Neuroscience 256 (2014) 36–42

INHIBITION OF MITOCHONDRIAL FISSION ATTENUATES Aβ-INDUCED MICROGLIA APOPTOSIS

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Abstract—Mitochondrial division inhibitor 1 (mdivi-1), a selective inhibitor of mitochondrial fission protein dynamin-related protein 1 (Drp1), has been reported to display neuroprotective properties in different animal models. In the present study, we investigated the protective effect of mdivi-1 on β-amyloid protein (Aβ)-induced cytotoxicity and its potential mechanisms in BV-2 and primary microglial cells. We found that mitochondrial fission was increased in Aβ treatment and inhibition of mitochondrial fission by mdivi-1 significantly reduced Aβ-induced expression of CD11b (a marker of microglial activation), viability loss and apoptotic rate increase in BV-2 and primary microglial cells. Moreover, we also found that mdivi-1 treatment markedly reversed mitochondrial membrane potential loss, cytochrome c (CytC) release and caspase-3 activation. Altogether, our data suggested that mdivi-1 exerts neuroprotective effects against Aβ-induced microglial apoptosis, and the underlying mechanism may be through inhibiting mitochondrial membrane potential loss, CytC release and suppression of the mitochondrial apoptosis pathway.

Key words: microglia, mdivi-1, β-amyloid, apoptosis, cytochrome c.

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder of the CNS characterized by progressive deterioration of memory and cognition. Excessive accumulation of β-amyloid protein (Aβ) in the brain is known to play a critical role in the pathogenesis of AD (Gilbert, 2013). There have been increasing studies on the effects of Aβ on nerve cells and the effects of Aβ on neurotoxicity have been implicated by several studies (Li et al., 2008; Feng et al., 2013). But there is a paucity of studies on the action of Aβ on non-neuronal brain cells.

Microglia are immunolike cells in CNS and participate in both innate and adaptive immune responses. Activated microglia are found at sites of neuronal degeneration in multiple pathological conditions such as cerebral ischemia and AD (Tambuyzer et al., 2009; Saijo and Glass, 2011). Microglia can be either neurotrophic or neurotoxic depending on the activation state (Luo et al., 2010); normally active microglia clear debris through phagocytosis to stimulate tissue repair and regulate transient inflammatory responses to pathogens, but sustained and excessive activation ("overactivation") leads to release of cytotoxins that cause neurotoxicity. Alternatively, overactivation can lead to microglial apoptosis, resulting in uncontrolled inflammatory responses (Kim and Li, 2013). It has been reported that Aβ could accelerate neurodegeneration by inducing the activation and apoptosis of microglial cells, which exert cytotoxic effects on neurons (Qin et al., 2002; Streit, 2004). However, the exact mechanisms of Aβ-mediated microglial "overactivation" and apoptosis remain obscure.

Mitochondrial fission has been reported to be involved in apoptosis and many neurodegenerative diseases (Cho et al., 2010). The Guanosine triphosphate (GTP)-binding protein, dynamin-related protein 1 (Drp-1) is a mitochondrial fission protein. It has been found that expression of Drp-1 promotes mitochondrial fragmentation, while the expression of a dominant-negative form of Drp-1 inhibits mitochondrial fission and thereby apoptosis (Karbowski, 2010). Mitochondrial division inhibitor (mdivi-1) is a highly efficient small molecule that selectively inhibits mitochondrial fission Drp1. It inhibits Drp1 GTPase activity by blocking the self-assembly of Drp1 *in vitro* and causes the rapid reversible and dose-dependent formation of netlike mitochondria in wild-type cells (Cassidy-Stone et al., 2008; Tanaka and Youle, 2008). Recent study has shown that pretreatment with mdivi-1 could provide neuroprotection against glutamate toxicity and oxygen-glucose deprivation (OGD) *in vitro* and ischemic brain damage *in vivo* (Grohm et al., 2012). However, the role of mdivi-1 in Aβ-induced microglia apoptosis remains unknown.

In this study, we investigated whether mdivi-1 might attenuate Aβ-induced microglia apoptosis. In addition, we also examined the potential protective mechanisms

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Abbreviations: AD, Alzheimer's disease; Aβ, β-amyloid protein; BALB, Bagg albino; CytC, cytochrome c; DMEM, Dulbecco's modified eagle medium; Drp1, dynamin-related protein 1; FBS, fetal bovine serum; GTP, Guanosine triphosphate; HD, Huntington's disease; Mdivi-1, mitochondrial division inhibitor 1; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-dipheny- ltetrazolium bromide; PBS, phosphate buffered saline; PD, Parkinson's disease; Rh-123, rhodamine 123; RT-PCR, Reverse transcription-PCR; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling.

0306-4522/13 $36.00
http://dx.doi.org/10.1016/j.neuroscience.2013.10.011

initiated by mdivi-1, including the mitochondrial membrane potential levels and cytochrome c (CytC)-dependent mitochondrial apoptosis pathway.

EXPERIMENTAL PROCEDURES

Reagents

All media components used in cell culture were obtained from Gibco Invitrogen Corporation (Carlsbad, CA, USA). Antibodies, including Drp1, total and cleaved caspase-3, were purchased from Cell Signaling Technology (Beverly, MA, USA). The antibodies of β-actin, CytC and CD11b were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

Microglial cultures. Bagg albino (BALB)/c mice were purchased from Experimental Animal Center of the Zhengzhou University (Zhengzhou, China). Mice were housed, bred, and euthanized in accordance with protocols reviewed and approved by the Commission of the Zhengzhou University for ethics of experiments on animals in accordance with international standards. Mouse primary microglial cells were isolated from mixed glial cultures, as described previously (Deierborg, 2013). Briefly, cortices were dissected from newborn BALB/c mice and dissociated by mechanical disruption and trypsinization. Primary microglia were co-cultured with astrocytes in poly-D-lysine-coated 75-cm^2 culture flasks in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin. On days 10–14, microglial cells were harvested by shaking the cultures and collecting the floating cells. The cells were seeded into plastic tissue culture flasks. After incubation at 37 °C for 1 h, non-adherent cells were removed by replacing culture medium. The cells were grown in DMEM with 10% FBS and maintained at 37 °C and 5% CO_2.

BV-2 cell culture. The cells were purchased from Cell Center of the Peking Union Medical College (Beíjing, China) and cultured in DMEM medium with 5% FBS and 1% Penicillin/Streptomycin. Cultures were incubated at 37 °C and 5% CO_2 in a fully humidified incubator.

Cell viability assay

Cell viability was assessed by the reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-dipheny- ltetrazolium bromide (MTT). Briefly, 20 μl MTT (5 mg/ml, Sigma–Aldrich) was added to each well, and plates were incubated at 37 °C for 4 h and then quantifying the color formation by means of an Elisa plate reader at 570-nm wavelength using 200 μl MTT solubilization solution.

TUNEL assay

The apoptotic cells were determined by terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay using a situ cell death detection kit (Roche Diagnostic, Indianpolis, IN, USA) according to the manufacturer's instructions as described in our previous studies (Xie et al., 2010a,b). The percentage of apoptotic cells was calculated by counting approximately 500 cells.

Flow cytometry analysis

Apoptotic cells were examined by flow cytometry as described previously (Wu et al., 2013). Briefly, cells (1×10^6) were washed once with phosphate buffered saline (PBS), and then suspended cells in 160 μl of Annexin V binding buffer (1×) and 4 μl of Annexin V-fluorescein isothiocyanate at room temperature for 20 minutes and then counterstained with 4 μl propidium iodide, and finally analyzed using a FACScan flow cytometer (BECTON DICKINSON FACSCalibur). All Annexin V-positive cells were considered to have apoptosis. All data are representative of three independent experiments.

Reverse transcription-PCR (RT-PCR)

Total RNA was extracted from the cells by use of the VERSA GENE RNA Tissue Kit (Gentra SYSTEMS; Minnesota) and RT-PCR was performed as described (Xie et al., 2010a). Briefly, first-strand cDNA was synthesized from 1 μg of total RNA using a Reaction Ready first-strand cDNA synthesis kit (SABioscience Corporation, Frederick, MD, USA). After incubation at 70 °C for 3 min and cooling down to 37 °C for 10 min, RT cocktail was added to the annealing mixture and further incubated at 37 °C for 60 min. The following primer pair was used: for CD11b: 5-GATGCTTACCTG GGTTATGCTTCT-3 (forward) and 5-CCGAGGTGC TCCTAAAACCA-3 (reverse).

Western blot analysis

To obtain cytosolic and mitochondrial protein extracts, the cells were subfractionized in homogenization buffer. The cytosolic and mitochondrial fractions were separately isolated by centrifugation as described previously (Jing et al., 2012). Thirty micrograms of protein were separated by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membrane. After blocking in 5% fat-free milk for 1 h, the membrane was incubated with the blocking solution containing the first antibody overnight at 4 °C. After washing, the blot was then incubated with a second antibody. The blot was washed again before being analyzed by the enhanced chemiluminescence (ECL) system (Amersham Pharmacia). The signals were quantified by densitometric analysis using a densitometer.

Mitochondrial membrane potential

The mitochondrial membrane potential was detected using rhodamine 123 (Rh-123) fluorescent dye. Rh-123 can enter the mitochondrial matrix and cause photoluminescent quenching dependent on mitochondrial transmembrane potential. Primary microglia and BV-2 cells were incubated with Rh-123 for 30 min at 37 °C.