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A possible pivotal role of mitochondrial free calcium in neurotoxicity mediated by *N*-methyl-D-aspartate receptors in cultured rat hippocampal neurons

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ABSTRACT

We have previously shown that mitochondrial membrane potential disruption is involved in mechanisms underlying differential vulnerabilities to the excitotoxicity mediated by *N*-methyl-D-aspartate (NMDA) receptors between primary cultured neurons prepared from rat cortex and hippocampus. To further elucidate the role of mitochondria in the excitotoxicity after activation of NMDA receptors, neurons were loaded with the fluorescent dye calcein diffusible in the cytoplasm and organelles for determination of the activity of mitochondrial permeability transition pore (mPTP) responsible for the leakage of different mitochondrial molecules. The addition of $CoCl_2$ similarly quenched the intracellular fluorescence except mitochondria in both cultured neurons, while further addition of NMDA led to a leakage of the dye into the cytoplasm in hippocampal neurons only. An mPTP inhibitor prevented the NMDA-induced loss of viability in hippocampal neurons, while an activator of mPTP induced a similarly potent loss of viability in cortical and hippocampal neurons. Although NMDA was more effective in increasing rhodamine-2 fluorescence as a mitochondrial calcium indicator in hippocampal than cortical neurons, a mitochondrial calcium uniporter inhibitor significantly prevented the NMDA-induced loss of viability in hippocampal neurons. Expression of mRNA was significantly higher for the putative uniporter uncoupling protein-2 in hippocampal than cortical neurons. These results suggest that mitochondrial calcium uniporter would be at least in part responsible for the NMDA neurotoxicity through a mechanism relevant to promotion of mPTP orchestration in hippocampal neurons.

1. Introduction

Glutamate (Glu) is believed to play an excitatory amino acid neurotransmitter role in the mammalian CNS through activation of different Glu receptors, which are categorized into two major subclasses, such as ionotropic and metabotropic receptors, on the basis of intracellular signal transduction systems as well as nucleotide sequential homology. Among ionotropic subtypes, *N*-methyl-D-aspartate (NMDA) receptor (NMDAR) is a subtype composed of a heteromeric protein complex between different NMDAR subunits toward the orchestration of a ligand-gated ion channel with higher permeability to Ca^{2+} than Na^{+} ions. Activation of NMDAR leads to an elevation of intracellular free Ca^{2+} concentrations in CNS neurons (MacDermott et al., 1986; Mayer and Westbrook, 1987), which is at least in part responsible for a variety of physiological and pathological events in the brain. These include neuronal development (Scheetz and Constantine-Paton, 1994), neuronal plasticity (Collingridge and Bliss, 1995) and neuronal cell death (Choi et al., 1988; Sattler and Tymianski, 2000).

Abbreviations: AM, acetoxymethyl ester; ANT, adenine nucleotide translocater; BSA, bovine serum albumin; CX, cerebral cortex; CypD, cyclophilin D; COX, cytochrome *C* oxidase; Cyt, cytochrome; $\Delta\Psi$, mitochondrial membrane potential; DH, dehydrogenase; DIV, days *in vitro*; DMEM, Dulbecco's modified Eagle medium; DMEM/F-12, Dulbecco's modified Eagle's medium: nutrient mixture F-12 (1:1) mixture; DNP, 2,4-dinitrophenol; ER, endoplasmic reticulum; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; Glu, glutamate; HC, hippocampus; LDH, lactate dehydrogenase; MAP2, microtubules-associated protein-2; mCU, mitochondrial calcium uniporter; mPTP, mitochondrial permeability transition pore; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide; NMDA, *N*-methyl-D-aspartate; NMDAR, *N*-methyl-D-aspartate receptor; PAO, phenylarsine oxide; Rhod, rhodamine; ROS, reactive oxygen species; RT-PCR, reverse transcription polymerase chain reaction; RuRed, ruthenium red; Syn, synthetase; UCP, uncoupling protein; VDAC, voltage dependent anion channel.

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Although these Glu receptors are widely distributed in the CNS (Kaneko and Fujiyama, 2002), neuronal loss is often seen in particular restricted brain structures under some neurodegenerative conditions. For instance, CA1 pyramidal neurons are highly vulnerable to an ischemic insult in the hippocampus (HC) (Kirino et al., 1990). Blood flow is similarly arrested in cortical and hippocampal regions in 4 vessel-occlusion model animals, whereas different profiles are seen with neuronal cell death in some neuronal populations several days after reperfusion (Pulsinelli et al., 1982). This ischemic delayed neuronal death is believed to be mainly mediated by extracellular Glu accumulated through the reverse transportation by Glu carrier proteins during ischemia (Szatkowski et al., 1990). Extracellular Glu levels are similarly increased in ischemic telencephalic regions such as cerebral cortex (CX) and HC, in fact, whereas neuronal cell death is selectively seen in the hippocampal CA1 subfield (Smith et al., 1984).

In our previous studies (Kambe et al., 2008), brief exposure to either Glu or NMDA leads to a loss of cellular viability in cultured rat hippocampal neurons without markedly affecting that in cortical neurons, along with similarly effective cell death by the endoplasmic reticulum (ER) stressor tunicamycin, the mitochondrial stressor 2,4-dinitrophenol (DNP), the oxidative stressor hydrogen peroxide and the calcium stressor A23187 in both hippocampal and cortical neurons. Although NMDA is more efficient in increasing intracellular free Ca^{2+} levels in resistant cortical neurons than in vulnerable hippocampal neurons, NMDA is more effective in disrupting mitochondrial membrane potential ($\Delta\Psi$) in hippocampal neurons than in cortical neurons (Kambe et al., 2008). Neurotoxic NR2B subunit protein is similarly expressed, in addition to the NR1 subunit essentially required for the heteromeric assembly to functional channels, in cortical and hippocampal neurons, whereas neurotrophic NR2A subunit protein expression is significantly higher in vulnerable hippocampal neurons than in resistant cortical neurons (Kambe et al., 2008). On the basis of these findings, we have proposed that hippocampal neurons would be highly vulnerable to the neurotoxicity mediated by NMDAR through a mechanism related to $\Delta\Psi$ disruption, rather than increased intracellular free Ca^{2+} levels.

Mitochondria are known as an organelle essential for cellular respiration as well as a key mediator of cell death through apoptotic and/or necrotic processes (Goldstein et al., 2000; Green and Kroemer, 2004). Cytosolic free Ca^{2+} ions would induce an opening of the mitochondrial permeability transition pore (mPTP) (Hunter et al., 1976; Halestrap, 2006), toward an increased permeability of mitochondrial outer membranes for different cytotoxic molecules, such as cytochrome (Cyt)-*C*, apoptosis inducing factor and Smac/DIABLO (Baines et al., 2005; Shalbuyeva et al., 2006). Accordingly, mPTP would mediate a decrease in $\Delta\Psi$ and subsequent mitochondrial swelling. Calcium entry would easily occur into mitochondria after activation of NMDAR rather than other Ca^{2+} gates including kainate receptors and voltage-sensitive Ca^{2+} channels (Peng and Greenamyre, 1998; Young et al., 2008). The inhibition of Ca^{2+} transport toward mitochondria is shown to protect neurons from cell death mediated by Glu, suggesting that Glu-induced neuronal death requires Ca^{2+} entry into mitochondria (Stout et al., 1998). In fact, mitochondrial dysfunction is a primary determinant of the fate of neurons exposed to Glu (Schinder et al., 1996). Reversible nuclear oxidative DNA damage occurs in cerebral cortical neurons in response to transient Glu receptor stimulation (Yang et al., 2010).

In this study, therefore, we have attempted to demonstrate the possible role of mitochondrial Ca^{2+} signaling in the neurotoxicity mediated by NMDAR in cultured neurons derived from the two distinct structures in order to elucidate the mechanism underlying different vulnerabilities seen in a variety of neurodegenerative disorders associated with the overactivation of NMDAR *in vivo*.

2. Materials and methods

2.1. Materials

Wistar rats were supplied by SANKYO LABO SERVICE (Tokyo, Japan). Poly-L-lysine, cytosine arabinoside, antibodies against microtubules-associated protein-2 (MAP2) and glial fibrillary acidic protein (GFAP), cyclosporine A, tunicamycin, A23187, phenylarsine oxide (PAO), ruthenium red (RuRed), DNP and NMDA were purchased from Sigma Chemical (St. Louis, MO, USA). Rhodamine (Rhod)-2 acetoxymethyl ester (AM) was provided by Molecular Probes (Eugene, OR, USA). Rhod-123 AM was supplied by Invitrogen (San Diego, CA, USA). Calcein AM was purchased from Dojindo (Kumamoto, Japan). Dulbecco's modified Eagle medium (DMEM) and Dulbecco's modified Eagle's medium: nutrient mixture F-12 (1:1) mixture (DMEM/F-12) were obtained from Gibco BRL (Grand Island, NY, USA). ECL Advance™ was purchased from GE Healthcare (England, UK). An anti-Cyt-*C* antibody was obtained from Cell Signaling Technology (Danvers, MA, USA). Tacrolimus (FK506; m.w. 822.03) was kindly donated by Astellas Pharma, Inc. (Tsukuba, Japan). Other chemical used were all of the highest purity commercially available.

2.2. Neuronal cultures

The protocol employed here meets the guideline of the Japanese Society for Pharmacology and was approved by the Committee for the Ethical Use of Experimental Animals at Kanazawa University. All efforts were invariably made to minimize animal suffering, to reduce the number of animals used and to utilize alternatives to *in vivo* techniques. Primary cortical and hippocampal neuronal cultures were obtained from 18-day-old embryonic Wistar rats according to the procedures of di Porzio et al. (1980), with minor modifications (Kambe et al., 2008). In brief, each brain structure was dissected and incubated with 0.25% trypsin and 0.01% DNase at 37 °C for 20 min. Cells were then dissociated mechanically using a Pasteur pipette with a fire-narrowed tip in the culture medium, and dissociated cells were plated at a density of 3×10^5 cells/cm^2 on plastic dishes (6 or 24-well plate) coated with 75 μg/ml poly-L-lysine in DMEM/F-12 with 10% fetal bovine serum (FBS) after a Trypan Blue dye exclusion test. After 3 days *in vitro* (DIV), medium was changed to defined medium; DMEM supplemented with 33 mM glucose, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 mM HEPES, 13 mM sodium bicarbonate, 50 μg/ml apotransferrin, 500 ng/ml insulin, 1 pM β-estradiol, 3 nM triiodothyronine, 20 nM progesterone, 8 ng/ml sodium selenite and 100 μM putrescine. Culture media were invariably filtered through a polyethersulfone membrane with a pore size of 0.2 μm before each use. Cells were cultured in DMEM with the aforementioned supplementation for different days up to 9 DIV at 37 °C in a 5% CO_2/95% air humidified incubator with medium change every 3 days. Cultures were treated with 10 μM cytosine arabinoside for 24 h during 2–3 DIV to reduce the number of proliferating cells. Under these culture conditions, approximately 95% of cells were immunoreactive for the neuronal marker MAP2 on double immunocytochemical analysis using antibodies against MAP2 and GFAP (Hirai et al., 2005).

2.3. mPTP imaging

mPTP imaging analysis was done according to the procedures described by Petronilli et al. (1999), with a minor modification, using calcein AM diffusible to different intracellular organelles such as mitochondria and nucleus. Neurons were loaded with 1 μM calcein AM for 15 min in recording medium, followed by