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Zinc Translocation Causes Hypoglycemia-Induced Neuron Death

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1. Introduction

Hypoglycemia is a common but serious problem among type1 and type 2 diabetic patients receiving intensive treatment with glucose-lowering drugs such as insulin or sulfonylurea. Moderate hypoglycemia is occurring 0.1-0.3 episode/patient per day and is usually corrected by patients themselves or just ignored. However, severe hypoglycemia causes unconsciousness and it may lead to neuronal injury in the cerebral cortex and hippocampus. Hypoglycemic neuronal death is resulted from a cascade of several events after prolonged period of lack of glucose since brain exclusively use glucose (Auer et al., 1984a; Auer and Siesjo, 1993; Auer et al., 1984b). Sustained release of glutamate from presynaptic terminals into the extracellular space and activation of glutamate receptors has been suggested as a necessary upstream event in this neuron death cascade (Auer and Siesjo, 1993; Wieloch, 1985). Also mitochondrial membrane permeability (Friberg et al., 1998), calpain activation (Ferrand-Drake et al., 2003), PARP-1 activation (Suh et al., 2003) and NADPH oxidase activation-induced ROS production (Suh et al., 2007; Suh et al., 2008) have been shown to be possible downstream events. Our lab has undertaken studies to establish whether vesicular zinc release and subsequent zinc translocation into postsynaptic neurons is an important upstream step in this hypoglycemia-induced neuron death process. Using an animal model of insulin-induced hypoglycemia we have shown that: (I) vesicular zinc is released from hippocampal mossy fiber terminals; (II) intracellular zinc accumulation is induced in the hippocampal neurons; (III) neuronal death is reduced by zinc chelation or zinc transporter gene deletion; (IV) PARP-1 activation is reduced by zinc chelation; (V) ROS production is reduced by zinc chelation after hypoglycemia and glucose reperfusion (HG/GR); and (VI) hypothermia prevented hypoglycemia-induced zinc release and neuron death. Together, these results suggest that zinc translocation is an upstream step linking HG/GR to PARP-1 activation, to NADPH oxidase activation and neuronal death in brain regions containing high concentrations of vesicular zinc. Zinc translocation into postsynaptic neurons was also demonstrated in the hippocampal slice model with combined oxygen and glucose deprivation (OGD) where neuronal zinc accumulation into the hippocampal CA1 neurons is blocked by extracellular zinc chelator, CaEDTA (Yin et al., 2002). In addition, hippocampal slices prepared from zinc transporter 3 (ZnT3) knockout mouse, which have little or no vesicular zinc in neuronal terminals, showed no zinc accumulation in post-synaptic neurons following OGD and hypoglycemia (Suh et al., 2008). These hippocampal slice experiments

with zinc chelator or with ZnT3 KO mice suggest that the zinc signal observed in post-synaptic hippocampal neurons as shown in our previous study (Suh et al., 2003) was a result of zinc translocation from the presynaptic terminals.

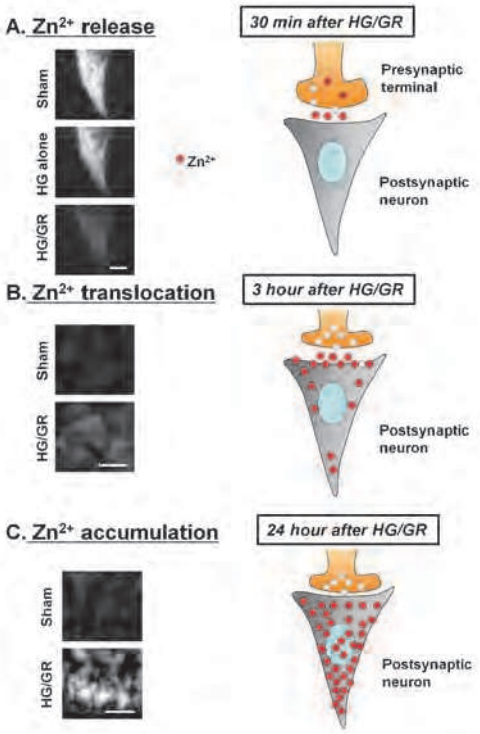

Fig. 1. Vesicular zinc release and translocation after hypoglycemia.
A) TSQ fluorescent images show vesicular zinc release from presynaptic terminals of hippocampal mossy fibers after hypoglycemia/ glucose reperfusion (HG/GR). Intense TSQ fluorescent signal (white color in the figure) in the mossy fiber of sham operated rats indicates high vesicular zinc contents in the vesicle. However, the diminished TSQ fluorescent intensity in the HG/GR rats indicates that bulk of vesicular zinc has been released and therefore presynaptic vesicular zinc contents are reduced at the time when the brain section was evaluated. TSQ fluorescent intensity in mossy fiber is decreased after 60 minutes of hypoglycemia (HG alone). TSQ fluorescent intensity is further decreased after 30 minutes hypoglycemia and 30 minutes glucose reperfusion (HG/GR), which represents mossy fiber vesicular zinc release from presynaptic terminals. A schematic drawing represents vesicular zinc release from presynaptic terminals after HG/GR. B) TSQ fluorescent images show zinc translocation into postsynaptic neurons of hippocampal CA1 pyramidal neurons 3 hours after hypoglycemia. Zinc accumulation in the intracellular space can be detected in this early time point. A schematic drawing represents intracellular zinc accumulation 3 hours after HG/GR. C) TSQ fluorescent images show zinc accumulation into postsynaptic neurons 24 hours after HG/GR. Intense zinc accumulation in the intracellular space is detected in this time point. A schematic drawing represents intracellular zinc accumulation 24 hours after HG/GR. Scale bar in (A) is 200 μm and in (B) and (C) are 20 μm.

2. Role of zinc in hypoglycemic neuronal death

Chelatable zinc (free or weakly bound to proteins) is present in a subset of glutamatergic axon terminals throughout the mammalian forebrain, especially in the hippocampus and in the cerebral cortex (Danscher et al., 1985) (Frederickson, 1989). The chelatable zinc is mainly localized in synaptic vesicles of excitatory presynaptic neuron terminals (Perez-Clausell and Danscher, 1985) and is released into the extracellular space during paroxysmal neuronal activity or membrane depolarization (Assaf and Chung, 1984; Howell et al., 1984). This zinc release has been suggested to contribute to neuronal death in several disease conditions, such as seizure (Frederickson et al., 1988; Suh et al., 2001), ischemia (Koh et al., 1996; Tonder et al., 1990) and traumatic brain injury (Suh et al., 2000). Zinc can induce the production of reactive oxygen species (ROS) and PARP-1 activation in cell cultures (Kim et al., 1999; Sensi et al., 1999a; Sheline et al., 2000), suggesting a possible role of zinc in hypoglycemia-induced neuronal death. Our previous study showed that hypoglycemia induces vesicular zinc release from the synaptic terminals. We also found that hypoglycemia increases neuronal zinc accumulation in postsynaptic neurons, which is prevented by intracerebroventricular injection of the Zn^{2+} chelator CaEDTA (Suh et al., 2004; Suh et al., 2008) or intraperitoneal injection of clioquinol (CQ) (Shin et al., 2010).

2.1 Vesicular zinc release and translocation after hypoglycemia

Oxidative stress and zinc release are both known to contribute to neuronal death after hypoglycemia; however, the temporal relationships between these events are not well established. Our study demonstrated that the vesicular zinc release from hippocampal mossy fiber and subsequent translocation into postsynaptic neurons occurs immediately after HG/GR. We used the fluorescent dye TSQ, which binds free zinc (Frederickson et al., 1987). The vesicular zinc signal detected by TSQ showed a partial decrease (release from mossy fiber terminal) after 60 minutes of hypoglycemia alone (HG alone), but was almost completely absent after 30 minutes of hypoglycemia followed by 30 minutes of glucose reperfusion (HG/GR) (Figure 1A) (Suh et al., 2004; Suh et al., 2007). This result suggests that vesicular zinc release from hippocampal mossy fiber is not caused by hypoglycemia itself but caused by a combination of hypoglycemia and subsequent glucose reperfusion. Conversely, TSQ staining in the postsynaptic pyramidal neuron bodies was absent under sham operated conditions or hypoglycemia alone, but TSQ intensity in the cytoplasm of CA1 neurons was increased 3 hours after 30 minutes of hypoglycemia and 30 minutes glucose reperfusion (HG/GR) (Figure 1B). This represents translocation of presynaptic zinc to postsynaptic neuron of CA1 pyramidal neurons. This initial cytoplasmic zinc increase was prevented by intracerebroventricular (i.c.v) injection of the zinc chelator, CaEDTA. Without zinc chelation, this intraneuronal zinc accumulation continued to increase until 24 hours after hypoglycemia and glucose reperfusion (Suh et al., 2008). However, CaEDTA treatment also prevented this continuous intracellular zinc accumulation when evaluated at 24 hours later, suggesting that released zinc from the synaptic vesicles translocated into the post-synaptic neurons during several hours after hypoglycemia and glucose reperfusion conditions (Figure 1C). From these findings, we speculate that zinc release/translocation is a key upstream step in the sequence of events leading to neuronal death after HG/GR (Suh et al., 2004; Suh et al., 2007). However, the identity of the factor(s) involved in the intermediating step(s) for HG/GR-induced vesicular zinc release and translocation process

is unknown. In our prior study, nitrotyrosine formation was detected shortly after glucose reperfusion, but not during hypoglycemia per se (Suh et al., 2003). Subsequently we found that a neuron specific NOS inhibitor, 7-NI, significantly inhibited hypoglycemia-induced vesicular zinc release from hippocampal mossy fiber (Fig 2A). 7-NI also prevented intracellular zinc accumulation and neuronal death at 24 hour post-HG/GR time point (Fig. 2B) (Suh et al., 2003). These findings suggest that nitric oxide production is an event upstream of vesicular zinc release and postsynaptic zinc accumulation. This observation is consistent with previous studies in which intra-hippocampal injection of nitric oxide donor (Spermino-NONOate) induced vesicular zinc release and intracellular zinc accumulation (Cuajungco and Lees, 1998; Frederickson et al., 2002).

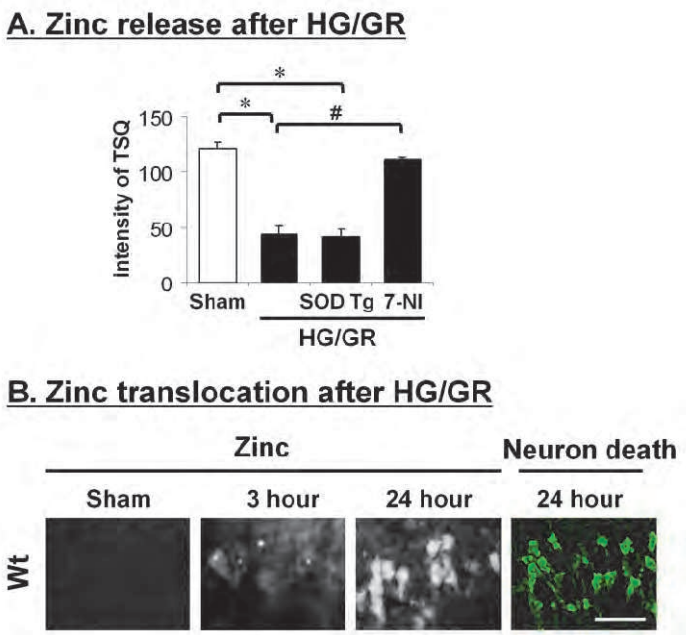

Fig. 2. A) Vesicular zinc release after hypoglycemia/glucose reperfusion is prevented by NOS inhibitor. Vesicular zinc release was evaluated at hippocampal hilus by TSQ fluorescence. The TSQ signal loss is apparent after 30 minutes of HG and 30 minutes of GR. SOD-1 over-expressing rats (SOD-1 Tg) show similar zinc release after HG/GR (HG+GR+SOD), whereas the NOS inhibitor 7-NI almost completely prevented vesicular zinc release from the hilus mossy fiber area. Graph shows TSQ fluorescence intensity. Data are mean + s.e.m; n = 10; * $P < 0.05$. # $P < 0.05$. B) Intracellular zinc accumulation and neuronal death after hypoglycemia/ glucose reperfusion (HG/GR). Images show neuronal zinc accumulation at 3 or 24 hours after HG/GR and neuronal death at 24 hours after HG/GR. TSQ intensity in CA1 pyramidal neurons is increased compared to sham operated rats by 3 hours after HG, and further increased at 24 hours. CA1 pyramidal neurons show Fluoro-Jade B staining (green) at 24 hours after HG/GR. Scale bar = 50 µm. n = 3-4. This figure is modified from our previous published paper (Suh et al., JCBFM, 2008).

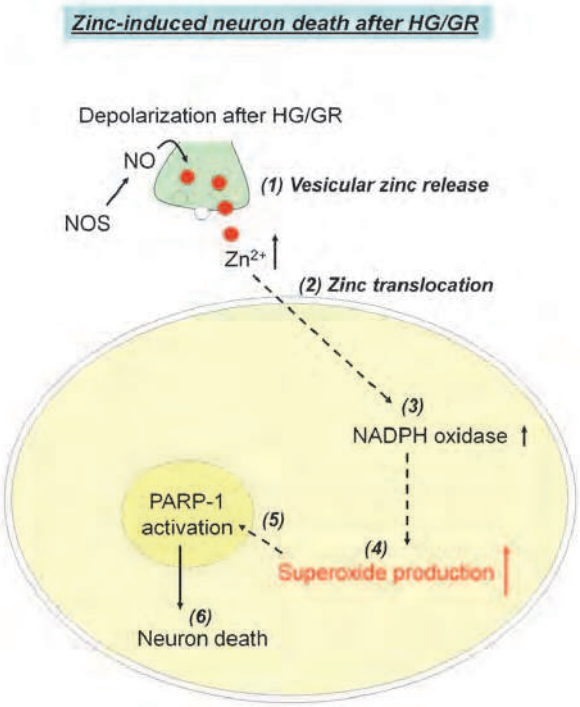

Fig. 3. Key aspects of hypoglycemia-induced neuronal death by zinc.
1) Nitric oxide (NO) production after hypoglycemia/ glucose reperfusion leads to release of zinc together with glutamate from presynaptic terminals. 2) Zinc translocates into intracellular space. 3) Translocated zinc activates NADPH oxidase. 4) NADPH oxidase activation induces ROS production. 5) Production of superoxide from NADPH oxidase induces DNA damage and activation of poly(ADP-ribose) polymerase-1 (PARP-1) in the nucleus. 6) Neuron death.

Since peroxynitrite (highly neurotoxic) is formed by reaction of nitric oxide (NO) with superoxide (Beckman and Koppenol, 1996), our previous study also sought to clarify the role of superoxide formation on presynaptic zinc release from hippocampal mossy fiber and postsynaptic zinc accumulation in the hippocampal CA1 neurons after hypoglycemic insult. This study showed that over-expression of SOD-1 significantly reduced hypoglycemia-induced neuronal death (Suh et al., 2007). To determine whether the neuroprotective role of SOD-1 over-expression was due to reduced release of vesicular zinc, SOD-1 transgenic rats were subjected to hypoglycemia. From this study, we concluded that SOD-1 overexpression had no effect on hypoglycemia-induced vesicular zinc release or on the initial zinc translocation into hippocampal postsynaptic neurons when evaluated at 3 hours after hypoglycemia, but that SOD-1 overexpression did reduce neuronal death and neuronal zinc accumulation when evaluated at 24 hours after hypoglycemia. These results suggest that vesicular zinc release occurs upstream of ROS production, but that ROS production continues to promote to zinc accumulation in post-synaptic neurons at later time points

(Figure 2, 3). This suggests that protein-bound zinc can be liberated by reactive oxygen species (ROS) such as superoxide. Thus, if neuronal SOD concentrations are adequate for clearance of superoxide, further intracellular free zinc release can be prevented even though initial zinc translocation event has occurred. Conversely, if superoxide production is not cleared or stabilized, intracellular free zinc will continue to increase to the point of neuronal demise. This result suggests that in addition to presynaptically-released Zn^{2+}, hippocampal neurons also have a pool of intracellularly releasable Zn^{2+}. Intracellularly derived zinc may arise from metallothionein (MTs) or other zinc binding proteins. MTs play a major role in modulating neuron death after seizure or ischemia as these proteins release a substantial amount of Zn^{2+} under conditions of oxidative stress. This notion is supported by prior studies suggesting that non-vesicular zinc may be also important in promoting brain injury (Lee et al., 2000).

2.2 The role of zinc on hypoglycemia-induced ROS production

The mechanism by which ROS production is aggravated by intracellular zinc influx has not been firmly established. Several lines of evidence suggest that zinc induces increased mitochondrial ROS production (Sensi et al., 1999b). However, in cell culture models, zinc has been identified as an activator of NADPH oxidase, an enzyme that produces superoxide. NADPH oxidase is present in many cell types including neurons (Kim and Koh, 2002; Noh and Koh, 2000). NADPH oxidase is a multi-component enzyme comprising a plasma membrane-bound subunit, gp91; a membrane-associated flavocytochrome, cytochrome b558; and at least three cytosolic subunits, $p47^{phox}$, $p67^{phox}$ and the small G protein Rac2 (Groemping and Rittinger, 2005). During activation, the $p47^{phox}$ component is phosphorylated and translocates to the plasma membrane, where it associates with the other subunits to form the active enzyme complex. The methoxy-substituted catechol, apocynin, blocks this assembly but does not inhibit mitochondrial dehydrogenases (Dodd and Pearse, 2000; Stolk et al., 1994). Interestingly, our previous studies examining the production of ROS in the brain during hypoglycemic insult suggest that superoxide is formed primarily during the glucose reperfusion period. The mechanism by which NADPH oxidase is activated in non-phagocytic cells is not well understood, but zinc has been identified as both an inducer of neuronal NADPH oxidase activity (Kim and Koh, 2002; Noh and Koh, 2000) and a contributor to hypoglycemic neuronal death (Suh et al., 2008). High concentrations of presynaptic zinc are present in the brain regions most vulnerable to hypoglycemic injury (Frederickson et al., 2005; Suh et al., 2004). Recently, we published that vesicular zinc release is required for NADPH oxidase activation in HG/GR (Suh et al., 2007). Rats pre-treated with an intracerebroventricular injection of the zinc chelator CaEDTA showed reduced neuronal ROS formation, suggesting that vesicular zinc release is an upstream event of NADPH oxidase activation. ZnEDTA, used as a control, showed no effect on ROS production. The translocation of NADPH oxidase subunits, $p47^{phox}$ or $p61^{phox}$, to the plasma membrane in cortical neuronal cultures subjected to glucose deprivation followed by glucose reperfusion was blocked by CaEDTA, but not by ZnEDTA (Figure 4). Moreover we demonstrated that zinc-induced ROS production in neuron cultures was almost completely absent in cultures from mice deficient in the $p47^{phox}$ subunit of NADPH oxidase and in wt neurons treated with the NADPH oxidase assembly inhibitor apocynin (Stolk et al., 1994; Suh et al., 2008). These results suggest that NADPH oxidase subunit assembly is triggered

by glucose reperfusion through a process requiring extracellular zinc signaling. To further confirm that vesicular zinc release is involved in HG/GR-induced ROS production and neuron death, we used the ZnT3$^{-/-}$ mouse, which has no vesicular zinc in the presynaptic terminals (Suh et al., 2007). The ZnT3$^{-/-}$ mice showed diminished ROS production at 3 hours after HG/GR and reduced neuronal death 7 days after HG/GR (Figure 5). This result confirms prior reports that zinc chelation prevents ROS production and neuron death after HG/GR (Suh et al., 2004; Suh et al., 2007) and strongly suggests that it is the vesicular zinc pool that contributes to neuronal demise in this setting.

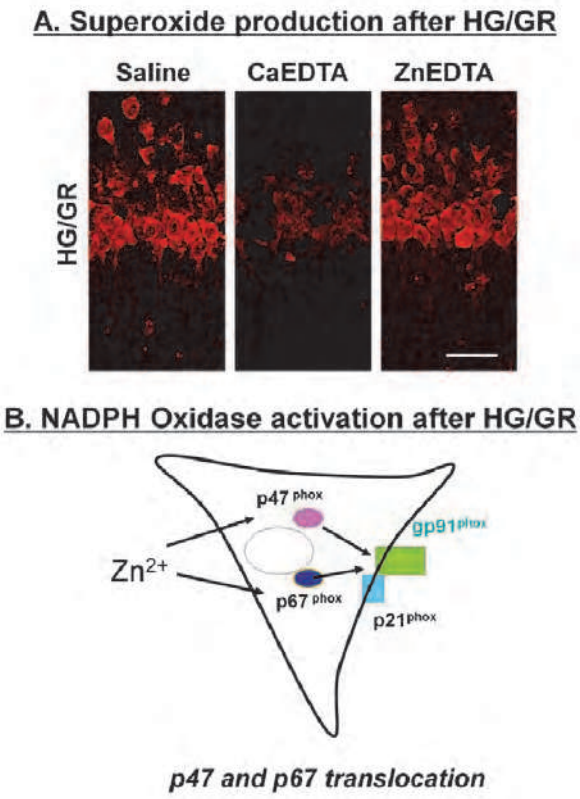

Fig. 4. Hypoglycemia/ glucose reperfusion-induced ROS production is mediated by zinc-induced NADPH oxidase activation. ROS production in neurons detected by ethidium (Et) fluorescence.
A) The zinc chelator, CaEDTA, reduces HG/GR-induced Et production in the CA1 neurons. ZnEDTA is the control. Rats were treated with saline, 100 mM CaEDTA, or 100 mM ZnEDTA. Scale bar is 50 μm. B) Schematic drawing of p47^{phox} and p67^{phox} translocation to plasma membrane by zinc translocation into neuron.

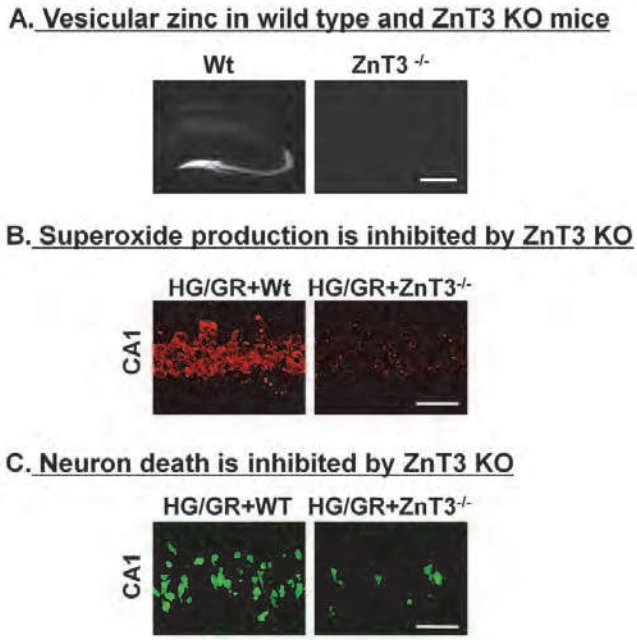

Fig. 5. Hypoglycemia/ glucose reperfusion-induced ROS production and neuronal injury is prevented by ZnT3 gene deletion in mice.
A) Vesicular zinc in the mouse hippocampus imaged with TSQ fluorescence (white) from wild-type mice and from ZnT3$^{-/-}$ mice. Scale bar is 500 μm. B) To characterize the source of ROS production in hypoglycemic neuronal injury, we used a rat model of insulin-induced hypoglycemia and evaluated the production of reactive oxygen species with dihydroethidium. Dihydroethidium is oxidized by superoxide and superoxide reaction products to form fluorescent ethidium (Et) species, which are then trapped within cells by DNA binding. In the ZnT3$^{-/-}$ mice, hypoglycemia-induced ROS production is almost completely prevented. Scale bar is 50 μm. C) Neuronal death (FJB (+) neurons) in ZnT3$^{-/-}$ mice was significantly less than wild type mice. Scale bar is 100 μm. Part of this figure is modified from our previous published paper (Suh et al., JCBFM, 2008).

2.3 The role of zinc on hypoglycemia-induced PARP-1 activation

PARP-1 activation has been shown to mediate neuronal death in a variety of disorders including ischemia, trauma, and inflammation (Virag and Szabo, 2002). PARP-1 uses the ADP-ribose group of NAD+ to form branched ADP-ribose polymers on specific acceptor proteins in the vicinity of DNA strand breaks or kinks (Burzio et al., 1979; D'Amours et al., 1999). Formation of these polymers facilitates DNA repair and prevents chromatid exchange, but extensive PARP-1 activation can promote cell death through a processes involving mitochondrial permeability transition and release of apoptosis inducing factor (Alano et al., 2004; Ha and Snyder, 1999; Yu et al., 2002). Our previous study showed that PARP-1 activation was substantially increased in hippocampal neurons after HG/GR. Rats treated with PARP-1 inhibitors after HG/GR showed a striking reduction in neuronal death, coupled with improved performance on the Morris water maze, a test of spatial learning

and memory (Suh et al., 2003). Administration of PARP-1 inhibitors at time points up to 3 hours after HG/GR was effective in reducing neuronal death, suggesting both that PARP-1 is a downstream event in the HG/GR cell death pathway and that PARP-1 inhibitors might be useful in the clinical treatment of hypoglycemic brain injury (Figure 6).

A link between zinc release and PARP-1 activation has been suggested by studies showing PARP-1 activation and PARP-1 mediated neuronal death after neuronal exposure to zinc in cell culture, and the ability of PARP-1 inhibitors to abrogate zinc-induced cell death (Kim and Koh, 2002; Sheline et al., 2000; Sheline et al., 2003; Virag and Szabo, 2002). How zinc leads to PARP-1 activation has not been firmly established, but zinc has been shown to induce formation of reactive oxygen species through actions on mitochondria (Ichord et al., 1999) and through up-regulation of NADPH oxidase and neuronal nitric oxide synthase (Kim et al., 2002). Our previous study showed that the zinc chelator CaEDTA attenuated poly(ADP-ribose) formation in the post-synaptic pyramidal cells after HG/GR, suggesting that zinc translocation may be an upstream event in hypoglycemia-induced PARP-1 activation. This result, coupled with the marked reduction in neuronal death observed with CaEDTA, and the prior observation that PARP-1 inhibitors reduce hypoglycemic neuronal death (Frederickson et al., 2002), suggests a sequential process of zinc entry, PARP-1 activation, and cell death triggered by HG/GR. These results do not, however, exclude other mechanisms by which vesicular zinc release could contribute to hypoglycemic neuronal death.

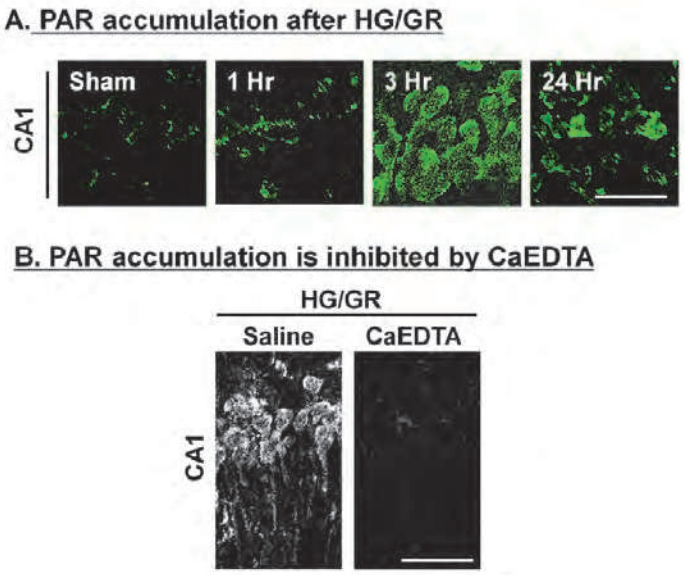

Fig. 6. Hypoglycemia/ glucose reperfusion-induced poly(ADP-ribose) formation in CA1 hippocampus in rats.

A) Poly(ADP-ribose) immunoreactivity was only modestly increased at termination of immediately after HG/GR (0 hr), but was markedly increased at 3 hr after insult, and then slowly declined after that point in the hippocampal CA1 and DG area. Scale bar is 50 μm.

B) Poly(ADP-ribose) formation was reduced by administration of zinc chelator, CaEDTA, at the time of glucose correction. Scale bar is 50 μm.

2.4 The role of zinc on hypoglycemia-induced microglia activation

Microglia is thought to be the resident immune cells of the central nervous system (CNS). Under physical conditions, resting microglia adopts the characteristic ramified morphological appearance and scatter throughout mature CNS to play role in the immune surveillance and host defense. The resting microglia transform into an activated states including amoeboid morphology, up-regulation of proliferation and release of proinflammatory mediators, when the cells bind to pathogen-derived molecules or other microglial activating agents. The pro-inflammatory cytokines such as interleukin-1 and tumor necrosis factor alpha, released from activated microglia following ischemia, brain trauma and the other brain damages (Clausen et al., 2005; Sairanen et al., 1997; Saito et al., 1996; Taupin et al., 1993), are thought to be associated with neuronal death (Loddick and Rothwell, 1996; Lu et al., 2005; Yamasaki et al., 1995). On the other hand, these cytokines have been reported to induce nerve growth factor expression or cell survival signaling (DeKosky et al., 1994), (Fontaine et al., 2002) (Herx et al., 2000). Moreover activated microglia have been reported to release neurotrophic factors such as brain-derived neurotrophic factor (Lee et al., 2002b). These reports are implying that microglia activation is not only neurotoxic but neurotrophic. However, the factors that trigger microglial activation have not been completely understood. Recently, poly (ADP-ribose) polymerase (PARP)-1 has been known to act as a coactivator of nuclear factor kappa B (NF-kB), which leads to microglial migration on excitotoxically damaged organotypic hippocampal slice culture, and neuronal cell death (Chiarugi and Moskowitz, 2003) and (Ullrich et al., 2001). Furthermore, in zinc-induced cell death of neuron cultures, PARP-1 has been reported to be activated by zinc through NADPH oxidase pathway (Sheline et al., 2003), (Kim and Koh, 2002). In our previous study, we sought to examine whether zinc induces microglial activation and how microglia is activated by zinc. We found that zinc can induce microglial activation which mediated by PARP-1 activation though NADPH oxidase pathway and that microglial activation in mice ischemic brain are blocked by zinc chelator (Kauppinen et al., 2008). During severe hypoglycemia, glucose reperfusion and its neurotoxic cascade may not only damage neurons directly, but may also promote neuronal injury indirectly via microglia activation. Microglia activation is a gradual process including change of morphology from highly ramified into an amoeboid shape, proliferation, migration to injury site, increased expression of surface molecules, increased secretion of cytokines, chemokines, free radicals and proteases, and assumption of phagocytotic activity (Kreutzberg, 1996). We tested whether zinc chelation prevents microglia activation after hypoglycemia. Both CaEDTA and CQ substantially decreased hypoglycemia-induced microglia activation in the hippocampal CA1 pyramidal area (Figure 7).

2.5 Prevention of hypoglycemia-induced neuronal death by hypothermia

Our previous study presented that mild hypothermia reduces hypoglycemia-induced neuronal death in the hippocampus, whereas hyperthermia aggravates those brain injuries. We suggested that hypothermia (lowering brain temperature) prevents hypoglycemia-induced neuronal death by reduction of vesicular zinc release, superoxide production and microglia activation, where temperature dependent vesicular zinc release was a key event upstream of hypoglycemia-induced superoxide production and microglia activation.

Mild hypothermia has been known as the most effective approach to prevent neuronal death after cerebral ischemia (Busto et al., 1987; Maier et al., 2002), traumatic brain injury (Clifton et al., 1991; Suh et al., 2006) and prolonged seizure (Liu et al., 1993). We found that

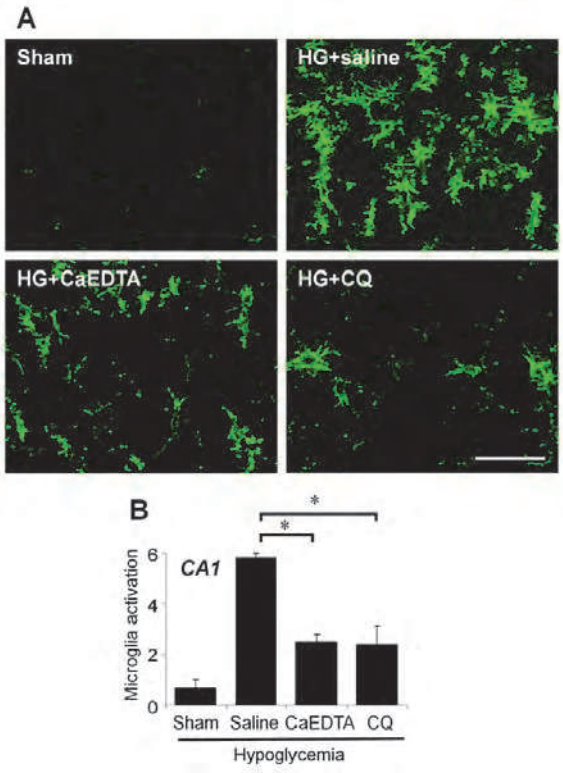

Fig. 7. Hypoglycemia-induced microglia activation is prevented by zinc chelation. (A) Morphological change and intensity of immunostaining of microglia after hypoglycemia is affected by zinc chelation. Hypoglycemia (HG+saline) substantially increased microglia activation in the hippocampal CA1 region. However, zinc chelation by CaEDTA (HG+CaEDTA) or clioquinol (HG+CQ) significantly reduced microglia activation in the above areas. Scale bar=100 μm. (B) Quantification of microglia activation was performed in the hippocampal CA1 area. As shown in the images, microglia activation is strongly prevented by zinc chelation. Data are mean±s.e.m. (n=3 to 6); $^{*}P$<0.05 compared with the saline treated group.

mild hypothermia also can prevent hypoglycemia-induced neuronal death. Neuronal death evaluated in hippocampal area shows that hypothermia significantly reduced neuronal death while hyperthermia applied after hypoglycemic events aggravated the neuronal death (Shin et al., 2010). The neuroprotective effects of hypothermia after hypoglycemia in our previous study, however, differ from those reported in previous studies (Agardh et al., 1992). Agardh et al. reported that mild hypothermia applied before and during of hypoglycemia (before and entire period of iso-EEG period) produced a similar degree of neuronal death compared to normothermic animals. No neuroprotective effect of hypothermia was seen in the hypoglycemic animals. The differences between our study and Agardh et al.'s may be explained by the onset of hypothermia application. Agardh et al. applied hypothermia before and during the iso-EEG period. However, in our study,

hypothermia applied after the iso-EEG period was terminated, i.e. brain temperature was decreased during the glucose reperfusion period after hypoglycemia. Since we have previously shown that hypoglycemia-induced neuronal death is not initiated during the period of glucose deprivation but instead during glucose reperfusion period, it may be that the hypothermic application before and during the isoelectric period was not sufficient to prevent neuronal death after hypoglycemic events. In our experimental setting we also found that hypothermia application before and during the iso-EEG period had no statistically significant neuroprotective effects as seen in the previous study (Agardh et al., 1992), strengthening our hypothesis that brain temperature is a critical factor during glucose reperfusion period after hypoglycemia.

Suggested neuroprotective mechanisms of mild hypothermia on several brain injuries are based on decreases in cerebral metabolic requirement (Erecinska et al., 2003), intracranial pressure (Soukup et al., 2002), glutamate release from presynaptic vesicles (Arai et al., 1993; Ichord et al., 1999), free radical generation (Globus et al., 1995; Horiguchi et al., 2003) and inflammatory reaction (Kumar and Evans, 1997; Wang et al., 2002). Previously, we have shown that hypothermia reduced vesicular zinc release and subsequent neuronal death after traumatic brain injury (Suh et al., 2006). We also have shown that hypoglycemia-induced neuronal death is mediated by vesicular zinc release and translocation (Suh et al., 2004; Suh et al., 2008). Therefore, we hypothesized that mild hypothermia has neuroprotective effects by reduction of the vesicular zinc release after hypoglycemia. Although zinc is released from presynaptic terminals as a component of normal physiologic signaling at zinc-modulated synapses (Li et al., 2001), a large amount of vesicular zinc released together with glutamate may enter postsynaptic neurons through glutamate receptors (Weiss and Sensi, 2000; Weiss et al., 2000) or voltage-sensitive calcium channels (Sensi et al., 1999b). Zinc translocation into post-synaptic neurons after hypoglycemia has been demonstrated by our lab (Suh et al., 2004; Suh et al., 2007; Suh et al., 2008). Many brain areas with high vesicular zinc level exhibit high vulnerability to hypoglycemia, but this correlation is not always true. Some brain areas with high vesicular zinc concentration are not correspondingly sensitive to hypoglycemia, and conversely some brain areas that are highly sensitive to hypoglycemia are not rich in vesicular zinc (Frederickson et al., 2000). Thus vesicular zinc is not the sole determinant of neuronal vulnerability to hypoglycemia, but may be a contributory factor in areas where vesicular concentrations are high. The zinc chelator CaEDTA was used to evaluate a causal role for extracellular zinc elevations in subsequent post-synaptic neuronal zinc accumulation and death after hypoglycemia. The utility of CaEDTA as a zinc chelator has been established in ischemia, brain trauma and epilepsy studies (Frederickson et al., 2002; Koh et al., 1996; Lee et al., 2002a). Interestingly, Aizenmann et al. suggested that the large fraction of zinc existing in the form of thiol-zinc-metalloproteins can be released from oxidation of intracellular zinc binding proteins (e.g. metallothionein) by oxidative stress. Zinc liberated in such a manner may then become cytotoxic (Aizenman et al., 2000). Our study showed that application of mild hypothermia significantly reduced hypoglycemia-induce neuronal death by reducing presynaptic zinc release and translocation into postsynaptic neurons (Figure 8) (Shin et al., 2010). Hyperthermia applied after hypoglycemia aggravates this zinc release and translocation compared to normothermia applied animals. From these results, we conclude that neuroprotective effects of mild hypothermia after hypoglycemia can be achieved by reduction of synaptic zinc release and subsequent zinc translocation. However, our study also found that zinc dependent DG neuron degeneration was prevented by the cell permeable zinc chelator, CQ. We therefore

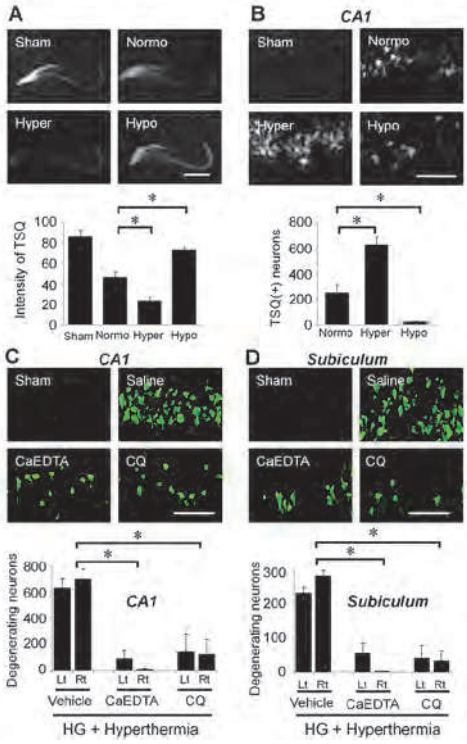

Fig. 8. Temperature dependent hypoglycemic neuronal death is mediated by zinc release and translocation.
(A-D) Vesicular zinc release and translocation is aggravated by hyperthermia but is prevented by hypothermia. (A) represents TSQ fluorescence images of hippocampus from sham operated (Sham) and hypoglycemia (HG) experienced rats. Hypothermia group (Hypo) almost completely prevented synaptic zinc release. Scale bar = 500 μm. (B) Bar graph shows quantitated TSQ fluorescence intensity from hilus area. Data are mean + s.e.m. (n=7-12). * $P <$ 0.05 compared with normothermic reperfusion group. (C) Photomicrographs of TSQ fluorescence staining shows zinc accumulation in the hippocampal CA1 neurons after hypoglycemia. Scale bar = 100 μm. (D) Bar graph shows quantitated TSQ (+) neurons in the CA1 area. Data are mean ± s.e.m. (n = 5-7). *$p < 0.05$ compared with normothermic glucose reperfusion group. (E-H) Zinc chelators, CaEDTA or clioquinol (CQ), prevents hypoglycemia-induced neuronal death. (E and G). FJB (+) neurons were reduced by CaEDTA or CQ injection even after hyperthermic reperfusion. Scale bar = 100 μm. (F and G) graphs represent quantitated neuronal death in the hippocampal CA1 and subiculum area after hypoglycemia. Data are the mean ± s.e.m (n=5-7) *$p < 0.05$ compared with saline treated rats. Part of this figure is modified from our previous published paper (Suh et al., JCBFM, 2010).

cannot exclude the possibility that intracellularly originated free zinc also contributes to hippocampal neuron cell death after hypoglycemia as previously suggested (Aizenman et al., 2000). Anatomical and physiological studies have shown that DG neurons contain a high concentration of vesicular zinc in their synaptic terminals which is released with neuronal activity. Intraneuronal accumulation of zinc may arise from cytoplasmic organelles or

proteins rather than from presynaptic terminals of stratum moleculare. However, the source of intraneuronal accumulation of zinc in DG neurons still requires further study. An additional unsolved question arises regarding how the extracellular zinc chelator, CaEDTA also prevented DG neuron death if intraneuronal zinc accumulation originates from cytoplasmic sources.

Taken together, the present study shows that post-hypoglycemic (glucose reperfusion period) brain temperature can modulate the outcome of brain injury, i.e. hypothermia significantly reduces, while hyperthermia aggravates, neuronal death after hypoglycemia through inhibition of vesicular zinc release, reduction of ROS production and prevention of microglia activation. Therefore, cautious brain temperature monitoring and maintaining lower brain temperature during glucose reperfusion period may predict a better clinical outcome after a severe hypoglycemic episode.

3. Proposed intervention strategies for hypoglycemia-induced neuron death

Taken together the present book chapter suggests a sequence of events that lead to neuronal death after HG/GR. Glucose reperfusion initiates nitric oxide production, which leads to vesicular zinc release, which in turn activates neuronal NADPH oxidase. ROS produced by NADPH oxidase leads to increased zinc accumulation, PARP-1 activation, and resultant cell death. Therefore, based on these studies, the present review suggests that following intervention strategies for preventing hypoglycemia-induced neuron death. As we described in schematic drawing (Figure 9), there are at least 6 different possible approaches.

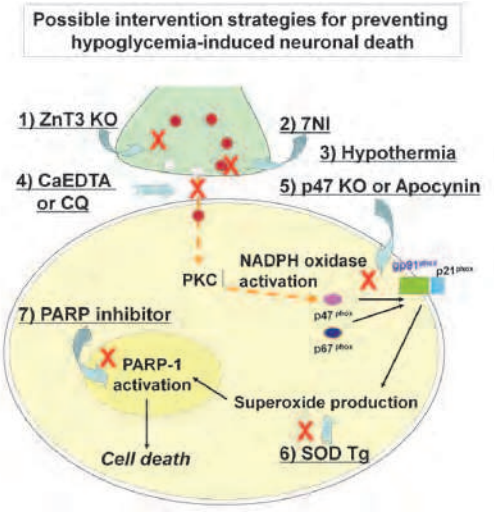

Fig. 9. Proposed intervention strategies for preventing hypoglycemia/ glucose reperfusion-induced neuron death. This schematic drawing indicates that hypoglycemia/ glucose reperfusion-induced neuron death can be prevented by several intervention methods. 1) Vesicular zinc content modulation by gene or chemical manipulation. 2) Vesicular zinc release inhibition by NO inhibitor. 3) Vesicular zinc release inhibition by hypothermia. 4) Zinc chelation in the extracellular space. 5) Inhibition of NADPH oxidase activation. 6) Scavenging or dismutating of reactive oxygen species. 7) Inhibition of PARP-1 activation. Round red colored dot represents ionic zinc. Symbol X represents intervention.

1) Modulation of vesicular zinc release by gene manipulation; 2) Prevention of vesicular zinc release by NOS inhibition; 3) hypothermia; 4) Chelation of extracellular zinc by zinc chelators; 5) Inhibition of NADPH oxidase activation; 6) Increase of SOD function; 7) PARP-1 inhibition. Among them, we speculate that prevention of vesicular zinc release and translocation would be the most promising intervention strategies. However, this intervention strategy requires a highly zinc specific chelator, which also can permeate blood brain barrier and has no side effects. No such agent is currently available and further investigation will be necessary to identify and develop candidate drugs for this purpose.

4. Conclusion

Vesicular zinc release and subsequent translocation of this ion into postsynaptic neurons has been known as a key upstream event of hypoglycemia-induced neuron death. Thus, zinc chelation is a promising target for the treatment of severe hypoglycemia-induced neuron death. However, still further studies will be needed to apply this concept to human.

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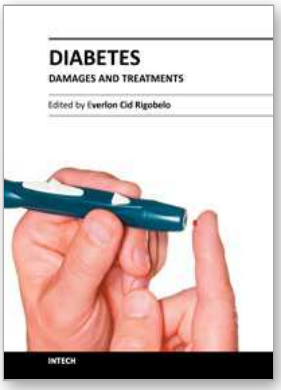

Diabetes - Damages and Treatments
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Over the last few decades the prevalence of diabetes has dramatically grown in most regions of the world. In 2010, 285 million people were diagnosed with diabetes and it is estimated that the number will increase to 438 million in 2030. Hypoglycemia is a disorder where the glucose serum concentration is usually low. The organism usually keeps the serum glucose concentration in a range of 70 to 110 mL/dL of blood. In hypoglycemia the glucose concentration normally remains lower than 50 mL/dL of blood. Hopefully, this book will be of help to many scientists, doctors, pharmacists, chemicals, and other experts in a variety of disciplines, both academic and industrial. In addition to supporting researcher and development, this book should be suitable for teaching.

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