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Synaptic and nonsynaptic mitochondria demonstrate a different degree of calcium-induced mitochondrial dysfunction

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ABSTRACT

Aims: Since variety in response to Ca^{2+}-induced mitochondrial dysfunction in different neuronal mitochondrial populations is associated with the pathogenesis of several neurological diseases, we investigated the effects of Ca^{2+} overload on synaptic (SM) and nonsynaptic mitochondrial (NM) dysfunction and probed the effects of cyclosporin A (CsA), 4′-chlorodiazepam (CDP) and Ru360 on relieving mitochondrial damage.
Main methods: SM and NM mitochondria were isolated from rats' brains (n = 5/group) and treated with various concentrations (5, 10, 100, and 200 μM) of Ca^{2+}, with and without CsA (mPTP blocker), CDP (PBR/TSPO blocker) and Ru360 (MCU blocker) pretreatments. Mitochondrial function was determined by mitochondrial swelling, ROS production and mitochondrial membrane potential changes ($\Delta\Psi m$).
Key findings: At 200-μM Ca^{2+}, SM presented mitochondrial swelling to a greater extent than NM. At 100 and 200-μM Ca^{2+}, the ROS production of SM was higher than that of NM and $\Delta\Psi m$ dissipation of SM was also larger. CsA, CDP and Ru360 could reduce ROS production of SM and NM with exposure to 200-μM Ca^{2+}. However, only Ru360 could completely inhibit ROS generation in both SM and NM, whereas CsA and CDP could only partially reduce the ROS level in SM. Moreover, CsA and CDP pretreatments were not able to restore $\Delta\Psi m$. However, Ru360 pretreatment could protect $\Delta\Psi m$ dissipation in both SM and NM, with complete protection observed only in NM.
Significance: Our findings suggested that mitochondrial calcium uniporter is a possible major pathway for calcium uptake in both mitochondrial populations. However, SM might have additional pathways involved in the calcium uptake.

Introduction

Mitochondria are critical regulators of neuronal cell survival and cell death (Boitier et al., 1999; Hyder et al., 2006; Murchison and Griffith, 2007; Yuan and Yankner, 2000). The pathophysiology of numerous neurological disorders, such as ischemic-reperfusion injury (Blomgren et al., 2003; Pandya et al., 2011), traumatic brain injury (Friberg and Wieloch, 2002; Norenberg and Rao, 2007) and neurodegenerative diseases (Reddy and Reddy, 2011; Reeve et al., 2008), is related to mitochondrial dysfunction, which leads to neuronal apoptosis. The most important factor inducing mitochondrial dysfunction is Ca^{2+} overload, which primarily occurs during neuroexcitotoxicity (Duchen, 2004; Nicholls, 2009; Starkov et al., 2004). Ca^{2+} overload is involved with mitochondrial membrane potential depolarization ($\Delta\Psi m$ dissipation), which has been proposed as an initiator as well as a consequence of mitochondrial transition pore (mPTP) opening (Ly et al., 2003; Wadia et al., 1998). Reactive oxygen species (ROS) are also crucial players in neuronal mitochondrial dysfunction (Wang et al., 2011). However, the relationship between Ca^{2+} overload and ROS in brain mitochondria is diverse depending on experimental conditions (Gyulkhandanyan and Pennefather, 2004; Komary et al., 2008; Panov et al., 2007; Petrosillo et al., 2004; Schonfeld and Reiser, 2007; Votyakova and Reynolds, 2005). Moreover, the correlation between Ca^{2+}-induced mitochondrial damage, $\Delta\Psi m$ dissipation, and ROS production is still controversial (Adam-Vizi and Starkov, 2010).

Brain mitochondria are classified into two groups, the synaptic mitochondria (SM) and the nonsynaptic mitochondria (NM). The properties of these two types of mitochondria especially in Ca^{2+} handling are different (Guo et al., 2005; Li et al., 2004). SM, which are located around the synapse, are exposed to extensive Ca^{2+} fluctuations and are at high risk for oxidative stress and Ca^{2+} accumulative damages (Banaclocha et al., 1997; Martinez et al., 1996). A previous study reported that the difference in the Ca^{2+}-induced mPTP opening could be due to the higher level of cyclophilin D (CypD) in SM (Naga et al., 2007). However, direct inhibition of CypD by cyclosporine A (CsA) cannot increase the Ca^{2+} accumulation capacity in SM (Brown et al., 2006), suggesting that additional mechanisms are likely

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responsible for the differences in Ca^{2+} handling in SM and NM. The main portal pathway for Ca^{2+} uptake of neuronal mitochondria proposed by previous studies is the mitochondrial calcium uniporter (MCU). However, subsequent studies of other tissues have discovered additional Ca^{2+} uptake mechanisms such as the rapid mode of Ca^{2+} uptake (RAM) (Buntinas et al., 2001; Sparagna et al., 1995), and the mitochondrial ryanodine receptor (mRYR) (Altschafl et al., 2007; Beutner et al., 2001; Beutner et al., 2005). Moreover, the Ca^{2+} uptake mechanism of SM and NM in the Ca^{2+} overload condition has not been investigated and whether Ca^{2+} entry via MCU in the SM and NM is different is not known. Therefore, in this study, we tested the hypothesis that 1) SM respond to Ca^{2+} overload conditions in a different way from NM, and 2) the mechanisms for Ca^{2+} entry via MCU in the SM and NM are different.

Materials and methods

Reagents

All of the reagents used in this study were purchased from Sigma (St. Louis, MO., USA), except Ru360, which was purchased from Calbiochem (San Diego, CA., USA).

Bovine serum albumin (BSA) and pyruvic acid were purchased from Amresco (Solon, OH., USA). CsA and CDP were prepared in DMSO and further diluted to final concentrations by 2% DMSO. Ru360 was prepared in deionized water.

Animal preparation

This study was approved by the Institutional Animal Care and Use Committee at the Faculty of Medicine, Chiang Mai University. Wistar rats (300–400 g) were obtained from the National Laboratory Animal Center, Mahidol University, Bangkok, Thailand. All animals were housed in a controlled room temperature maintained between 22 and 25 °C in a constant 12-h light/dark cycle. They were fed with standard pellet rat diet and water ad libitum.

Experimental protocols

Isolated mitochondria from synaptosomes and nonsynaptosomes of rat cortical brains were used as in a previous study (Chelli et al., 2001; Novalija et al., 2003; Thummasorn et al., 2011; Tong et al., 2005). The first protocol was to investigate the effect of Ca^{2+}-induced mitochondrial dysfunction on synaptic versus nonsynaptic mitochondria in the morphological aspects of mitochondrial swelling, ROS production and $\Delta\Psi m$ dissipation. In this study, $CaCl_2$ at concentrations of 5, 10, 100 and 200 μM were used (n = 6/group for ROS and $\Delta\Psi m$ measurements). Various doses of $CaCl_2$ were applied for 10 min to isolated mitochondria before the assessment of all parameters. In the second protocol, the mechanism underlying the differences in Ca^{2+} responses of SM and NM was investigated by several pharmacological interventions: cyclosporine A (CsA), 4′-chlorodiazepam (CDP) and Ru360 (as shown in Fig. 1). CsA is known as a mitochondrial permeability transition pore blocker, CDP is the specific peripheral benzodiazepine receptor (or presently known as translocator protein; TPSO) antagonist, and Ru360 is the mitochondrial calcium uniporter blocker. In the second protocol, both SM and NM were randomly assigned into eight groups: control (vehicle), CsA (5 μM), CDP (100 μM), Ru360 (10 μM), $CaCl_2$ (200 μM), $CaCl_2$ pretreated with CsA, $CaCl_2$ pretreated with CDP and $CaCl_2$ pretreated with Ru360 (n = 5/group). CsA and CDP were applied to the mitochondria for 30 min prior to exposure to Ca^{2+} or vehicle for 30 min, while Ru360 was added at 3 min before Ca^{2+} exposure. Doses of all blockers were used according to those reported previously (Thummasorn et al., 2011; Zhang et al., 2006).

Isolation of nonsynaptic brain mitochondria

Nonsynaptic brain mitochondria were isolated from 300–350 g male Wistar rats using a method modified from that described in a previous study (Lai and Clark, 1979; Clark and Nicklas, 1970; Krasnikov et al., 2005). Following decapitation, brains were rapidly removed and placed in ice-cold isolation buffer containing 320 mM sucrose, 10 mM HEPES, and 0.5 mM EGTA at pH 7.4. All homogenization and centrifugation steps were carried out at 4 °C. The cortices were chopped into small pieces with razors. Then, the brains were homogenized by the homogenizer containing 10 ml of isolation buffer. The homogenate was centrifuged at 1300 *g* for 3 min. The supernatant was centrifuged at 1300 *g* for 3 min. The supernatant was collected and centrifuged at 17,000 *g* for 8 min. The pellets obtained from this step were suspended in 3 ml of isolation buffer and applied to the top of 3 ml of 10% (w/v) Ficoll overlaid by 3 ml of 7.5% (w/v) Ficoll. After centrifugation at 99,000 *g* for 20 min, aliquots were removed. The nonsynaptic mitochondria-containing pellets at the bottom of the tube were resuspended in isolation buffer and recentrifuged at 12,000 *g* for 8 min. The pellet was resuspended in an isolation buffer supplemented with 0.5 mg/ml BSA and centrifuged at 12,000 *g* for 8 min. Protein concentration was determined using a Bicinchoninic Acid (BCA) assay with bovine serum albumin used as a concentration standard (Walker, 1994).

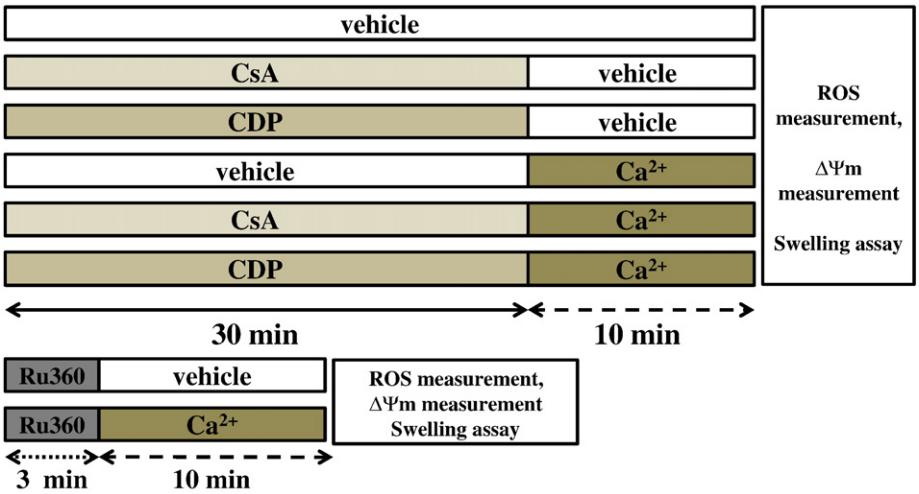

Fig. 1. Study protocol to determine the effects of cyclosporine A (CsA), 4′-chlorodiazepam (CDP) and Ru360 on Ca^{2+}-induced mitochondrial dysfunction.