

Cite this article as: Neural Regen Res. 2012;7(2):109-113.

# The calcium uniporter regulates the permeability transition pore in isolated cortical mitochondria<sup>★</sup>

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## Abstract

To investigate the influence of the mitochondrial calcium uniporter on the mitochondrial permeability transition pore, the present study observed mitochondrial morphology in cortical neurons isolated from adult rats using transmission electron microscopy, and confirmed the morphology and activity of isolated mitochondria by detecting succinic dehydrogenase and monoamine oxidase, two mitochondrial enzymes. Isolated mitochondria were treated with either ruthenium red, an inhibitor of the uniporter, spermine, an activator of the uniporter, or in combination with cyclosporin A, an inhibitor of the mitochondrial permeability transition pore. Results showed that ruthenium red inhibited CaCl<sub>2</sub>-induced mitochondrial permeability transition pore opening, spermine enhanced opening, and cyclosporin A attenuated the effects of spermine. Results demonstrated that the mitochondrial calcium uniporter plays a role in regulating the mitochondrial permeability transition pore in mitochondria isolated from the rat brain cortex.

**Key Words:** mitochondria; calcium uniporter; permeability transition pore; neuron; cortex; cell death; cerebroprotection

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Received: 2011-10-19  
Accepted: 2011-12-20  
(N20110927001/WLM)

Yu N, Wang SL, Wang P, Li Y, Li SH, Wang L, Chen HB, Wang YT. The calcium uniporter regulates the permeability transition pore in isolated cortical mitochondria. Neural Regen Res. 2012;7(2):109-113.

www.crter.cn  
www.nrronline.org

doi:10.3969/j.issn.1673-5374.2012.02.005

## INTRODUCTION

Mitochondria play an important role in energy metabolism, Ca<sup>2+</sup> signaling, aging and apoptotic cell death by releasing several apoptotic factors such as cytochrome c, apoptosis-inducing factor and procaspases<sup>[1]</sup>. Mitochondria are spatiotemporal modulators of cytosolic Ca<sup>2+</sup> as they have the ability to both sequester and release Ca<sup>2+</sup><sup>[2-5]</sup>. Moreover, calcium signals have been identified as one of the major signals that converge on mitochondria to trigger the mitochondrial-dependent pathway of apoptotic cell death<sup>[1, 6-12]</sup>. Physiological mitochondrial Ca<sup>2+</sup> efflux is attributed to either activation of the permeability transition pore (PTP) or reversal of the mitochondrial calcium uniporter (MCU)<sup>[4, 6, 10]</sup>. The MCU, located on the inner mitochondrial membrane, is the primary influx pathway for Ca<sup>2+</sup> in mitochondria, and hence is a key regulator of mitochondrial Ca<sup>2+</sup><sup>[5, 13-16]</sup>. The MCU controls the rate of energy production, shapes the amplitude and spatiotemporal patterns of intracellular Ca<sup>2+</sup> signals, and is instrumental to cell death<sup>[5]</sup>. The mitochondrial PTP (mPTP) is defined as a voltage-dependent, cyclosporine A-sensitive, high-conductance inner membrane

channel<sup>[17-21]</sup>. If a Ca<sup>2+</sup> concentration gradient exists between the matrix and the external medium, onset of permeability transition will lead to Ca<sup>2+</sup> release<sup>[22-23]</sup>. However, ischemia/reperfusion injury of neurons was closely related to opening of the mPTP<sup>[24-25]</sup>, which has been recognized as a critical area in mitochondrial-mediated apoptosis<sup>[19, 26-30]</sup>. Recent evidence has shown that the perfusion of Ru360, which is a selective and potent MCU blocker, prevented opening of the mPTP in mitochondria isolated from reperfused hearts<sup>[31]</sup>. In addition, it has been demonstrated that the MCU is involved in the cardioprotection conferred by ischemic preconditioning, and that activity of the mPTP may be related to MCU activity<sup>[32]</sup>. Accordingly, we conclude that the MCU may play a role in regulating the mPTP, and this regulatory relationship could be of great significance to cerebral protection. The present study attempted to verify the conclusion using electron microscopic observation, enzyme activity detection and spectrophotometry.

## RESULTS

### Ultrastructure of isolated mitochondria from cortical neurons

Following transmission electron microscopy,

normal mitochondria showed intact membranes and a dense matrix space (Figure 1A). However, incubation with CaCl<sub>2</sub> induced mitochondrial swelling (Figure 1B), separated the inner and outer mitochondrial membrane, ruptured cristae, and a non-dense matrix was evident<sup>[32-35]</sup>. At the end of the experiment, we examined the treated mitochondria by transmission electron microscopy. The degree of swelling in the ruthenium red group (Figure 1C) was attenuated compared with the control group. Mitochondrial electron photomicrographs from the spermine group (Figure 1D) and cyclosporine A + spermine group (Figure 1E) were similar to the control group, and in some cases they were difficult to distinguish.

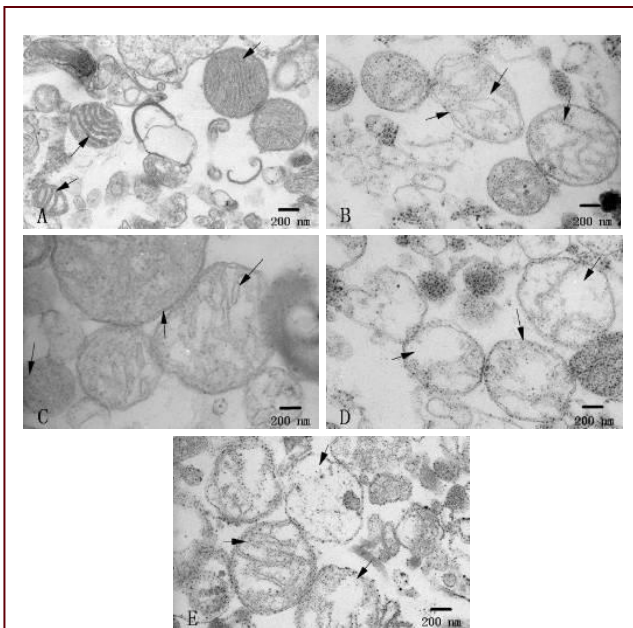


Figure 1 Electron photomicrographs of mitochondria (scale bars: 200 nm).

(A) Normal mitochondria: following fixation, intact membranes and dense matrix were observed (arrows).

(B) Control group: incubation with CaCl<sub>2</sub> induced mitochondrial swelling, separated inner and outer mitochondrial membrane, ruptured cristae, and a non-dense matrix was observed (arrows).

(C) Ruthenium red group: some comparatively intact membranes and dense matrix space were also observed (arrows).

(D) Spermine group: separated inner and outer mitochondrial membrane, ruptured cristae, and a non-dense matrix space were clearly observed (arrows).

(E) Cyclosporine A plus spermine group: the degree of swelling (arrows) was similar to that of the control group.

### Succinic dehydrogenase (SDH) and monoamine oxidase (MAO) activities in isolated mitochondria from cortical neurons

The activities of the mitochondrial marker enzymes were assessed as soon as possible to prevent enzyme inactivation. The activities of SDH and MAO were higher in isolated mitochondria than in the cytosol ( $P < 0.01$ ),

especially SDH activity (Table 1), suggesting that isolated mitochondria have normal structure and function.

Table 1 Activities of succinic dehydrogenase (U/min/mg) and monoamine oxidase (U/h/mg) in mitochondria and the cytosol (specific activity)

Mitochondrial marker enzyme	Isolated mitochondria	Cytosol
Succinic dehydrogenase	115.07±9.56 <sup>a</sup>	7.80±2.02
Monoamine oxidase	20.31±2.22 <sup>a</sup>	5.02±1.64

Values represent mean ± SD of eight independent experiments. <sup>a</sup> $P < 0.01$ , vs. cytosol (Student's *t*-test).

### Regulatory effect of MCU activity on mPTP in isolated mitochondria (Figure 2)

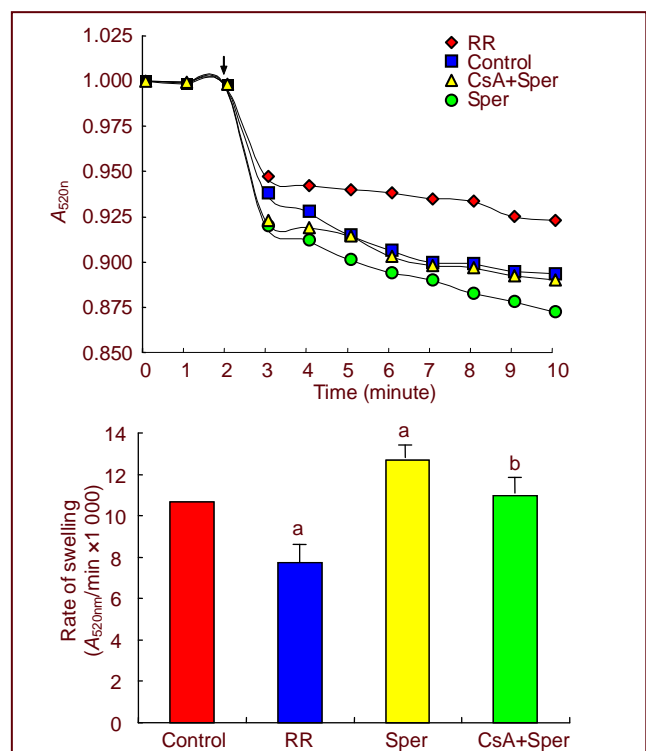


Figure 2 Effects of ruthenium red (RR), spermine (Sper), and cyclosporine A (CsA)+Sper treatment on mitochondrial permeability transition pore opening.

(A) Pore opening was determined by an absorbance decrease at 520 nm ( $A_{520nm}$ ) after the addition of CaCl<sub>2</sub> (200 μM). RR (5 μM) or CsA (2 μM) was added 2 minutes before Ca<sup>2+</sup> addition, Sper (100 μM) and Ca<sup>2+</sup> were added together<sup>[32]</sup>. The amplitude of swelling was observed for 10 minutes. All data are expressed relative to an initial absorbance of 1<sup>[36]</sup>.

(B) Maximal swelling rates of mitochondria. Values represent mean ± SD of 8 independent experiments. <sup>a</sup> $P < 0.01$ , vs. control group; <sup>b</sup> $P < 0.01$ , vs. Sper group (one-way analysis of variance).

Induction of mitochondrial swelling in isolated mitochondria was determined by absorbance decreases at 520 nm. Pore opening was initiated by the addition of CaCl<sub>2</sub> (200 μM). The absorbance decreased rapidly after the addition of Ca<sup>2+</sup>. To explore the possibility that mPTP

opening may be regulated by the activity of the MCU, a group of isolated mitochondria were preincubated (2 minutes) with the MCU inhibitor ruthenium red (5  $\mu$ M). This treatment inhibited pore opening induced by  $Ca^{2+}$ . Meanwhile, spermine (100  $\mu$ M), an activator of the MCU, added together with  $Ca^{2+}$  accelerated the opening of the pore, and this was attenuated by cyclosporine A (2  $\mu$ M), a specific pore inhibitor (Figure 2).

## DISCUSSION

In the present study, we detected that inhibition of the MCU with ruthenium red diminished the opening of the mPTP in isolated brain mitochondria, and activation of the MCU with spermine increased pore opening. Furthermore, cyclosporin A, a specific pore inhibitor, attenuated the effect of spermine. In addition, the effect of Ru360, a highly specific MCU inhibitor, was similar and more obvious than ruthenium red (data not shown). Accordingly, the MCU may be involved in the regulation of the mPTP in mitochondria isolated from the rat brain. Regulation of the mPTP by the MCU in liver and heart isolated mitochondria has been mentioned. For example, the MCU and rapid mode of calcium uptake, a mechanism of calcium uptake, mediated the two physiological roles-control of the rate of cellular adenosine triphosphate (ATP) production and induction of permeability transition and apoptosis. In addition, a previous study suggested that the MCU, but not the rapid mode, played the primary role in permeability transition induction<sup>[3]</sup>. Inhibition of the MCU by Ru360 could prevent opening of the mPTP in post-ischemic rat hearts<sup>[31]</sup>, demonstrating that the MCU was likely to be involved in the regulation of the mPTP. This hypothesis has been preliminarily proven by another study that showed activity of the mPTP may be related to MCU activity in mitochondria isolated from the heart<sup>[32]</sup>. MCU is a key regulator of mitochondrial  $Ca^{2+}$ , and is instrumental to cell death<sup>[5, 13-16]</sup>. In addition, the mPTP has been shown to signal necrotic and apoptotic cell death<sup>[37-38]</sup>. Previous studies of rat heart mitochondria indicated that preventing or reducing mitochondrial accumulation by inhibiting the MCU may benefit the heart during ischemia and reperfusion, *i.e.*, blockade of the MCU during reperfusion provided protection against ischemia/reperfusion injury. In contrast, activating the MCU prohibited myocardial protection of ischemic preconditioning, and cyclosporin A abolished this effect<sup>[32]</sup>. In addition, regulation of the mPTP by the MCU was involved in myocardial protection of remote preconditioning. In the present study, two properties of brain mPTP were detected, which were different to those observed in mitochondria from other tissues, as described previously<sup>[33, 39-41]</sup>. Rat brain mitochondrial swelling was slower and milder; moreover, rat brain mitochondria were less sensitive to cyclosporin A, compared with the rat liver and heart mPTP<sup>[33, 39-41]</sup>.

However, the exact relationship between the MCU and the mPTP in isolated brain mitochondria remains unclear. The mPTP open-closed states are modulated by the transmembrane electrical potential, by matrix pH, by redox potential, by adenine nucleotides, and by divalent cations (*e.g.*  $Me^{2+}$ ,  $Mg^{2+}$ ,  $Sr^{2+}$  and  $Mn^{2+}$ )<sup>[21, 28, 42-45]</sup>. Zhang *et al.*<sup>[32]</sup> speculated that the activity of the MCU was related to the mitochondrial membrane *via* mitochondrial ATP-sensitive potassium channel activation, *i.e.*, mitochondrial membrane depolarization inhibited MCU activity, and subsequently mPTP opening was inhibited. Similarly, in one of our previous studies, we found that inhibiting the MCU alleviated ischemia/reperfusion injury, which may be related to the decline in reactive oxygen species generation and caspase-3 activation<sup>[46]</sup>. Accordingly, we assume that the mechanism may include oxidation-reduction status, calcium content of the mitochondrial matrix, and transmembrane electrical potential. Further studies should verify the hypothesis at the tissue and cellular levels, and clarify the significance of the regulatory effect on cerebral protection.

## MATERIALS AND METHODS

### Design

An *in vitro* controlled animal experiment.

### Time and setting

The study was performed at Cerebrovascular Disease Institute of Qingdao University Medical College and Central Laboratory of the Affiliated Hospital of Qingdao University Medical College, China, from August 2010 to April 2011.

### Materials

Eight male Wistar rats of clean grade, aged 3 months and weighing 250–300 g, were purchased from the Laboratory Animal Center of Qingdao (License No. SCXK (Lu) 20090007). Animals were housed in a temperature-controlled (22–24°C) room under a 12-hour light/12-hour dark cycle and allowed free access to water and food. All procedures used in this study were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China<sup>[47]</sup>.

### Methods

#### Extraction and quantification of mitochondria

Mitochondria were isolated from rats according to a standard differential centrifugation procedure using a mitochondria isolation kit (Genmed Scientifics Inc., Shanghai, China). Briefly, rats were anesthetized by intraperitoneal injection of 10% (v/v) chloral hydrate (35 mg/100 g) and sacrificed by decapitation, then the cerebral cortex was rapidly removed. The following experimental operation was performed according to previously described methods with some modifications<sup>[33, 35-36]</sup>. All procedures were conducted at 0–4°C using a 3K30 refrigerated centrifuge (Sigma, St. Louis, MO, USA). Mitochondrial protein was measured by the Bradford method using the total protein

quantification kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), with bovine serum albumin as a standard.

#### **Transmission electron microscopy for monitoring the ultrastructure of isolated mitochondria**

The ultrastructure of isolated mitochondrial preparations was observed by transmission electron microscopy using a JEM-1200EX transmission electron microscope (JEOL, Tokyo, Japan)<sup>[33]</sup>. Mitochondrial pellets were fixed in a 3.0% (v/v) solution of glutaraldehyde and the sample was fixed in a 1% (w/v) solution of osmium tetroxide. The fixed mitochondria were embedded in Epon812-Araldite and ultrathin slices (50 nm thick) were obtained.

#### **Spectrophotometry for measuring mitochondrial enzyme activities**

The marker enzyme activities of mitochondria were assessed spectrophotometrically using the SDH detection kit and MAO detection kit (Nanjing Jiancheng Bioengineering Institute). The activities of SDH were measured as an inner membrane marker enzyme, and MAO as an outer membrane marker enzyme. Enzyme activity was expressed as the specific activity units per hour or minute per milligram of protein<sup>[32, 34, 48]</sup>. Enzyme activities were assessed after mitochondrial lysis without delay.

#### **Mitochondrial swelling for the detection of mPTP opening**

To measure mPTP opening, isolated brain mitochondria were suspended in reaction buffer (150 mM KCl, 10 mM Tris, 20 mM 3-(N-Morpholino) propanesulfonic acid, 2  $\mu$ M rotenone (Sigma), and 2  $\mu$ M A23187 (Sigma), pH 7.4) to a final concentration of 0.5 mg/mL<sup>[34, 44-45]</sup>. mPTP opening was determined by an absorbance decrease in light scattering at 520 nm using a Safire2 multimode microplate reader (Tecan, Zurich, Switzerland). The temperature was maintained at room temperature. Ruthenium red (5  $\mu$ M; Sigma) or cyclosporine A (2  $\mu$ M; Sigma) was added 2 minutes before  $\text{Ca}^{2+}$  addition, spermine (100  $\mu$ M; Sigma) and  $\text{Ca}^{2+}$  were added together. Mitochondrial swelling was initiated by addition of  $\text{CaCl}_2$  (200  $\mu$ M), and the extent of mPTP opening was expressed in terms of the maximal rate of mitochondrial swelling<sup>[34-35, 49-51]</sup>. In the present study, the swelling rate was calculated from the initial linear change in light scattering following the addition of  $\text{CaCl}_2$ <sup>[35]</sup>.

#### **Statistical analysis**

All data were expressed as mean  $\pm$  SD. Statistical significance was determined by one-way analysis of variance, using SPSS 13.0 software (SPSS, Chicago, IL, USA). A value of  $P < 0.01$  was considered statistically significant.

**Author contributions:** Ning Yu participated in data acquisition, study design and manuscript drafting. Shilei Wang, Peng Wang, Yu Li, Shuhong Li, and Hongbing Chen were responsible for study design, manuscript revision and instruction. Li Wang and Yanting Wang provided data support.

**Conflict of interest:** None declared.

**Funding:** This work was supported by the National Natural Science Foundation of China, No. 30972855/C160203 and Natural Science Foundation of Shandong Province No. ZR2009CM062.

**Ethical approval:** This study was approved by the Animal Ethics Committee of the Affiliated Hospital of Qingdao University Medical College, China.

**Acknowledgments:** We thank Dr. Ruyong Yao, Central Laboratory of the Affiliated Hospital of Qingdao University Medical College, Prof. Yunliang Guo, Cerebrovascular Disease Institute, Associate professors Yingyi Hou and Jinshan Tan, Electron Microscopy Research Office of Qingdao University Medical College, China for providing technical support.

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(Edited by Zhang H, Zhou YF/Su LL/Wang L)