certain intracellular stores such that the Zn\(^{2+}\) was previously inaccessible to FluoZin-3. Fig. 4E shows that the perfusion of 100 µM EDTA in Locke’s buffer alone did not cause an increase of fluorescence. Thus, perfusion with 100 µM TPEN and the subsequent rapid drop in fluorescence indicated that the increased fluorescence signal was due to changes in intracellular free Zn\(^{2+}\). To demonstrate that the drop in fluorescence intensity was not caused only as a response to removing spermine NONOate, after perfusion with spermine NONOate, EDTA alone was perfused, but no dramatic decrease of fluorescence was observed. Thus, after washout of spermine NONOate, a persistent (at least for 5 min) increase in intracellular free Zn\(^{2+}\) was observed. Fluorescence intensity decreased only when TPEN was perfused subsequently (Fig.4F).
Figure 4: Effect of brief perfusion with 1 µM spermine NONOate: Coverslips containing cortical neurons were prepared as in Fig.1. Different treatments were applied to the neuronal cells sequentially. The images were taken after different treatment for the same region. A. 5 min after perfusion with 100 µM EDTA. B. 5 min after perfusion with 1 µM spermine NONOate. C. 5 min after perfusion with 100 µM TPEN. Images were quantified and normalized to F/F₀ (see methods). Legend bar = 10 µm. Each represented pictures selected from twenty different pictures in six different coverslips. D. The fluorescent changes after perfusion with 1 µM spermine NONOate. After 5 min perfusion with Locke’s buffer, neurons were perfused with 100 µM EDTA. Then, neurons were perfused for 5 min with 1 µM spermine NONOate. Finally, neurons were perfused with 100 µM TPEN for 5 min. E. The fluorescent changes after perfusion with 100 µM EDTA. After 5 min perfusion with Locke’s buffer, neurons were perfused with 100 µM EDTA. Then, neurons were perfused for another 5 min with 100 µM EDTA. Finally, neurons were perfused with 100 µM TPEN for 5 min. F. The washout experiment. After 5 min perfusion with Locke’s buffer, neurons were perfused with 100 µM EDTA. Neurons were perfused with 100 µM EDTA for another 5 min. Finally, neurons were perfused with 100 µM TPEN for 5 min. Each point represents the mean±SEM (n=20 neurons) from three different coverslips from at least two rats.

Next, the inactive analog of spermine NONOate (spermine) was tested. The results show that the cellular fluorescence intensity did not increase after perfusion with 1 µM spermine and 100 µM EDTA (Fig.5A). The fluorescence intensity decreased because of Zn²⁺ efflux and/or photobleaching. This result confirmed that the observed fluorescence increases were likely caused by NO released from spermine NONOate. Was the NO released from spermine NONOate responsible for the observed increase in intracellular fluorescence? To test this, 10 µM hemoglobin, (an NO scavenger that can react with NO at nearly diffusion-limited rates to produce nitrate and iron-nitrosylhemoglobin), was coadministred with spermine NONOate (Herold et al. 2001). The results showed that the fluorescence intensity did not increase after perfusion with 10 µM hemoglobin and 1 µM spermine NONOate together (Fig.5B). Again, the fluorescence intensity decreased
because 100 μM EDTA was present in the perfusion solution. These data confirmed that the fluorescence change observed was caused by NO produced by spermine NONOate only.

Figure 5: Effect of brief perfusion with spermine and NO scavenger. Coverslips were prepared as before. A. After 5 min perfusion with Locke’s buffer, neurons were perfused with 100 μM EDTA. Then, neurons were perfused for 5 min with 1 μM spermine. Finally, neurons were perfused with 100 μM TPEN for 5 min. B. After 5 min perfusion with Locke’s buffer, neurons were perfused for 5 min with 100 μM EDTA. Then, neurons were perfused for 5 min with 10 μM hemoglobin and 1 μM spermine NONOate. Finally, neurons were perfused with 100 μM TPEN for 5 min. Each point represents the mean±SEM (n=20 neurons) from three different coverslips from at least two rats.
Dose response analysis of extracellular spermine NONOate addition

To determine if the effect of spermine NONOate was dose dependent, neurons were treated with various concentrations of spermine NONOate. The treated neurons showed a concentration-dependent increase in fluorescence (Fig.6A). The fluorescent change observed with 0.5 µM spermine NONOate was significant compared to before the perfusion with spermine NONOate (35.35±9.61% mean ±SEM, p<0.001, n=18). The increase in fluorescence intensity peaked at 1 µM spermine NONOate (123.8±28.47% p<0.001, n=20), and decreased as the spermine NONOate concentration was further elevated. 2 µM spermine NONOate caused an increase of 108.6±28.45% (p<0.001, n=18) and 5 µM spermine NONOate increased the fluorescence by only 67.17±12.27% (p<0.001, n=20). Neurons were perfused with 100 µM Zn$^{2+}$ and 25 µM pyrithione after the perfusion with 1 µM spermine NONOate. The fluorescence further increased with the perfusion of Zn$^{2+}$ and pyrithione (Fig.6B). The results show that although 1 µM spermine NONOate produced a maximal fluorescence response, relative to addition of exogenous NO generator, the fluorophore was not saturated, as evidenced by the increase in fluorescence observed when Zn$^{2+}$ and pyrithione were subsequently added. This signal was completely reversed by perfusing with 100 µM TPEN. Higher concentrations of spermine NONOate (1 mM) were tested, but saturation of fluorophore was not observed since the addition of 100 µM Zn$^{2+}$ and 25 µM pyrithione produced similar further fluorescence increase (Fig.6C).
Figure 6: A: Dose effect of spermine NONOate. Each bar represents the average maximal response (see methods) after perfusion with 0.5, 1, 2 and 5 µM spermine NONOate (n > 18). B: Neurons were perfused with 100 µM EDTA for 5 min. Next, neurons were perfused with 1 µM spermine NONOate for 5 min. Then, neurons were perfused with 100 µM Zn^{2+} and 25 µM pythione. Finally, neurons were perfused with 100 µM TPEN. Each point represented the mean±SEM (n=18 neurons) from three different coverslips. C: Neurons were perfused with 100 µM EDTA for 5 min. Next, neurons were perfused with 1 mM spermine NONOate for 5 min. Then, neurons were perfused with 100 µM Zn^{2+} and 25 µM pythione. Finally, neurons were perfused with 100 µM TPEN. Each point represented the mean±SEM (n=20 neurons) from three different coverslips.

Brief treatment with extracellular spermine NONOate did not cause cell death

To determine whether the treatments used for the experiments described were neurotoxic, a cell viability assay with MTT was performed. The high-density cortical neuronal cultures were incubated under various conditions and times, then the medium was changed back to NB media and the neurons were incubated overnight at 37 °C. The next day the MTT assay was performed to quantify viable cells. Since the treatment with Locke’s buffer for 3 h did cause a significant decrease in cell viability (78.04±2.64%, p<0.01, n=3) (Fig.7), longer time exposures to spermine NONOate were compared to the same period treated with Locke’s buffer. No significant increase in cell death occurred after treatment with 1 µM spermine NONOate (95.99±2.76%, n=3) for 3 h, but 1 mM spermine NONOate for 3 h did show a significant increase in cell death (58.85±5.34%, n=3, p<0.001). These data suggested that the incubation with 1 µM spermine NONOate did not trigger apoptosis pathways in neurons, instead certain physiological pathways might be activated after the exposure. Incubation in 1 mM Zn^{2+} overnight showed nearly complete death of all neurons compared to the cells incubated in NB media (5.23±0.95%, n=6, p<0.001).
Figure 7: Cell death after different concentrations of NO generator treatments. High-density cortical neuronal cultures were treated with 1 µM and 1 mM spermine NONOate and 100µM TPEN and 1mM Zn$^{2+}$ for different time periods and then incubated with NB media overnight. The MTT assay was performed the next day to assess the amount of cell death. There was no statistically significant increase in cell death for 1 µM NO generator treated with 5 min or 3 hrs. Only 1mM NO generator treated with 3 hrs showed significant cell death. 1mM Zn$^{2+}$ incubation overnight caused nearly complete cell death (n≥4).
**Induction of Tolerance to OGD by Exogenous NO**

**Brief pretreatment with spermine NONOate protected neurons from subsequent OGD treatment**

Several studies have suggested that exposure to sublethal stimuli can induce tolerance to ischemic injury (Kitagawa et al., 1990; Gonzalez-Zulueta et al., 2000). NO is a key mediator in processes leading to tolerance against lethal ischemia. In my previous studies, brief exposure of exogenous NO caused intracellular Zn\(^{2+}\) increase. To determine if this Zn\(^{2+}\) increase is involved in the mechanism of tolerance caused by NO, OGD was used as an in vitro model to test ischemic injury. Neurons were pretreated with 1 µM spermine NONOate in Locke’s buffer (pH 7.4) containing 100 µM EDTA for 5 min to induce tolerance to OGD. 100 µM EDTA was added to prevent any actions of extracellular Zn\(^{2+}\). After 24 hrs, the neurons were exposed to OGD treatment for 90 min. Cell survival could be qualitatively determined by simple visual inspection using an inverted microscope. Images were captured just after 5 min incubation in Locke’s buffer with or without addition of 1 µM spermine NONOate (Fig. 8A&B), after 90 min OGD (Fig. 8C&D) or 24 hrs after a 90 min OGD treatment (Fig. 8E&F). Pretreatments had no immediate visible effect on neuron morphology or survival. However 24 hrs after OGD treatment, neurons pretreated (24 hrs prior to OGD treatment), with Locke’s buffer for 5 min, showed significant cell death (Fig 8E). On the other hand, the neurons pretreated with NO for 5 min did not show significant cell death 24 hrs after OGD treatment (Fig 8F).
Figure 8: Visual inspection of the difference in neuron survival after induction of tolerance to OGD. A. Day 1 after 5 min Locke’s (pH 7.4) buffer incubation. B. Day 1 after 5 min 1 µM spermine NONOate incubation. C. Day 2 after 90 min OGD treatment from day 1 in Locke’s buffer incubation. D. Day 2 after 90 min OGD treatment from day 1 in spermine NONOate incubation. E. Day 3 24 hrs after 90 min OGD treatment from day 1 in Locke’s buffer incubation. F. Day 3 24 hrs after 90 min OGD treatment from
day 1 in spermine NONOate incubation. The Hoffman modulation condenser was used to show the cellular morphology. Different represented regions were selected after different treatments. Size bar: 10 µm.

Neuron survival was quantified by using alamar blue reduction. Alamar blue is a sensitive oxidation-reduction indicator that fluoresces and changes color upon reduction by living cells. Studies showed that alamar blue was more sensitive to detect cell viability compared to MTT assay (Hamid et al., 2004). In all experiments, visual inspection confirmed that experimental wells contained similar cell densities and the differences observed in alamar blue reduction were not the result of the original plating density. A 5 min preincubation with 1µM spermine NONOate nearly completely prevented the cell death caused by 90 min of OGD (Fig. 9A). The coadministration of 10 µM TPEN and NO generator blocked the induction of tolerance to OGD suggesting intracellular Zn\(^{2+}\) was necessary for the induction of tolerance to OGD. The effects were obtained in the presence of 100 µM EDTA (extracellular Zn\(^{2+}\) chelator), thus ruling out any effect of extracellular Zn\(^{2+}\). The effect of TPEN was concentration dependent. Tolerance to OGD was prevented by 10 µM and greater TPEN but not by lower concentrations (Fig. 9B). The inactive analog of spermine NONOate, spermine (Fig. 9A) or DMSO was without effect (data not shown). The coadministration of NO scavenger hemoglobin also blocked the induction of tolerance to OGD. Since in our previous observations, the exposure of neurons to NO caused an increase of intracellular free Zn\(^{2+}\), I next tested if an increase in intracellular Zn\(^{2+}\) concentration was sufficient to induce tolerance to OGD in the absence of NO.
Figure 9: A. Demonstration of the induction of tolerance to OGD by treatment of cortical neurons with 1 µM spermine NONOate for 5 min. Cell viability was measured by alamar blue as described in methods. Induction of tolerance to OGD was not affected by addition of 100 µM EDTA to the Locke’s buffer. Spermine showed no effect on tolerance. The coadministration of hemoglobin with spermine NONOate blocked the induction of tolerance to OGD. B. The induction of tolerance to OGD was prevented by the coadministration of either 10 or 100 µM TPEN for 5 min. Induction was not affected by addition of 1 and 3 µM TPEN. All experimental treatments were compared to the treatment with Locke’s buffer containing 100 µM EDTA. The difference in cell survival rate was considered significant when \( p \leq 0.01 \) and marked by asterisks. Each column represents a mean obtained from cells from at least two different rats \((n \geq 5)\).

**Evidence that intracellular Zn\(^{2+}\) was necessary but not sufficient for the induction of tolerance to OGD by exogenous NO**

In previous studies, 5 min treatment with 10 µM Zn\(^{2+}\) or coadministration of 10 µM Zn\(^{2+}\) and 5 µM pyrithione (Zn\(^{2+}\) ionphore) resulted in measurable increases in intracellular Zn\(^{2+}\) levels in cultured cortical neurons (Colvin et al., 2008). The coadministration of 10 µM Zn\(^{2+}\) and 5 µM Zn\(^{2+}\) ionphore pyrithione did not induce tolerance to OGD showing that although intracellular Zn\(^{2+}\) was necessary for NO induction of tolerance to OGD, increases in intracellular Zn\(^{2+}\) alone were not sufficient to induce tolerance in the absence of NO (Fig. 10). Coadministration of TPEN and spermine NONOate blocked the induction of tolerance to OGD by spermine NONOate. Next, it is tested if TPEN was effective only after coadministration with NO. 10 µM TPEN was added to the neurons for 5 min at different time points after preincubation with spermine NONOate. TPEN could still effectively block the induction of tolerance to OGD when added as long as 6 hrs after the addition of spermine NONOate. However, longer delays (12 or 24 hrs) resulted in a loss in the ability of TPEN to block the induction of tolerance...
to OGD (Fig. 10). When 10 µM Zn\(^{2+}\) was coadministered with 10 µM TPEN, the actions of TPEN were blocked (Fig. 11). This finding further supports the contention that Zn\(^{2+}\) was necessary for the induction of tolerance to OGD. Since TPEN has high affinity for other metals, it is investigated whether 10 µM Cu\(^{2+}\) or Fe\(^{2+}\) alone caused any protective effects or if when coadministered with 10 µM TPEN had the ability to block the actions of TPEN. There is no effect of administration of Cu\(^{2+}\) or Fe\(^{2+}\) alone to induce the tolerance to OGD. The coadministration of Cu\(^{2+}\) or Fe\(^{2+}\) showed no effect on the actions of TPEN (Fig. 11).
Figure 10: Increased intracellular Zn\(^{2+}\) alone was ineffective at inducing tolerance to OGD. No tolerance to OGD was observed after neurons were treated with either 10 µM Zn\(^{2+}\) or 10 µM Zn\(^{2+}\) with 5 µM Zn\(^{2+}\) ionphore pyrithione for 5 min. The action of TPEN to block the induction of tolerance to OGD did not require coadministration with spermine NONOate. The protective effect of 1 µM spermine NONOate after 90 min OGD was blocked by TPEN when administrated even 6 hrs after preconditioning. All the treatments were present in the media for 5 min but the time of addition varied. All the treatments were compared to the cells treated with Locke’s buffer containing 100 µM EDTA for 5 min then with 90 min OGD. 100 µM EDTA was present in all the conditions except Zn\(^{2+}\) and Zn\(^{2+}\) with Pyrithione. The difference in cell survival rate was considered
significant when $p \leq 0.01$ and marked by asterisks. Each column represents a mean obtained from cells from at least two different rats ($n \geq 8$).

Figure 11: Addition of 10 µM Zn$^{2+}$ blocked the effects of 10 µM TPEN. The addition of 10 µM Cu$^{2+}$ or Fe$^{2+}$ alone did not induce tolerance to OGD. The coadministration of 10 µM Cu$^{2+}$ or Fe$^{2+}$ with 10 µM TPEN showed no effect on the actions of TPEN. All the treatments were compared to the treatment with Locke’s buffer containing 100 µM EDTA for 5 min then with 90 min OGD. The difference in cell survival rate was
considered significant when \( p \leq 0.01 \) and marked by asterisks. Each column represents a mean obtained from cells from at least two different rats (\( n \geq 7 \)).

The universal protein synthesis inhibitor CHX blocked induction of tolerance to OGD

Although the cellular mechanisms underlying the induction of neuronal tolerance to OGD are still in question, the synthesis of new protein is required for this process (Matsuyama et al, 2000; Puisieux et al, 2004). The effect of addition of the universal translation inhibitor CHX was tested. The results showed that the presence of 10 \( \mu \)M CHX in the NB media during the period between NO exposure and OGD treatment prevented the induction of tolerance to OGD (Fig. 12). The critical steps requiring protein synthesis occur between 0 hr and 12 hrs after NO pretreatment since the addition of CHX 12 hrs after NO pretreatment could not block the induction of tolerance. However, the addition of CHX 0 to 6 hrs or 6 to 12 hrs both prevented the induction of tolerance to OGD (Fig. 12). The effects of CHX are rapidly reversed by just simply removing it from the media. These results strongly suggest that new protein synthesis was required for the induction of tolerance to OGD triggered by addition of exogenous NO. No significant effect of CHX in the absence of OGD was observed on cell viability.
Figure 12: 10 µM CHX, a translation inhibitor, blocked the induction of tolerance to OGD by 1 µM spermine NONOate. CHX was added immediately or at different time points after spermine NONOate and was present in the NB media as indicated by the labels in the figure. All the treatments were compared to the treatment with Locke’s buffer containing 100 µM EDTA. The difference in cell survival rate was considered significant when p≤0.01 and marked by asterisks. Each column represents a mean obtained from cells from at least two different rats (n≥8).
The addition of TPEN inhibited new protein synthesis

It has been shown that the protein synthesis inhibitor, CHX reversed the tolerance by NO. In the mean time, the effect of TPEN to reverse tolerance to OGD showed similar time pattern with CHX. This suggested that TPEN might share similar mechanism with CHX to reverse the tolerance to OGD. To investigate whether TPEN can inhibit translation, a $^{35}$S incorporation assay was used to quantify the rate of nascent protein synthesis. Neurons were preexposed to 10 µM CHX or 10 µM TPEN for 5 min. Then the cells were metabolically labeled with $[^{35}\text{S}]$ Met/Cys in a methionine/cysteine-free minimal essential medium with the same concentration of CHX or TPEN present. After washing with PBS, neurons were lysed. The same amount of protein was loaded and analyzed by SDS-PAGE. The nascent protein synthesis was determined by autoradiography. 10 µM CHX incubated for 5 min completely inhibited new protein synthesis. The incubation of 10 µM TPEN for 5 min also showed inhibition of nascent protein synthesis (Fig. 13), but not to the same degree.
Figure 13: Neurons were exposed to different treatments for 5 min. Then, the total cell extracts from 5 min 10 µM CHX (C), 10 µM TPEN (T), Neuro basal (N) and DMSO (D) treatment and [³⁵S]Met/Cys pulse-labeled neurons were resolved by 12% SDS-PAGE, stained with Coomassie Blue R-250 (upper left panel), treated with Enhance (PerkinElmer Life Sciences), and dried. The nascent protein synthesis was determined by autoradiography (upper right panel). A, Total protein levels for different treatments; B, The new protein synthesis level of different treatments; C, The relative average intensity of DMSO, TPEN and CHX. The intensity of different treatments was quantified by
Image J. The mean signal intensity of DMSO treatment was set as 100%. TPEN treatment significantly decreased new protein synthesis (P<0.05). The CHX treatment showed no new protein synthesis activity (P<0.01) (n=3).

The effect of TPEN to inhibit protein synthesis could be blocked by coadministration of zinc

To further investigate the effect of TPEN to inhibit translation, neurons were preexposed to 1 µM spermine NONOate, or coadministration with 1 µM spermine NONOate and 10 µM TPEN or 10 µM TPEN and 10µM Zn$^{2+}$ for 5 min. Then, cells were back into NB media in the incubator. Four hrs later, cells were metabolically labeled with [$^{35}$S] Met/Cys in a methionine/cysteine-free minimal essential medium. After washing with PBS, cells were lysed in lysis buffer. The protein concentrations were checked by BioRad protein assay. The same amount of protein was loaded and analyzed by SDS-PAGE. The nascent protein synthesis was determined by autoradiography. 10 µM TPEN incubated for 5 min showed inhibition of new protein synthesis compared to the control with DMSO addition. The coadministration of 10 µM TPEN and 10 µM Zn$^{2+}$ for 5 min completely blocked the effect of TPEN (Fig. 14).
Figure 14: Neurons were exposed to different treatments for 5 min. Four hrs later, the total cell extracts from $^{35}$S Met/Cys pulse-labeled neurons were resolved by 12% SDS-PAGE, stained with Coomassie Blue R-250, treated with En3Hance, and dried. The nascent protein synthesis was determined by autoradiography. A, Total protein levels for different treatments; B, The new protein synthesis level of different treatments; C, The relative average intensity of NO generator with DMSO, TPEN and TPEN&Zn. The intensity of different treatments was quantified by Image J. The mean signal intensity of DMSO treatment was set as 100% (n=2).
The protective effect by exogenous NO was prevented by ERK1/2 and RSK inhibitors

Previous reports indicate that ERK1/2 signaling may be important for the development of ischemic preconditioning. Hypoxia stimulates rapid ERK phosphorylation in the cortex of rats in a NMDA receptor-dependent manner (Gozal et al., 1999). Increased phosphorylation of ERK has been observed in the CA1 region of the hippocampus in a rat model of global cerebral ischemic preconditioning (Shamloo et al., 1999). Other research has demonstrated that all of the ERK signaling mediators, Ras, Raf, Mek and ERK are required for the development of tolerance to ischemia (Gonzalez-Zulueta et al., 2000). Two ERK1/2 specific inhibitors U0126 and PD98059 were applied to test whether the ERK1/2 pathway was involved in the induction of ischemic tolerance triggered by spermine NONOate. 10 µM U0126 and 10 µM PD98059 were added 30 min prior to the preconditioning stimulus and present in the media during and after the preconditioning stimulus until OGD treatment to block the activation of ERK1/2 signaling pathways. The inactive analog of U0126, U0124 was added in the same manner as U0126. Both U0126 and PD98059 prevented the induction of ischemic tolerance triggered by 1 µM spermine NONOate (Fig. 15). But the inactive analog of U0126, 10 µM U0124, failed to reverse the induction of tolerance. These results implied that the activation of ERK1/2 signaling pathways was a necessary step for the induction of tolerance to OGD triggered by addition of exogenous NO. A substrate for ERK1/2 phosphorylation includes RSKs. An inhibitor of RSK, SL0101-1, was tested. 30 µM SL0101-1 was added 30 min prior to the preconditioning stimulus and present in the
media during and after the preconditioning until OGD treatment. SL0101-1 prevented the induction of tolerance caused by spermine NONOate (Fig. 15).
Figure 15: 10 μM U0126 and PD98059, inhibitors of ERK1/2, prevented the induction of tolerance caused by 1 μM spermine NONOate. 10 μM U0124, the inactive analog of U0126 failed to prevent the protective effect. 30 μM SL0101-1, an inhibitor of RSK prevented the induction of tolerance caused by 1 μM spermine NONOate. The difference in cell survival rate was considered significant when $p \leq 0.01$ and marked by asterisks. Each column represents a mean obtained from cells from at least two different rats ($n \geq 10$).
Studies of the duration of exogenous NO-induced tolerance to OGD

Although pretreatment with exogenous NO 24 hrs before OGD induced a robust tolerance to OGD, whether 24 hrs is necessary or optimum to induce tolerance is still unknown. What is the time point for the neurons most tolerated to OGD was investigated. Neurons were treated with 1 µM spermine NONOate for 5 min. Then, the cells were placed back into the incubator for 1, 12, 24, 48 and 72 hrs before 90 min OGD. Cell viability was checked 24 hrs after OGD. At 12, 24, 48 72 hrs before 90 min OGD, the cell viability was increased with incubation with spermine NONOate compared to the incubation with Locke’s buffer only. The cell viability was not increased by incubation with 1 µM spermine NONOate 1 hr before OGD. Statistical analysis was carried to test the significance of time effect. It was shown that only exposure to spermine NONOate 24 hrs before 90 min OGD resulted in significant protection (Fig. 16). The 12, 48 and 72 hrs all showed significant difference compared to the pretreatment 24 hrs before OGD. These data indicate that the addition of spermine NONOate showed tolerance to OGD from 12 hrs after the preconditioning stimulus and until 72 hrs. But the tolerance effect reached a maximal at 24 hrs after exposed to NO and decreased after 24 hrs.
Figure 16: 1 µM spermine NONOate was added at different times before 90 min OGD. Cell viability increased for the exposure of exogenous NO at 72, 48, 24 and 12 hrs before 90 min OGD compared to Locke’s buffer only treatment. Only the exposure to spermine NONOate at 24 hrs before OGD treatment did not cause significant cell death. The difference in cell survival rate was considered significant when \( p \leq 0.01 \) and marked by asterisks. Each column represents the cells from at least two different rats (\( n \geq 8 \)).

**Summary of NO-induced tolerance to OGD**

In this study, intracellular \( \text{Zn}^{2+} \) was involved in the mechanism of exogenous NO-induced tolerance to OGD. It was shown in these experiments that the heavy metal chelator TPEN reversed the tolerance caused by NO. The effect of TPEN was inhibited by addition of same amount of \( \text{Zn}^{2+} \) but not by \( \text{Fe}^{2+} \) or \( \text{Cu}^{2+} \). The addition of TPEN showed inhibition to tolerance induced by NO even applied to neurons 6 hrs after
pretreatment. The protein synthesis inhibitor CHX also blocked the tolerance to OGD and showed similar time pattern as TPEN. A $^{35}$S incorporation assay showed that TPEN significantly inhibited nascent protein synthesis and confirmed that the effect of TPEN to block tolerance could be explained at least in part by an inhibition of translation. The downstream pathway of NO-induced tolerance was confirmed to be ERK1/2-RSK pathway. The coincubation of ERK1/2 inhibitors reversed the induction of tolerance by NO. The downstream mediator of ERK1/2 cascade, RSK was first found to be required for the induction of tolerance by NO since the coincubation of RSK inhibitor was found to block induction of tolerance.

Small lipophilic metal chelators induced tolerance to OGD in cultured cortical neurons

The tolerance to OGD induced by DP-b99 and Cq

In this study, neurons were pretreated for 5 min with 50µM DP b-99 (125mM stock in ethanol, diluted to 5 mM in 10%BSA in 50 mM chelex treated HEPES buffer) or 1 µM Clioquinol (Cq) (1mM in methanol) in Locke’s buffer 37°C for 5 min as preconditioning stimuli to induce ischemic tolerance. Then, 24 hours later neurons were exposed to OGD for 90 min as described before. The cell survival rate was measured by alamar blue 24 hrs after OGD. The results show that DP-b99 and Cq are both highly efficacious small molecule inducers of ischemic tolerance in cultured neurons (Fig. 17).
Figure 17: The induction of tolerance by 5 min pretreatment with 1 µM Clioquinol and 50 µM DP-b99 to 90 min OGD in neurons. The cell survival rate was measured by alamar blue as described in methods. The difference in cell survival rate was considered significant when $p \leq 0.01$ and marked by asterisks. Each column represents a mean obtained from cells from at least two different rats ($n \geq 10$).

Once again, 100 µM EDTA was added to prevent any actions of extracellular Zn$^{2+}$.

The coadministration of 10 µM TPEN completely blocked the induction of tolerance by
DP-b99 and Cq suggesting intracellular Zn\(^{2+}\) was necessary for the induction of tolerance to OGD (Fig. 18). These results, which are similar to that seen with exogenous NO, suggest that DP-b99 and Cq might share certain steps with NO to trigger tolerance to OGD.

Figure 18: The induction of tolerance by pretreatment with 50 µM DP-b99 and 1 µM Cq to 90 min OGD was prevented by coadministration with 10 µM TPEN. The induction of
tolerance was not affected by coadministration with 100 µM EDTA. The difference in cell survival rate was considered significant when \( p \leq 0.01 \) and marked by asterisks. Each column represents a mean obtained from cells from at least two different rats (\( n \geq 10 \)).

**The universal protein synthesis inhibitor CHX blocked the induction of ischemic tolerance by DP-b99**

The effect of addition of the universal translation inhibitor CHX was tested. The results show that the presence of 10 µM CHX in the NB media during the period after DP-b99 exposure and OGD treatment prevented the induction of ischemia tolerance to OGD (Fig. 19). Also, the addition of CHX 12 hrs after DP-b99 pretreatment could not block the induction of ischemic tolerance just like that seen with NO induction. The addition of CHX right before OGD showed no effect on the induction of ischemic tolerance as well. These results imply that new protein synthesis was required for the induction of tolerance to OGD triggered by addition of DP-b99.
Figure 19: 10 µM CHX, a protein synthesis inhibitor prevented the induction of tolerance by 50 µM DP-b99 to 90 min OGD. CHX was added at different times after preconditioning stimulus and present in the media until OGD treatment. Addition of CHX 12 hrs after preconditioning stimulus did not affect induction of tolerance suggesting that the induction of ischemic tolerance by DP-b99 was complete at 12 hrs. The addition of CHX right before OGD did not prevent the induction of tolerance. The difference in cell survival rate was considered significant when p≤0.01 and marked by asterisks. Each column represents a mean obtained from cells from at least two different rats (n≥8).
The dose dependence effects of DP-b99 to induce tolerance

As a metal chelator, how DP-b99 binds and transports metal ion across plasma membrane is still unknown. 50µM DP-b99 (125mM stock in ethanol, diluted to 5 mM in 10%BSA in 50 mM chelex treated HEPES buffer) was diluted in Locke’s buffer or Ca\(^{2+}\) free Locke’s buffer. The aggregation induced light-scattering was visible in the regular Locke’s buffer, higher concentration of DP-b99 induced more light scattering (Fig.20). There is no visible aggregation of all different concentration of DP-b99 in Ca\(^{2+}\) free Locke’s buffer. The light-scattering of different concentrations of DP-b99 was also tested by spectrometry. The data from the spectrometer confirmed the results that DP-b99 was shown to exhibit visible aggregation dependent on the concentration of DP-b99 added in Locke’s buffer (Fig. 21). Whereas in Ca\(^{2+}\) free Locke’s buffer, even 100 µM DP-b99 failed to cause any aggregation. This result confirmed the ability of DP-b99 to bind Ca\(^{2+}\) and form aggregates in solution. This result also gave possible hints for the mechanism of DP-b99 induced tolerance to OGD.
Figure 20: Digital images of DP-b99 in Locke’s buffer or Ca\(^{2+}\) free Locke’s buffer. A. Different concentrations of DP-b99 in regular Locke’s buffer containing 2.3mM Ca\(^{2+}\). B. Different concentrations of DP-b99 in Ca\(^{2+}\) free Locke’s buffer. In regular Locke’s buffer, DP-b99 was shown to exhibit visible aggregation dependent of the concentration of DP-b99 added in Locke’s buffer. But in Ca\(^{2+}\) free Locke’s buffer, all concentrations of DP-b99 failed to show any visible aggregation.
Figure 21: The light-scattering analysis of Ca\(^{2+}\) dependent of DP-b99 aggregation in Locke’s buffer or Ca\(^{2+}\) free Locke’s buffer. All concentrations of DP-b99 tested in Locke’s buffer showed some level of light-scattering. The most light-scattering was observed with 100 µM DP-b99 in Locke’s buffer. In Ca\(^{2+}\) free Locke’s buffer, even 100 µM DP-b99 failed to show aggregation. The light-scattering level is stable for at least 5 min.

To determine the concentration dependence of the protective effect induced by DP-b99, neurons were pretreated with 10, 30, 50 and 100 µM DP-b99. 30 and 50 µM both showed an increase in cell viability, but only the effects caused by 50 µM DP-b99 showed significant tolerance to OGD (Fig. 22). Previous studies have shown that DP-b99 is a membrane-active lipophilic BAPTA diester able to chelate divalent metal ions (Angel et al., 2002). Besides the chelating ability to Cu\(^{2+}\), Zn\(^{2+}\) and Fe\(^{2+}\), DP-b99 was also found to mediate the transfer of Ca\(^{2+}\) ions from aqueous to organic phase. The difference of
protective effects by different concentrations of DP-b99 might be caused by the \( \text{Ca}^{2+} \) ionophoric activity at the plasma membrane. It was shown that higher concentrations of DP-b99 in the solution would cause more aggregates in the solution with \( \text{Ca}^{2+} \). Thus, lower concentrations of DP-b99 failed to induce tolerance to OGD. But when the concentration of DP-b99 is too high, such as 100 µM, DP-b99 might likely also work as TPEN to chelate intracellular \( \text{Zn}^{2+} \). This will prevent the induction of tolerance.

![Figure 22](image)

Figure 22: The induction of tolerance by pretreatment with 10, 30, 50 and 100 µM DP-b99 to 90 min OGD in neurons. The cell survival rate was measured by alamar blue as described in methods. The difference in cell survival rate was considered significant when \( p \leq 0.01 \) and marked by asterisks (n\geq 10). Each column represents a mean obtained...
from cells from at least two different rats.

**The protective effects by DP-b99 and Cq were affected differently by extracellular Ca$^{2+}$**

Recently published experiments from our laboratory (Colvin et al., 2008) suggested that lipophilic metal chelators like DP-b99 and Cq can have ionophoric actions. To test whether Ca$^{2+}$ entry was required for the action for DP-b99 and Cq, neurons were exposed to DP-b99 and Cq in Ca$^{2+}$ free Locke’s buffer and Locke’s buffer (containing 2.3mM Ca$^{2+}$). The results showed that in Ca$^{2+}$ free Locke’s buffer, DP-b99 was ineffective at inducing ischemic tolerance (Fig. 23). Since 100 µM EDTA was present in the buffer to chelate extracellular Zn$^{2+}$, there is no evidence showing that DP-b99 requires extracellular Zn$^{2+}$ to induce ischemic tolerance. On the other hand, although there were slight differences observed between Cq induced tolerance and Locke’s buffer and Ca$^{2+}$ free Locke’s buffer, there are no statistical differences between these treatments. Cq showed no dependence on extracellular Ca$^{2+}$ to induce tolerance to OGD. Under the same conditions, exogenous NO also showed no dependence on extracellular Ca$^{2+}$ to induce tolerance to OGD (data not shown). But the coadministration of TPEN could prevent the induction of tolerance to OGD. These results suggested that Ca$^{2+}$ entry is required for DP-b99 induced ischemic tolerance.
DP-b99 and Cq showed different effects on the induction of tolerance to 90 min OGD. DP-b99 could not induce tolerance when extracellular Ca$^{2+}$ was removed. The removal of extracellular Ca$^{2+}$ did not cause any significant effect on the induction of tolerance by Cq to 90 min OGD in the neurons. This suggests that although both compounds showed a requirement for intracellular Zn$^{2+}$, some steps involved in induction of tolerance differ between Cq and DP-b99. The difference in cell survival rate was considered significant when $p \leq 0.01$ and marked by asterisks. Each column represents a mean obtained from cells from at least two different rats ($n \geq 10$).
It has been shown that DP-b99 formed visible aggregates with Ca\(^{2+}\) in Locke’s buffer. To investigate whether the interaction of DP-b99 with Ca\(^{2+}\) would cause the intracellular Ca\(^{2+}\) content to change, neurons were incubated in different concentrations of DP-b99 in Locke’s buffer or Ca\(^{2+}\) free Locke’s buffer for 5 min as a preconditioning stimulus. The cells were then lysed and the protein concentrations were checked by the BioRad protein assay. The total Ca\(^{2+}\) content was analyzed by ICP-OES. Total Ca\(^{2+}\) content was normalized to the protein amount in the sample. As expected, the incubation of DP-b99 in Ca\(^{2+}\) free Locke’s buffer with neurons caused no changes in intracellular Ca\(^{2+}\) content. Only the incubation of DP-b99 in Locke’s buffer significantly increased intracellular Ca\(^{2+}\) content (Fig. 24A). The incubation of DP-b99 failed to change intracellular Zn\(^{2+}\) content (Fig. 24B). Other common metal ions, such as Al\(^{3+}\), Cu\(^{2+}\), Fe\(^{2+}\), Mg\(^{2+}\) and Mn\(^{2+}\) were also tested. None of these ion contents were changed with DP-b99 incubation (Data not shown). This result suggests that although aggregation is related to the presence of Ca\(^{2+}\), the aggregation is not the only factor since Ca\(^{2+}\) uptake does not follow the same trend as light-scattering.
Figure 24: A. The treatment of DP-b99 in Locke’s buffer significantly increased intracellular Ca\(^{2+}\) contents. B. The intracellular Zn\(^{2+}\) content was stable for different concentrations of DP-b99. The difference in cellular ion concentrations was considered significant when p≤0.05 and marked by asterisks. Each column represents a mean obtained from cells from at least two different rats (n=4).

The induction of tolerance by DP-b99 was blocked by ERK1/2 and RSK inhibitors

Two ERK1/2 specific inhibitors U0126 and PD98059 were applied to test whether ERK1/2 activated pathways were involved in the induction of ischemic tolerance. 10 µM U0126 and 10 µM PD98059 were added 30 min prior to the addition of DP-b99 and present in the media during and after the preconditioning stimulus until OGD treatment (the same protocol that was used for NO induced protection). The inactive analog of U0126, U0124 was added in the same manner as U0126. Both U0126 and PD98059 prevented the induction of ischemic tolerance triggered by DP-b99 addition (Fig.24).
However, although the incubation of the inactive analog of U0126, U0124 did show slight decrease of cell survival rate, there is still significant increase of cell survival rate compared to the control. Since the downstream activator of ERK1/2 was involved in the pathway of NO-induced tolerance, 30 µM SL0101-1, the inhibitor of RSK was added 30 min prior to the preconditioning stimulus and present in the media during and after the preconditioning until OGD treatment. SL0101-1 prevented the induction of tolerance caused by DP-b99 (Fig. 25). These results implied that the activation of ERK1/2 and RSK was a necessary step for the induction of tolerance to OGD triggered by addition of DP-b99. Previous studies have shown that intracellular Ca\(^{2+}\) increase could activate NOS and result in the production of endogenous NO. We’ve shown that the 5 min incubation of DP-b99 increase intracellular Ca\(^{2+}\) content. It would be interesting to test whether NO is the downstream target of DP-b99 to induce tolerance. To investigate the effect of NOS inhibitor on the tolerance induced by DP-b99, 50 µM L-NAME was added 30 min prior to the preconditioning stimulus and present in the media during and after the preconditioning until OGD treatment. The addition of L-NAME prevented the tolerance to OGD caused by DP-b99 (Fig. 25).
Figure 25: 10 μM U0126 and PD98059, inhibitors of ERK1/2, prevented the induction of tolerance by 50 μM DP-b99. U0124, the inactive analog of U0126 failed to prevent the induction of tolerance. 30 μM SL0101-1, the inhibitor of RSK prevented the induction of tolerance caused by DP-b99. 50 μM L-NAME was able to prevent the induction
tolerance to OGD caused by DP-b99. The difference in cell survival rate was considered significant when \( p \leq 0.01 \) and marked by asterisks. Each column represents a mean obtained from cells from at least two different rats (\( n \geq 11 \)).
DISCUSSION

I. Intracellular Zn$^{2+}$ increase caused by exogenous NO

In this study, evidence is provided that exposure of cultured neurons to a broad range of concentrations of exogenous NO resulted in a rapid and persistent increase in intracellular free Zn$^{2+}$.

In resting cells, the free Zn$^{2+}$ concentration is within picomolar levels (Krezel & Maret, 2006), but the free Ca$^{2+}$ concentration is in nanomolar range which is much higher than Zn$^{2+}$ (Rizzuto et al., 2002). It was suggested that FluoZin-3 responds robustly to small Zn$^{2+}$ loads with $K_{D_{Zn}}$ is about 15 nM, but is relatively insensitive to high Ca$^{2+}$ or Mg$^{2+}$ (Devinney et al., 2005). In a recent study, it was demonstrated that as high as 10 mM Ca$^{2+}$ did not prevent FluoZin-3 from detecting Zn$^{2+}$ elevations as low as 100 picomolar (Zhao et al., 2008). Previous studies (Horn et al., 2002) have shown that treatment of cultured neurons with 4-10 μM NO produced an increase in intracellular Ca$^{2+}$ levels as detected by Fura-2 fluorescence. In this experimental condition, the possible changes of intracellular Ca$^{2+}$ should have no effect on the observed signal of FluoZin-3. In addition, all observed increases in FluoZin-3 were reversible with 100 μM TPEN, which shows a high degree of selectivity for metals (including Zn$^{2+}$) over Ca$^{2+}$. Thus, the fluorescence changes observed in the present study were most likely to changes in intracellular free Zn$^{2+}$. In the current study, the perfusion of exogenous NO did not induce any significant cell death suggesting that the intracellular Zn$^{2+}$ changes observed are physiologically significant.
It is now well established that several “classical” Ca\(^{2+}\) indicators are sensitive to both Ca\(^{2+}\) and Zn\(^{2+}\) (Thompson et al., 2002; Devinney et al., 2005; Stork & Li, 2006). Similar results (i.e., an increase in intracellular Zn\(^{2+}\)) have been observed after the administration of NO generators to the dorsal hippocampus of intact rats (Cuajungco & Lees, 1998) and these increases were prevented by preadministration of TPEN. It is interesting to note that the Fura-2 fluorescence changes observed by Horn and colleagues could be due to changes in either Ca\(^{2+}\), Zn\(^{2+}\) or both ions. By the fluorescence indicator used in this study, the exposure of exogenous NO is more likely to induce changes of intracellular Zn\(^{2+}\) than Ca\(^{2+}\).

The source of released Zn\(^{2+}\) in the present study is clearly intracellular, since incubation with 100 μM extracellular EDTA had no effect on the observed increase in fluorescence. However, it was observed that the maximal response of FluoZin-3 to exogenous NO did not represent saturation of the fluorophore, since addition of pyrithione/Zn\(^{2+}\) could further increase the observed fluorescent signal. Thus, the rise in intracellular Zn\(^{2+}\) had characteristics of a ceiling phenomenon. What could limit the rise in intracellular Zn\(^{2+}\)? One possibility would be compartmentalization of FluoZin-3 as reported for other fluorescent indicators (Colvin, 2002; Devinney et al., 2005). Previous studies suggest that FluoZin-3 could be used to detect lysosomal labile Zn\(^{2+}\) (Hwang et al., 2008; Chung et al., 2009). The Zn\(^{2+}\) signal could also be reversed by addition of TPEN. The compartmentalization of Zn\(^{2+}\) fluorescent signal might result in the absence of free indicator for available free Zn\(^{2+}\). However, there is no evidence showing that FluoZin-3 is concentrated in certain cytoplasmic compartments, such as lysosomes. In
this study, no evidence of compartmentalization was observed in our experiments. Another possibility is that the intracellular levels of NO are limited in some way. Several studies now suggest that NO exerts much, if not all of its actions, in the form of S-nitrosothiols especially S-nitrosoglutathiones (GSNOs). It has been shown that GSNO can react with MT in a transnitrosation reaction causing the release of Zn$^{2+}$ (Chen et al., 2002). Recently it has been shown that most cells (including neurons) contain GSNO reductase activity (Jensen et al., 1998; Shah et al., 2007), one of the more interesting being protein disulfide isomerase (PDI) activity (Sliskovic et al., 2005). Thus, neurons are likely to self-limit the levels of intracellular GSNO produced by exogenous NO administration, producing an apparent “ceiling” effect. The observed decline in intracellular Zn$^{2+}$ release at 2 and 5 μM spermine NONOate could then be the result of increasing GSNO reductase activity. Finally, it is possible that available Zn$^{2+}$ for release is limited and the “ceiling” effect might represent depletion of intracellular pools. It is not known for certain which MT isoforms are predominately expressed in cultured neurons (Aschner et al., 1997). Thus it is possible that small pools of MT-III (which is particularly sensitive to transnitrosation (Chen et al., 2002) might exist in cultured neurons and be depleted of Zn$^{2+}$ under the conditions produced in these experiments. It was observed that 1 mM NO generator caused increases in intracellular Zn$^{2+}$, similar to that observed by exposure to 1 μM NO generator. It is quite possible that whatever factors were causing a decline in the Zn$^{2+}$ response when NO was raised from 1 to 5μM would be overwhelmed by large amounts of NO. It should be noted that exposure to 1 mM NO still did not produce saturation of FluoZin-3.
Previous studies have investigated the toxic effects of NO on cultured cells and neurons (Zhang et al., 2004; Bossy-Wetzel et al., 2004). This study shows that brief exposures (5 min) to even 1 mM NO generator were not toxic. This observation is in agreement with previous studies that showed that NO toxicity could be at least partially prevented by early chelation of Zn\textsuperscript{2+} but not after a certain amount of time had elapsed (Zhang et al., 2004). The present study showed that 1 mM NO generator was toxic when exposed to cultured neurons for 3 h, but had no effect when the exposure was only 5 min. During long term exposure to NO, it as been suggested that peroxynitrite is formed (Bossy-Wetzel et al., 2004; Mander & Brown, 2005) and increased Zn\textsuperscript{2+} acts to activate 12-LOX and p38 MAPK (Zhang et al. 2004; Bossy-Wetzel et al., 2004). Both 12-LOX and p38 MAPK were shown to be activated by increased Zn\textsuperscript{2+} and increase peroxynitrite and ROS production. Different signaling pathways were activated by the increased Zn\textsuperscript{2+} and both led to apoptotic neuronal cell death. The toxicity caused by increased intracellular Zn\textsuperscript{2+} was blocked by addition of 1 µM TPEN indicating that the change of intracellular Zn\textsuperscript{2+} is in nanomolar range. It is clearly evident from the present study that after a short term exposure (5 min) to a nearly physiological concentration (lower than 10 µM) of NO (with concomitant increases in intracellular Zn\textsuperscript{2+}), these cell death pathways are not activated. Thus, this raises the interesting possibility that increases in intracellular free Zn\textsuperscript{2+} caused by NO, might have a physiological signaling function in neurons. However, it is important to note that for a signaling function to exist, a highly effective mechanism to lower intracellular free Zn\textsuperscript{2+} must exist also. Such mechanisms have yet to be characterized in neurons.
There are many published papers which have characterized the actions of Zn\(^{2+}\) on various signal transduction pathways. For example, Zn\(^{2+}\) induced IL-8 expression or caused AP-1, JNK, and ERK activation in human airway epithelial cells (Kim et al., 2006). ERK activation is also shown by ZIP mediated intracellular Zn\(^{2+}\) in breast cancer cells (Taylor et al., 2008). The addition of micromolar levels of Zn\(^{2+}\) induced phosphorylation of MAPK, which regulated AP-1 and NF-kappa activation in PC-3 human prostate cancer cells (Uzzo et al., 2006). In another study, 100 μM Zn\(^{2+}\) initiates the phosphorylation of p70 S6 kinase and glycogen synthase kinase 3beta cells in SH-SY5Y neuronblastoma cells (An et al., 2005). Zn\(^{2+}\) was used to induce modifications in Na\(^+\)/H\(^+\) exchange and pyruvate kinase activity through protein kinase C in isolated mantle/gonad cells of Mystilus galloprovincialis (Kaloyianni et al., 2005). On the other hand, previous studies also showed that Zn\(^{2+}\) suppressed mitogen-activated IL-2 production (Tanaka et al., 2005). Zn\(^{2+}\) was also shown to suppress IL-6 synthesis by inhibiting phospholipase C and phospholipase D (Hatakeyama et al., 2002).

In this study, the source and the route of intracellular Zn\(^{2+}\) release are not demonstrated. Although it is quite likely that the Zn\(^{2+}\) source is from intracellular stores, how and where the intracellular Zn\(^{2+}\) becomes free Zn\(^{2+}\) needs to be further investigated. Several stores were speculated as the source of Zn\(^{2+}\) including metallothionein and several organelles. In summary, evidence was provided that briefly exposing cultured neurons to physiological concentrations of exogenous NO resulted in persistent increases in intracellular free Zn\(^{2+}\) levels. In light of the observation that no significant neuronal death accompanied the increase in intracellular free Zn\(^{2+}\), it seems likely that such
increases could play a physiological signaling role in neurons. Studies presented here provides new evidence of crosstalk between NO and intracellular Zn$^{2+}$.

II. The tolerance to OGD caused by exogenous NO

Various studies have shown that oxidative stress and excitotoxic stimulus (Frederickson et al., 1989; Zhang et al., 2004; Lin et al., 2007) could result in increases in intracellular Zn$^{2+}$ concentrations. So it is likely that increases in intracellular Zn$^{2+}$ occur during stimuli that induce preconditioning or tolerance or OGD. However, it has never been shown if intracellular Zn$^{2+}$ is involved in the cellular pathways in induction of tolerance to OGD in cultured neurons.

The results presented here have shown that in cultured neurons, intracellular chelatable Zn$^{2+}$ is necessary for the induction of tolerance to OGD triggered by exogenously added NO or lipophilic metal chelators. However, the administration of 10 µM Zn$^{2+}$ or Zn$^{2+}$ and pyrithione failed to induce tolerance to OGD. Thus, Zn$^{2+}$ is not sufficient by itself to induce tolerance to OGD. It appears that an increase in intracellular Zn$^{2+}$ alone is not a trigger for the induction of tolerance to OGD. Rather, it would appear that Zn$^{2+}$ could be a cofactor at downstream steps where NO triggers induction of tolerance. On the other hand, the tolerance induced by exogenous NO did not show a requirement for the presence of extracellular Ca$^{2+}$. The mechanisms underlying induction of tolerance to OGD by NO are incompletely understood. Thus, it can only be speculated on the step(s) where Zn$^{2+}$ might be required. Previous studies showed that NO works by activating a Ras/ERK involved pathway (Gonzalez-Zulueta et al., 2000; Lin et al., 2004).
The mechanism of Ras activation by NO is possibly due to S-nitrosylation of a critical cysteine residue which stimulates guanine nucleotide exchange (Lander et al., 1995). Redox-agents were found to promote Ras activation by S-nitrosylation when transition metals such as Fe$^{2+}$ or Cu$^{2+}$ were present (Heo & Campbell, 2006). Therefore, the presence of Zn$^{2+}$ might be required for the S-nitrosylation of Ras as a result of elevated intracellular levels of NO.

The above conclusions are based on the ability of added TPEN to chelate intracellular Zn$^{2+}$. However, TPEN also may chelate other metals such as Fe$^{2+}$ or Cu$^{2+}$ that have important cellular functions as well. It was shown that only Zn$^{2+}$ when added in combination with TPEN was able to block the actions of TPEN. When Fe$^{2+}$ or Cu$^{2+}$ was coadministered with TPEN no effect was observed. Thus, it appears highly likely that Zn$^{2+}$ and not other metals are required for the induction of tolerance to OGD. The actions of TPEN in blocking the induction of tolerance to OGD, showed an interesting time-dependence. When TPEN was coadministered with NO, TPEN was effective to block induction. It was also shown that 5 min applications of TPEN during a 12 hrs window subsequent to NO administration was just as effective, whereas waiting longer (e.g., 12 or 24 hrs) to apply TPEN was ineffective. Although the results obtained when TPEN and NO were coadministrated indicates Zn$^{2+}$ may be required as a cofactor for NO S-nitrosylation of Ras as discussed above, it should be mentioned that it is uncertain the degree of TPEN washout after the 5 min exposure. Thus, it is possible that effective concentrations of TPEN might exist intracellularly long after the 5 min application even
after solution change. Therefore, at present it is uncertain at what step Zn\(^{2+}\) is required during the first 12 hrs after NO application.

NO was involved in the induction of tolerance by triggering p21\(^{ras}\) activation (Gonzalez-Zulueta et al., 2000). The downstream mediator of Ras action is the ERK1/2 cascade. ERK1/2 activation was also shown to be required for inducing tolerance to OGD. The presented studies demonstrate that the activation of ERK1/2 cascade is required to induce the tolerance to OGD by NO using ERK1/2 inhibitors. Besides the activation of ERK1/2, a downstream modulator of the ERK1/2 cascade, RSK was first shown to be involved in the induction of tolerance by NO. An inhibitor of RSK was also shown to prevent the tolerance induced by NO. These data indicate that NO activates the ERK1/2-RSK pathways leading to the induction of tolerance to OGD.

New protein synthesis is required for the induction of tolerance to OGD. This is demonstrated by the fact that 10 µM CHX present in the media during the 24 hrs between NO and OGD treatment blocked the induction of tolerance to OGD. CHX is a protein synthesis inhibitor in eukaryotic organisms. CHX exerts its effect by interfering with transferase II and polysome aggregation (Baliga et al., 1969). This action is consistent with previous studies showing that \textit{de novo} protein synthesis is required for induction of tolerance to OGD (Matsuyama et al., 2000; Puisieux et al., 2004). Studies with CHX showed that the new protein synthesis required for induction of tolerance to OGD occurred during the first 12 hrs after NO treatment. Addition of CHX 12 hrs after treatment with NO was ineffective. Previous studies have shown that Zn\(^{2+}\) is able to regulate translation. Using neuronal cell lines, it was demonstrated that Zn\(^{2+}\) binds Trk
receptors and can phosphorylate GSK3beta and P70S6K, thus affecting tau protein phosphorylation and synthesis (An et al., 2005; Hwang et al., 2005). Zn\(^{2+}\) can also inhibit translation in neuronal cells correlated with phosphorylation of initiation factor eIF-2 (Alirezaei et al., 1999). Zn\(^{2+}\) act at the initiation step of protein synthesis, it strongly decreased the amount of polyribosomes. The addition of extracellular Zn\(^{2+}\) was shown to regulate translation via disruption of polysome, aggregation of processing bodies, in which mRNA decapping and degradation take place, and impairment of the ribonucleoparticle (RNP)-polysome interaction in neuronal cells (Jacob & Irith, 2008). The time dependence of CHX action is similar to that observed with TPEN suggesting that Zn\(^{2+}\) might be required for efficient translation and new protein synthesis required for the induction of tolerance to OGD. In \(^{35}\)S incorporation experiment, TPEN showed a similar effect with CHX to inhibit new protein synthesis. The addition of Zn\(^{2+}\) reversed the inhibition protein synthesis caused by TPEN suggested that intracellular Zn\(^{2+}\) is necessary for the new protein synthesis. Recent studies demonstrated that zinc enhanced eIF 4B homodimerization and interaction with Poly(A)-binding protein (PABP) to organize the assembly of the translational machinery (Cheng et al., 2008).

The intermediate step between ERK1/2-RSK activation and new protein synthesis would be the activation of certain transcription factors and gene expression. Although it has not been identified which transcription factors are activated after the ischemic preconditioning. In a recent study, the transcription factor cyclic AMP response element-binding protein (CREB) was activated during ischemic preconditioning (Meller et al., 2005). CREB is a substrate for phosphorylation by multiple kinases, including ERK1/2
and RSK (Bonni et al., 1999; Pierrat et al., 1998; Creson et al., 2009). Although the MAPK inhibitor, U0126 did not block the phosphorylation of CREB, the inhibitor U0126 did inhibit the binding of CREB-binding protein (CBP) to Bcl-2 promoter. The binding of CBP to Bcl-2 promoter is a crucial step for the activation of CREB. Many studies have demonstrated that many genes with prosurvival effects are regulated by CREB. The expression of Bcl-2 has profound neuroprotective effects (Alberi et al., 1996). Inhibiting the expression of Bcl-2 after preconditioning blocks ischemic tolerance (Shimizu et al., 2001). The overproduction of endogenous and the addition of exogenous NO were shown to protect neuroblastoma cells from apoptosis through CREB activation (Ciani et al., 2002). The activation of CREB was also regulated by intracellular Zn$^{2+}$. Using TPEN to deplete intracellular Zn$^{2+}$, down-regulated CREB mRNA expression in cultured hippocampal neurons (Liu et al., 2008). The co-addition of Zn$^{2+}$ almost completely reversed TPEN-induced alteration of CREB expression. From these studies, it is clearly shown that the activation of CREB is related to NO, ERK1/2 and RSK pathway and intracellular Zn$^{2+}$ changes. More studies could be conducted to further investigate whether CREB is one of the key mediators in the mechanism of tolerance to OGD.

In this study, what kind of the genes were turned on after preconditioning stimulus is unknown. Upregulation of gene expression is an important mechanism for cell survival in tolerance. It would be interesting to use gene chips to screen the genes activated after the preconditioning stimulus. Another question that could be further discussed is the distribution of intracellular Zn$^{2+}$ after preconditioning stimulus. Although the presence of
intracellular Zn\(^{2+}\) is required for the induction of tolerance, whether and how the distribution would change after preconditioning is still in mystery.

### III. The tolerance to OGD caused by small molecular lipophilic chelators

Previous studies have shown that Cq has therapeutically beneficial effects on Alzheimer’s disease patients. In this study, Cq was demonstrated to induce tolerance by a 5 min pretreatment 24 hrs prior to OGD treatment. The tolerance induced by Cq was dependent to intracellular Zn\(^{2+}\) because coadministration of TPEN prevented the induction of tolerance. The presence of extracellular Ca\(^{2+}\) is not necessary for the induction of tolerance since the removal of extracellular Ca\(^{2+}\) in Locke’s buffer did not affect the ability of Cq to induce tolerance.

The lipophilic metal chelator, DP-b99 was shown to significantly improve the recovery rate in stroke patients. In this study, DP-b99 was also shown to induce a robust tolerance to OGD at 50 \(\mu\)M. Similar with the effect of exogenous NO and Cq; the coadministration of TPEN prevented the induction of tolerance suggesting that intracellular Zn\(^{2+}\) is necessary to induce tolerance. Although the addition of universal protein synthesis inhibitor CHX right before OGD or 12 hrs after DP-b99 treatment had no effect on the induction of tolerance, the incubation of CHX during the 24 hrs between DP-b99 and OGD treatment blocked the induction of tolerance suggesting that new protein synthesis is also required for the tolerance induced by DP-b99 during the first 12
hrs, as was observed for NO. Similar with exogenous NO induced tolerance, DP-b99 was found to require ERK1/2-RSK activation for induction of tolerance by the fact that ERK1/2-RSK inhibitors blocked the tolerance induced by DP-b99.

DP-b99 was found to form visible aggregates with Ca$^{2+}$ in Locke’s buffer. The extent of aggregation of DP-b99 with Ca$^{2+}$ increases as the concentration of DP-b99 increases. In Ca$^{2+}$ free Locke’s buffer, no aggregation of DP-b99 was formed. The aggregation of DP-b99 and Ca$^{2+}$ in Locke’s buffer may be related to the mechanism of its ionophoric action to transport Ca$^{2+}$ into cytoplasmic compartments. The incubation of cultured neurons with DP-b99 in Locke’s buffer resulted in an elevated intracellular Ca$^{2+}$ content. Whereas, other metal ions such as Cu$^{2+}$, Fe$^{2+}$ or Zn$^{2+}$ did not show similar increases after DP-b99 exposure. The intracellular Ca$^{2+}$ increase peaks at 50 µM, but is lower at both 30 µM and 100 µM concentrations of DP-b99. Although the total intracellular Ca$^{2+}$ content was increased by all the concentrations of DP-b99 tested, the size of the increase in intracellular free Ca$^{2+}$ for the incubation with different concentrations of DP-b99 is not unknown. The results suggested that the tolerance induced by DP-b99 required Ca$^{2+}$ entry from extracellular milieu; this effect was supported by the facts that DP-b99 in Ca$^{2+}$ free Locke’s buffer did not cause an increase of intracellular Ca$^{2+}$ content and failed to induce tolerance to OGD. Ca$^{2+}$ entry is not tightly related with the induction of tolerance since all different concentrations of DP-b99 incubation showed increase of intracellular Ca$^{2+}$ content but only the incubation with 50 µM DP-b99 induced robust tolerance. The tolerance induced by DP-b99 did not show typical concentration dependence. Both higher (100 µM) or lower (30 and 10 µM) concentration of DP-b99 failed to induce significant
tolerance to OGD. Thus, it is unlikely that DP-b99 acts at a specific receptor site, rather the concentration dependence showed a threshold-like effect. This type of effect could be explained by the ionphore mechanism, since one would expect a critical concentration would need to be reached to produce \( \text{Ca}^{2+} \) entry. But a few exceptions are present in the results. First is that the intracellular \( \text{Ca}^{2+} \) content is not increased in a dose dependent manner between 10, 30 and 50 \( \mu \text{M} \) DP-b99. One possible explanation is that a localized change in free \( \text{Ca}^{2+} \) is more important than the total uptake. Secondly, the induction of tolerance by DP-b99 goes away at 100 \( \mu \text{M} \). One possible reason is that the aggregation in Locke’s buffer is so great that \( \text{Ca}^{2+} \) uptake actually decreased. Although in ICP data the uptake of \( \text{Ca}^{2+} \) decreasing, statistical analysis did not show any significance compared to 50 \( \mu \text{M} \). Another possible explanation is that higher concentrations of DP-b99 have a secondary effect such as zinc chelation and begins to act like TPEN and prevents the induction of tolerance.

These results suggest that DP-b99 provides an ionophoric activity by carrying \( \text{Ca}^{2+} \) from extracellular to certain intracellular locations to induce tolerance. One possible effector of \( \text{Ca}^{2+} \) is NOS. Many studies have shown that an increase of intracellular \( \text{Ca}^{2+} \) leads to the activation of NOS (Busse & Mulsch, 1990; Heinzel et al., 1992; Mayer et al., 1990). Since the activation of NOS is \( \text{Ca}^{2+} \) dependent, it is logical to propose that the increased intracellular \( \text{Ca}^{2+} \) caused by DP-b99 exposure might lead to the activation of NOS in neurons. This effect might increase NO production which eventually would induce tolerance in same manner as exogenous NO addition. This conclusion was supported by the result that coadministration with 50 \( \mu \text{M} \) NOS inhibitor, L-NAME
prevented the induction of tolerance by DP-b99. Thus, the effects of DP-b99 and NO are linked together. It seems that DP-b99 is the upstream activator of NO although indirectly via an increase in intracellular Ca\(^{2+}\).

In this study, extracellular Ca\(^{2+}\) is shown to be required for the tolerance induced by DP-b99. But the exact role of Ca\(^{2+}\), especially the distribution of intracellular free Ca\(^{2+}\) is still a mystery. Further studies are needed to investigate the intracellular free Ca\(^{2+}\) changes during and after the incubation with DP-b99 to define the specific role of Ca\(^{2+}\) in DP-b99 induced tolerance.

From these results, a proposed signaling pathway for the induction of tolerance to OGD by exogenous NO and DP-b99 is shown in Fig. 26.
Figure 26: The proposed diagram of NO and DP-b99 induced tolerance to OGD. The incubation of DP-b99 would bring extracellular Ca$^{2+}$ into the cells. The increase of intracellular Ca$^{2+}$ activated NO synthase to increase the production of endogenous NO. NO activated p21ras and ERK/RSK signal pathway. One possible transcription factor involved in this signal pathway is CREB. Different possible neuroprotective genes were up-regulated and neuroprotective proteins were expressed resulting in increased cell survival.

IV. Summary of study

In this study, it was demonstrated that the brief exposure of exogenous NO to cultured cortical neurons increased intracellular Zn$^{2+}$ using a selective fluorophore,
FluoZin-3. The intracellular Zn$^{2+}$ change was not affected by extracellular EDTA but was eliminated by TPEN. The increase peaked at 1 µM spermine NONOate. The NO scavenger hemoglobin blocked the effects and the inactive analog of the NO generator, spermine, was without effect.

It is also shown that intracellular Zn$^{2+}$ was necessary for the induction of tolerance to OGD triggered by exogenously applied NO or small molecular lipophilic metal chelators such as DP-b99 and Cq. But Zn$^{2+}$ alone was not sufficient to induce tolerance in the absence of NO. The coadministration of TPEN and NO blocked the induction of tolerance and the effect of TPEN was blocked by coadministration with same amount of Zn$^{2+}$. TPEN effectively blocked the induction of tolerance to OGD when applied to the neurons up to 12 hrs after NO pretreatment. CHX blocked the induction of tolerance to OGD up to 12 hrs as well. TPEN inhibited new protein synthesis as evidence by $^{35}$S incorporation assay, the addition of same amount this suggests that Zn$^{2+}$ maybe required for translational activity needed for the induction of tolerance. The data provide evidence that several steps are required for induction to occur during a 12 hr window after the preconditioning stimulus (either NO or DP-b99), some of which could be Zn$^{2+}$ requiring. Using pharmacological inhibitors, it was shown that the ERK1/2-RSK signal pathway was involved. Different with the tolerance induced by NO and Cq, the tolerance by DP-b99 showed a requirement for extracellular Ca$^{2+}$. The addition of DP-b99 to Locke’s buffer was shown to form visible aggregates with Ca$^{2+}$. The treatment of cultured neurons with DP-b99 increased intracellular Ca$^{2+}$ content in at the same concentrations that induced tolerance. The effect of DP-b99 to induce tolerance at 50 µM but not at higher or
lower concentrations suggesting that there is a threshold effect. Incubation with NOS inhibitor, L-NAME blocked the tolerance induced by DP-b99 suggesting the production of NO is the downstream effector of DP-b99.
REFERENCES


"Metallothioneins in brain--the role in physiology and pathology." Toxicol Appl Pharmacol. 142(2): 229-42.


inhibits the proteasome and displays preclinical activity in leukemia and myeloma." Leukemia. 23(3): 585-90.


on delayed neuronal death in the gerbil hippocampus." Brain Res. 743(1-2): 362-5.


APPENDIX A: BUFFERS

1. **Hank’s Balanced Salt Solution (HBSS)**

One bag of HBSS powder (Sigma. H2387-1L) is dissolved in 950ml Mili-Q H₂O, then 0.35g of NaHCO₃ is added and the pH adjusted to 7.25. The volume is brought up to 1000ml with Mili-Q H₂O. The solution is sterilized by membrane filtration. Finally 0.5ml gentamycin (10mg/ml) is added to 500ml HBSS. The solution is stored at 0-4°C.

2. **Minimal Essential Medium**

One bag of MEM powder (Sigma. MO769-1L) is dissolved in 950 ml Mili-Q H₂O. Then 2.2 grams of NaHCO₃ and 10 grams of glucose are added and the pH adjusted to 7.25. The volume is brought to 1000 ml. The solution is sterilized by membrane filtration. 1ml of 10 mg/ml gentamycin (0.5 ml to 500 ml) is added. The solution is stored at 0-4°C.

3. **MEM+**

To make 200 ml MEM+, 20 ml sterile heat-inactivated fetal bovine serum (final concentration 10%), 22 mg of UV treated pyruvic acid (1 mM) (0.022 g), 32 mg L-glutamine and 220 mg KCl are mixed together, then MEM is added to bring the volume to 200 ml. The solution is sterilized by membrane filtration using an Acro 50A filter (0.2 µm, Gelman Sciences. Cat. No. 4260). The solution is stored at 0-4°C.

4. **Neurobasal Medium**
To make 200 ml Neurobasal medium, 14.6 mg L-Glutamine and 4 ml B-27 supplement (2%) was added to 46 ml warmed Neurobasal Medium (GIBCO. 21103-049) to the tube and mix gently. Once the L-glutamine is completely dissolved, the solution was filtered with a snap-cap syringe filter into a sterile glass bottle. 150ml Neurobasal was then added to get a final volume of 500 ml. The solution is stored at 0-4°C for up to two weeks.

5. Lockes’ Buffer

This 5x solution has the composition 770mM NaCl, 28mM KCl, 11.5mM CaCl$_2$, 5mM MgCl$_2$, 25mM HEPES and 50mM Glucose, pH7.4.

6. Low Ca$^{2+}$ Locke’s buffer (5x)

This 5x solution has the composition 770mM NaCl, 28mM KCl, 2.5mM CaCl$_2$, 5mM MgCl$_2$, 25mM HEPES and 50mM Glucose, pH8

7. BSA/NGS

To make 10 ml BSA/NGS solution, 1 ml 10×PBS, 10 mg BSA and 0.4 ml NGS were mixed together. Mili-Q H$_2$O was added to bring the volume to 10 ml.

8. PBS 10x (Dulbecco’s Phosphate Buffered Saline)

One bottle of DPBS (VWR. 55-031-PB) was dissolved into 950 ml Mili-Q H$_2$O in a 1000 ml beaker and pH was adjusted to 7.4. The solution volume was brought to 1 liter.
9. **polyethyleneimine coating solution**

First, Borate Buffer 10x (for plate coating, 500 ml) was made by filling a 500 ml beaker with about 450 ml Mili-Q H₂O. Then 1.55 g boric acid and 2.37 g borax were added, the pH was adjusted to 8.2, the volume was brought to 500 ml with water stored in a glass bottle and stored in 4 °C. To make 50 ml of coating solution, 50 µl of the polyethyleneimine (SIGMA, P-3143 50% solution) was added to 5 ml borate buffer and mixed completely. Mili-Q H₂O was added to bring volume to 50 ml. The coating solution should be made fresh just before coating the cell culture plates.

10. **Block solution**

To make 100 ml block solution, 10 ml goat serum, 1 g BSA, 10 ml and 0.1 ml Triton-X were mixed together, the volume was brought to 100 ml by Mili-Q H₂O.

11. **Borate Buffer**

To make 500 ml borate buffer, 1.55 g boric acid and 2.37 g borax were mixed well and added to 450 ml Milli-Q water in a 1000 ml beaker with gently stir until dissolved. The pH was adjusted to 8.2 with 1N NaOH or HCl. the volume was brought to 500 ml by Mili-Q H₂O.

12. **PBST**

0.5 ml Triton-x was added to 50ml 10×PBS, and the volume was brought to 500 ml by Mili-Q H₂O.
13. **Nonidet P-40 lysis buffer**

The solution has composition 2% Nonidet P-40, 80 mM NaCl, 100mM Tris-HCl, 0.1% SDS.