Experimental Neurology 302 (2018) 181–195

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Research Paper

Zn^{2+}-induced disruption of neuronal mitochondrial function: Synergism with Ca^{2+}, critical dependence upon cytosolic Zn^{2+} buffering, and contributions to neuronal injury

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ARTICLE INFO

Keywords:
Zinc
Calcium
Excitotoxicity
Mitochondria
Ischemia
Neuronal cultures
VSCC
Ca^{2+} channel
Metallothionein
Reactive oxygen species

ABSTRACT

Excitotoxic Zn^{2+} and Ca^{2+} accumulation contributes to neuronal injury after ischemia or prolonged seizures. Synaptically released Zn^{2+} can enter postsynaptic neurons via routes including voltage sensitive Ca^{2+} channels (VSCC), and, more rapidly, through Ca^{2+} permeable AMPA channels. There are also intracellular Zn^{2+} binding proteins which can either buffer neuronal Zn^{2+} influx or release bound Zn^{2+} into the cytosol during pathologic conditions. Studies in culture highlight mitochondria as possible targets of Zn^{2+}; cytosolic Zn^{2+} can enter mitochondria and induce effects including loss of mitochondrial membrane potential ($\Delta\Psi_m$), mitochondrial swelling, and reactive oxygen species (ROS) generation. While brief (5 min) neuronal depolarization (to activate VSCC) in the presence of 300 μM Zn^{2+} causes substantial delayed neurodegeneration, it only mildly impacts acute mitochondrial function, raising questions as to contributions of Zn^{2+}-induced mitochondrial dysfunction to neuronal injury.

Using brief high (90 mM) K^+/Zn^{2+} exposures to mimic neuronal depolarization and extracellular Zn^{2+} accumulation as may accompany ischemia *in vivo*, we examined effects of disrupted cytosolic Zn^{2+} buffering and/or the presence of Ca^{2+}, and made several observations: **1.** Mild disruption of cytosolic Zn^{2+} buffering—while having little effects alone—markedly enhanced mitochondrial Zn^{2+} accumulation and dysfunction (including loss of $\Delta\Psi_m$, ROS generation, swelling and respiratory inhibition) caused by relatively low (10–50 μM) Zn^{2+} with high K^+. **2.** The presence of Ca^{2+} during the Zn^{2+} exposure decreased cytosolic and mitochondrial Zn^{2+} accumulation, but markedly exacerbated the consequent dysfunction. **3.** Paralleling effects on mitochondria, disruption of buffering and presence of Ca^{2+} enhanced Zn^{2+}-induced neurodegeneration. **4.** Zn^{2+} chelation after the high K^+/Zn^{2+} exposure attenuated both ROS production and neurodegeneration, supporting the potential utility of delayed interventions. Taken together, these data lend credence to the idea that in pathologic states that impair cytosolic Zn^{2+} buffering, slow uptake of Zn^{2+} along with Ca^{2+} into neurons via VSCC can disrupt the mitochondria and induce neurodegeneration.

1. Introduction

Ischemic stroke is a leading cause of morbidity and mortality to the aging population, but no neuroprotective therapy exists, partly reflecting limited understanding of relevant injury mechanisms. Excitotoxicity, caused by excessive glutamate release, is considered to be a major contributor to neurodegeneration. Prior studies of excitotoxic injury have largely focused on rapid Ca^{2+} entry through N-methyl-D-aspartate (**NMDA**) receptors, and have suggested mitochondria to be critical targets of the cellular Ca^{2+} loads (Choi et al., 1988; Nicholls and Budd, 2000; Rothman and Olney, 1986), but NMDA receptor targeted therapies have shown limited clinical efficacy (Hoyte et al., 2004; Ikonomidou and Turski, 2002).

Additional studies have implicated another highly prevalent divalent cation, Zn^{2+}, which accumulates in neurons after ischemia and prolonged seizures, and contributes to neurodegeneration (Frederickson et al., 1989; Koh et al., 1996; Tonder et al., 1990). Zn^{2+} is stored in presynaptic vesicles, can be released upon activation, and

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; Ca-AMPA, Ca^{2+} permeable AMPA channels; FCCP, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; DTDP, 2,2′-dithiodipyridine; HEt, hydroethidine; MT, metallothionein; NMDA, N-methyl-D-aspartate; TPEN, N,N,N,N-tetrakis(2-pyridylmethyl)ethane-1,2-diamine; OGD, oxygen glucose deprivation; ROS, reactive oxygen species; Rhod123, rhodamine 123; VSCC, voltage sensitive Ca^{2+} channels

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https://doi.org/10.1016/j.expneurol.2018.01.012
Received 28 October 2017; Received in revised form 15 December 2017; Accepted 15 January 2018

enters postsynaptic neurons ("**Zn^{2+} translocation**") through routes including voltage sensitive Ca^{2+} channels (**VSCC**) (Weiss et al., 1993), and with greater rapidity, through the subset of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid channels that are both Ca^{2+} and Zn^{2+} permeable (**Ca-AMPA**) (Jia et al., 2002; Sensi et al., 1999a; Yin and Weiss, 1995). Zn^{2+} has potent effects on isolated mitochondria (Brown et al., 2000; Dineley et al., 2005; Gazaryan et al., 2007; Jiang et al., 2001; Link and von Jagow, 1995; Sensi et al., 2003; Skulachev et al., 1967), and neuronal Zn^{2+} loading triggered by rapid entry through Ca-AMPA channels induces acute mitochondrial dysfunction, including reactive oxygen species (**ROS**) generation (Sensi et al., 1999a; Sensi et al., 2000), with greater potency than Ca^{2+}, suggesting that mitochondria might be critical targets of Zn^{2+} effects. However, while slower Zn^{2+} entry through VSCC caused considerable delayed neurodegeneration, these exposures had relatively little impact on acute mitochondrial function (Sensi et al., 1999a; Weiss et al., 1993), raising doubt that Zn^{2+} translocation contributes importantly to mitochondrial dysfunction in pathological conditions (Pivovarova et al., 2014).

It is now apparent that in addition to direct entry of extracellular Zn^{2+}, another critical determinant of cytosolic (and mitochondrial) Zn^{2+} accumulation is the presence of Zn^{2+} buffering proteins—like metallothioneins (**MTs**)—which normally buffer Zn^{2+} loads, but can also provide a source from which bound Zn^{2+} can be released by oxidative stress/acidosis, as can occur during pathological conditions (Malaiyandi et al., 2004; Maret, 2011; Maret and Vallee, 1998). Indeed, strong disruption of these intracellular Zn^{2+} pools causes acute cytosolic and mitochondrial Zn^{2+} accumulation even without Zn^{2+} translocation (Sensi et al., 2003), and can trigger slow Zn^{2+} dependent neuronal injury (Aizenman et al., 2000).

However, little is known about the respective contributions of each of these sources to mitochondrial dysfunction; indeed, only few studies to date have begun to explore the idea that the integrity of cytosolic buffering may critically modulate the effects of exogenous Zn^{2+} entry on mitochondrial function in cultured neurons (Clausen et al., 2013; Sensi et al., 2003). Furthermore, as most early studies were carried out in Ca^{2+} free media to ensure observation of Zn^{2+} specific effects, there is debate about the respective contributions of Ca^{2+} and Zn^{2+} to mitochondrial dysfunction observed *in vivo*, with some proposing synergy between these ions (Gazaryan et al., 2007; Jiang et al., 2001; Sensi et al., 2000) while others see little evidence for Zn^{2+} contributions (Devinney et al., 2009; Pivovarova et al., 2014).

The present study seeks to model early Zn^{2+} dependent events in ischemic neuronal injury to quantitatively examine how disrupted cytosolic Zn^{2+} buffering and the presence of Ca^{2+} modulate the consequences of moderate exogenous Zn^{2+} loads on mitochondrial function and cell death. To this aim, we use brief high K^+/Zn^{2+} exposures (to mimic neuronal depolarization and extracellular Zn^{2+} accumulation as may accompany ischemia *in vivo*), and find that both disrupted buffering and the presence of Ca^{2+} strongly increase the impact of low Zn^{2+} exposures on mitochondrial function and cell death, with greater synergistic effects when combined. These findings support the hypothesis that slow Zn^{2+} entry into depolarized neurons could well contribute to mitochondrial dysfunction and neurodegeneration *in vivo*. Furthermore, Zn^{2+} chelation after the Zn^{2+} load diminishes both mitochondrial ROS generation and cell death, supporting the idea that delayed interventions targeting mitochondrial Zn^{2+} could provide therapeutic benefits.

2. Material and methods

2.1. Ethics statement

This study was carried out in accordance with the recommendations from the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

2.2. Cortical cultures

Primary mixed cortical cultures were prepared as described previously (Yin et al., 1994). Briefly, cell suspensions from neocortical regions of CD-1 mouse embryos (gender not determined) from 15 to 16 gestational day timed-pregnant mice (ordered from Charles River Crl:CD1[ICR]) were extracted and plated on astrocytic monolayers in glass-bottomed dishes, on culture treated 24 well microplates, or on Seahorse XF24 cell culture microplates. Cells were maintained in media consisting of Minimum Essential Medium (**MEM**) supplemented with 10% heat-inactivated horse serum, 10% fetal bovine serum, 2 mM glutamine, and 25 mM glucose, and kept in 37 °C/5% CO_2 incubator. 2–3 days after dissection, the cultures were switched to an identical maintenance medium lacking fetal bovine serum and non-neuronal cell division halted by adding 10 μM cytosine arabinoside for 24 h. To prepare glial cultures (used to establish astrocytic monolayers described above), the same protocol was used, except the following: 1) tissues were obtained from 1 to 3 days old postnatal mice (gender not determined), 2) plating media was supplemented with epidermal growth factor (10 ng/ml), and 3) suspensions were directly plated on poly-D-lysine and laminin-coated coverslips and/or tissue culture treated plates.

2.3. Reagents and indicators

Hydroethidine (**HEt**) was purchased from Assay Biotech (Sunnyvale, CA). Newport Green, FluoZin-3 AM, MitoTracker Green, Pluronic F-127, MEM, fetal bovine serum, glutamine, and horse serum were purchased from Life Technologies (Grand Island, NY). N-methyl-D-aspartate (**NMDA**), 2,2′-dithiodipyridine (**DTDP**), Rhodamine 123 (**Rhod123**), and N,N,N,N-tetrakis(2-pyridylmethyl)ethane-1,2-diamine (**TPEN**) were purchased from Sigma-Aldrich (St. Louis, MO). Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (**FCCP**) was purchased from Tocris Bioscience (Ellisville, MO), apocynin obtained from Acros Organics (Morris Plains, NJ), and XF Base Medium (minimal Dulbecco's Modified Eagle's Medium) from Agilent Technologies (Santa Clara, CA). All other chemicals and reagents were purchased from common commercial sources.

2.4. Zn^{2+}/Ca^{2+} loading

Prior to all experiments, cultured neurons were removed from the incubator and placed in HEPES-buffered media (consisting of [in mM] 120 NaCl, 5.4 KCl, 0.8 $MgCl_2$, 20 Hepes, 15 glucose, 10 NaOH, in pH 7.4) with either 1.8 mM $CaCl_2$ (**1.8 Ca^{2+} HSS**) or 0 mM $CaCl_2$ (**0 Ca^{2+} HSS**) at room temperature. Cultures were maintained in HSS (± Ca^{2+}) for 10 min, followed where indicated by addition of "pre-treatment" (with DTDP and/or TPEN) for 10 min prior to induction of Zn^{2+} and/or Ca^{2+} loading. To do so, neurons were exposed to indicated levels of Zn^{2+} (0–300 μM) and/or 1.8 mM Ca^{2+} in 90 mM K^+ HSS ("**high K^+**"; HSS modified with 90 mM K^+ and Na^+ adjusted to 35 mM to maintain osmolarity) for 5 min to depolarize neurons, inducing ion entry through VSCC. When Ca^{2+} was present during exposure (with or without Zn^{2+}), the NMDA antagonist MK-801 (10 μM) was added to inhibit Ca^{2+} entry through NMDA receptors. After the 5 min exposure in high K^+, neurons were washed 3 times into HSS (± Ca^{2+}, DTDP and TPEN as present before the exposure) for durations indicated. In addition, as we have found highly Ca^{2+} or Zn^{2+} permeable Ca-AMPA channels to be expressed in a small subset (~13%) of cultured cortical neurons (Yin et al., 1994), we carried out additional controls using the AMPA channel antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (**NBQX**; 10 μM) during the exposures (Sensi et al., 1999a), and found no differences.