ELSEVIER

Mitochondrion 5 (2005) 55–65

Mitochondrion

www.elsevier.com/locate/mito

Zinc causes loss of membrane potential and elevates reactive oxygen species in rat brain mitochondria

Kirk E. Dineley[1], Lauren L. Richards, Tatyana V. Votyakova, Ian J. Reynolds*

Department of Pharmacology, University of Pittsburgh, W1351 Biomedical Science Tower, Pittsburgh, PA 15261, USA

Received 26 July 2004; received in revised form 11 November 2004; accepted 18 November 2004

Abstract

Emerging evidence suggests that Zn^{2+} may impair neuronal metabolism. We examined how Zn^{2+} affects the activity of isolated brain mitochondria fueled with glutamate + malate, succinate or glycerol 3-phosphate. Submicromolar levels of Zn^{2+} dissipated membrane potential and inhibited oxygen utilization in all three substrate conditions. Zn^{2+}-induced depolarization was reversed by the membrane-impermeant metal chelator, EGTA, and was inhibited by uniporter blockade. Cyclosporin A did not block Zn^{2+}-induced depolarization. Added Zn^{2+} increased accumulation of reactive oxygen species (ROS) in glutamate + malate or glycerol 3-phosphate conditions, but inhibited succinate-supported ROS accumulation. These results show that Zn^{2+} blocks mitochondrial function in all physiologically relevant substrate conditions.

Keywords: ROS; Spectrofluorometry; Permeability transition; Calcium uniporter; Excitotoxicity; Neurodegeneration

1. Introduction

Mitochondria are critical for maintaining neuronal homeostasis. In addition to producing large quantities of ATP required for neuronal function, mitochondria are important regulators of intracellular calcium, the major site of reactive oxygen species (ROS) production, and are thought to be key activators of programmed cell death. While mitochondrial uptake of calcium as well as limited production of ROS appear necessary for normal cellular functions, either in excess contributes to neuronal injury. Under such stresses, mitochondria can initiate cell death by releasing pro-apoptotic factors such as cytochrome C and apoptosis inducing factor (AIF). Given their central role in both normal and pathological cellular processes, it is not surprising that mitochondrial disruption is considered a critical event in various neurodegenerative schemes, including models of stroke, amyotrophic lateral sclerosis, and Parkinson's (for reviews, see Nicholls and Budd, 2000; Zamzami and Kroemer, 2001).

Abbreviations used: CsA, cyclosporin A; $\Delta\Psi_m$, mitochondrial membrane potential; ETC, electron transport chain; G+M, glutamate and malate; G3P, glycerol 3-phosphate; MPT, mitochondrial permeability transition; ROS, reactive oxygen species; $[Zn^{2+}]$, free Zn^{2+} concentration.

* Corresponding author. Tel.: +1 412 648 2134, fax: +1 412 624 0794

E-mail address: iannmda@pitt.edu (I.J. Reynolds).

[1] Department of Biology, Francis Marion University, Florence, SC 29501, USA.

1567-7249/$ - see front matter © 2004 Elsevier B.V. and Mitochondria Research Society. All rights reserved.
doi:10.1016/j.mito.2004.11.001

Zinc is present in cells at concentrations up to approximately 200 μM. The vast majority of cellular zinc is bound to intracellular sites such as proteins, consequently there is very little free ionic zinc (Zn^{2+}) in the cytoplasm. Tight regulation of intracellular free Zn^{2+} is necessary, because excessive elevation of free zinc is toxic (Weiss et al., 2000). Live cell estimation of free zinc is difficult (Dineley et al., 2002), however it has been reported that free zinc in the range of several hundred nanomolar kills neurons (Canzoniero et al., 1999). While Zn^{2+} can enter neurons through membrane channels and transporters, increasing evidence suggests that zinc-mediated neuronal death is caused by liberating zinc from intracellular sites. For instance, several reports suggest that oxidant-mediated neuronal injury involves mobilization of excessive amounts of zinc (Cuajungco and Lees, 1998; Aizenman et al., 2000), presumably from metalloproteins (Maret and Vallee, 1998). Other reports suggest that zinc causes ROS accumulation in live cells, possibly from multiple mechanisms including inhibition of mitochondria (Sensi et al., 1999) and activation of NADPH oxidase (Noh et al., 1999).

Precisely how excess $[Zn^{2+}]_i$ kills neurons remains unclear, but increasing evidence indicates that Zn^{2+} impedes cellular energy production (reviewed by Dineley et al., 2003). The impact of Zn^{2+} on the activity of isolated mitochondria has been explored with varying results. Zn^{2+} probably inhibits electron transport at the bc_1 complex (Lorusso et al., 1991; Link and von Jagow, 1995; Berry et al., 2000), but there may be additional sites of inhibition in the electron transport chain (Skulachev et al., 1967; Nicholls and Malviya, 1968) or the tricarboxylic acid cycle (Brown et al., 2000). Also, there is conflicting evidence with respect to Zn^{2+}-induced permeability transition (Wudarczyk et al., 1999; Brown et al., 2000; Jiang et al., 2001). A number of factors could contribute to these disparate conclusions including assay conditions using ion versus sucrose-based recording solutions, or substantial variations in the amount of Zn^{2+} used (from $\sim 10^{-9}$ to $> 10^{-5}$ M). In any case, because the majority these studies used non-neural mitochondria they may be of limited relevance to neurodegeneration.

The present study employs fluorometric and polarographic techniques to directly determine how Zn^{2+} alters membrane potential ($\Delta\psi_m$), O_2 utilization, and ROS accumulation in isolated brain mitochondria fueled by three different substrate conditions. Because these substrates enter the electron transport chain at different sites, this approach may provide insight regarding the important site(s) of Zn^{2+} inhibition. More importantly, this approach reveals what alternate catabolic pathways, if any, mitochondria may use when challenged by Zn^{2+}. We found that $[Zn^{2+}]$ in the approximate range of 100–200 nM inhibits O_2 utilization, dissipates $\Delta\psi_m$, and alters ROS accumulation in a substrate dependent fashion. Together, these results show that neurotoxic concentrations of zinc profoundly inhibit mitochondrial activity and suggest several important mechanisms for Zn^{2+}-mediated neuronal death. Some of these results have been presented previously in an abstract (Dineley et al., 2001).

2. Materials and methods

2.1. Isolation of rat brain mitochondria

All procedures using rats were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh and are consistent with guidelines provided by the National Institutes of Health. Rat brain synaptosomal mitochondria were isolated from the cortex of adult Sprague Dawley rats by conventional differential centrifugation as described by Rosenthal et al. (1987) with minor modifications. Following removal, the brain was homogenized at 0–2 °C in an isolation buffer (IB) containing (in mM): 225 mannitol; 75 sucrose; 5 HEPES; 0.5 EDTA; with 1 mg/mL BSA and adjusted to pH 7.35 with KOH. The supernatant was collected after two rounds of centrifugation at 2000*g* (three minutes each), then was then centrifuged for 10 min at 10,500*g* to yield the first mitochondrial pellet. The first pellet was resuspended in IB containing digitonin (0.013%) and centrifuged again. The supernatant was discarded, the pellet was resuspended in IB with 10 μM EGTA and no EDTA, and the final pellet was retrieved after a final round of centrifugation. Supernatant was discarded, and the pellet collected in ~100 μL of the EGTA buffer, typically yielding a final concentration of 20–30 mg protein/mL as