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THE NEUROPROTECTIVE ROLE AND MECHANISMS OF TERT IN NEURONS WITH OXYGEN–GLUCOSE DEPRIVATION

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Abstract—Telomerase reverse transcriptase (TERT) is reported to protect neurons from apoptosis induced by various stresses including hypoxia–ischemia (HI). However, the mechanisms by which TERT exerts its anti-apoptotic role in neurons with HI injury remain unclear. In this study, we examined the protective role and explored the possible mechanisms of TERT in neurons with HI injury *in vitro*. Primary cultured neurons were exposed to oxygen and glucose deprivation (OGD) for 3 h followed by reperfusion to mimic HI injury *in vivo*. Plasmids containing TERT antisense, sense nucleotides, or mock were transduced into neurons at 48 h before OGD. Expression and distribution of TERT were measured by immunofluorescence labeling and western blot. The expression of cleaved caspase 3 (CC3), Bcl-2 and Bax were detected by western blot. Neuronal apoptosis was measured with terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL). The mitochondrial reactive oxygen species (ROS) were measured by MitoSOX Red staining. Fluorescent probe JC-1 was used to measure the mitochondrial membrane potential ($\Delta\Psi$m). We found that TERT expression increased at 8 h and peaked at 24 h in neurons after OGD. CC3 expression and neuronal apoptosis were induced and peaked at 24 h after OGD. TERT inhibition significantly increased CC3 expression and neuronal apoptosis after OGD treatment. Additionally, TERT inhibition decreased the expression ratio of Bcl-2/Bax, and enhanced ROS production and $\Delta\Psi$m dissipation after OGD. These data suggest that TERT plays a neuroprotective role via anti-apoptosis in neurons after OGD. The underlying mechanisms may be associated with regulating Bcl-2/Bax expression ratio, attenuating ROS generation, and increasing mitochondrial membrane potential.

Key words: telomerase reverse transcriptase, neuron, hypoxia–ischemia, apoptosis, reactive oxygen species, mitochondrial membrane potential.

INTRODUCTION

Hypoxic–ischemic (HI) brain injury is a pathological condition caused by low arterial oxygen tension and insufficient blood flow. It is one of the severe complications of perinatal asphyxia in newborns with a high mortality, especially in preterm newborns (Selway, 2010). Survivors usually have long-lasting neurological deficits including cerebral palsy, learning disability and epilepsy in childhood (Ferriero, 2004; Hossain, 2008; Derganc and Osredkar, 2008). Although numerous studies have focused on the mechanisms and therapeutic targets of neonatal HI brain injury, effective clinical treatments are still rare. Therefore, studies for therapies with a definite protective effect are quite necessary.

It is widely reported that apoptosis is the major cell death pathway after HI in developing brains (Kawamura et al., 2005; Northington et al., 2005; Park et al., 2006; Hossain, 2008; Derganc and Osredkar, 2008). Therefore, inhibition of apoptosis may provide an effective therapeutic intervention in neonatal HI brain injury (Zhang et al., 2010; Li et al., 2010; Zhao et al., 2013).

Telomerase reverse transcriptase (TERT), a catalytic unit of telomerase, is the key regulator and rate-limiting determinant of telomerase activity (Fujiki et al., 2010; Wang et al., 2010). TERT plays a significant role in maintaining the length of telomere and thus preventing genome instability-promoting events (Blackburn, 2005; Wyatt et al., 2010). Previous studies have reported that TERT has many other functions in addition to its classical function in telomerase, such as protecting against apoptosis in different types of cells including neurons (Chung et al., 2005; Blackburn, 2005; Wyatt et al., 2010). TERT can protect neurons from apoptosis induced by various injuries including DNA damage, oxidative stress, ischemia, β-amyloid or trophic factor withdrawal (Zhu et al., 2000; Fu et al., 2000; Lu et al., 2001; Kang et al., 2004; Kong et al., 2009). Meanwhile, accumulating data suggest that the anti-apoptotic effect of TERT is mitochondrial (Fu et al., 2000; Del Bufalo

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Abbreviations: AI, apoptotic index; CC3, cleaved caspase 3; DIV, day in vitro; HBSS, hank's buffered salt solution; HI, hypoxia–ischemia; IPP, image pro plus; MAP-2, microtubule associated protein 2; NB, neurobasal; OD, optical density; OGD, oxygen and glucose deprivation; PBS, phosphate-buffered Saline; ROS, reactive oxygen species; rTdT, terminal deoxynucleotidyl transferase, recombinant; SD, Sprague–Dawley; SD, standard deviation; TERT, telomerase reverse transcriptase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling; $\Delta\Psi$m, mitochondrial membrane potential.

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et al., 2005; Massard et al., 2006; Ahmed et al., 2008; Haendeler et al., 2009; Kovalenko et al., 2010). The underlying mechanisms include regulating the expression of Bcl-2 family proteins (Del Bufalo et al., 2005; Massard et al., 2006), decreasing cellular reactive oxygen species (ROS) generation, or increasing the mitochondrial membrane potential (Ahmed et al., 2008; Haendeler et al., 2009; Kovalenko et al., 2010).

Recently, *in vivo*, we have demonstrated that TERT is induced in a neonatal rat model of HI brain injury and may play an anti-apoptotic role by inhibiting caspase-3 activation (Zhao et al., 2012). Based on these findings, we hypothesized that TERT can protect neurons from apoptosis during HI injury by interacting with the mitochondrial pathway. To test this hypothesis, we exposed cultured neurons to oxygen and glucose deprivation (OGD) to mimic HI *in vivo*, and examined the possible mechanisms involved in the anti-apoptotic effect of TERT.

EXPERIMENTAL PROCEDURES

Primary cortical neuron cultures

Sprague–Dawley (SD) rats were purchased from the Experimental Animal Center of Sichuan University. All animal research was approved by the Animal Ethics Committee of Sichuan University. Primary cortical neuron cultures were established from the cerebral cortices of newborn SD rats (< 24 h). Brains from the newborns were cleaned of meninges and blood vessels. The whole cerebral cortex was isolated and cells were dissociated in 0.25% trypsin solution for 10 min at 37 °C. Fetal bovine serum was added and the dissociated cells were forced through a mesh. After centrifuging, the cortex cells were resuspended and plated in poly-D-lysine (Sigma) coated six-well plates, and grown in Neurobasal (NB) (Gibco) with 500 μM glutamine (Gibco) and 2% B27 supplement (Gibco) in a humidified incubator with 5% CO_2 at 37 °C. The cell culture medium was replaced with fresh culture medium on day in vitro (DIV) 3 and DIV5. The following experiments were performed at DIV5 and DIV7. The purity of neurons was measured by staining with anti-microtubule associated protein 2 (MAP-2) antibody (Santa Cruz, 1:50) using immunofluorescence staining.

OGD treatment

To induce OGD, cultured cells at DIV7 were gently washed twice with phosphate-buffered saline (PBS), and then the culture medium was replaced with dulbecco's modified eagle medium (DMEM) without glucose. Cells were exposed to hypoxia in the airtight chamber with 95%N_2, 5%CO_2 at 37 °C for 3 h. After 3 h of OGD treatment, the culture medium was changed back to normal NB medium and then cultured in an incubator with normal condition at 37 °C to form reoxygenation.

Plasmid administration

Plasmids including pcDNA3.1-AS carrying the antisense nucleotides (5′-GGGATGGCAT CATAGGCCCC TGTCA CATCT GCCTTAACAA AGTACATCCT GGGTGTCT GG-3′) against rat telomerase reverse transcriptase (rTERT), pcDNA3.1-SE carrying the sense nucleotides (5′-CCAGACACCC AGGATGTACT TTGTTAAGGC AG ATGTGACA GGGGCCTATG ATGCCATCCC-3′) against rTERT and pcDNA3.1 were synthesized and constructed by Jinsite Biotechnology.

.Neuron transduction was conducted at DIV5. To inhibit TERT expression, pcDNA3.1-AS (As) was transduced into neurons, and the control neurons were transduced with pcDNA3.1-SE (Se). As a mock control, neurons were transduced with pcDNA3.1 (Mo). Gene transduction was performed using X-tremeGENE HP DNA Transfection Reagent (Roche) according to the manufacturer's instructions. At 48 h after transduction, cells were exposed to OGD treatment. Inhibition of TERT expression was confirmed by immunofluorescence staining and western blot analysis.

Western blot analysis

Cells were lysed in Radio Immunoprecipitation Assay (RIPA) with protease inhibitor cocktail (Roche), and then centrifuged at 14,000 rpm for 30 min at 4 °C. The supernatant was collected. The protein concentration in the supernatant was measured by the Bio-Rad Bradford kit (Bio-Rad Laboratories, Hercules). Proteins were denatured in gel sample buffer by boiling for 5 min at 100 °C. Equal amounts of protein samples (60 μg) were loaded per lane and electrophoresed on 8% Sodium dodecyl sulfate/Polyacrylamide gel electrophoresis (SDS/PAGE) gels. Then, proteins were electrotransferred onto a polyvinylidene fluoride (PVDF) membrane (Roche). After blocking in 5% nonfat milk buffer for 1 h at room temperature, the membranes were incubated with rabbit anti-TERT polyclonal antibody (Santa Cruz Biotechnology, USA, 1:200), rabbit anti-cleaved caspase 3 polyclonal antibody (Cell Signaling Technology, USA, 1:1000), rabbit anti-Bcl-2 polyclonal antibody (Abcam, 1:200), and rabbit anti-Bax polyclonal antibody (Abcam, 1:1000) separately overnight at 4 °C. Mouse β-actin monoclonal antibody (Santa Cruz Biotechnology, 1:3000) was measured as the internal control. After being washed three times with Tris-buffered saline containing 0.1% Tween-20, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, 1:3000) in blocking solution at room temperature for 2 h. The signals of bound antibodies were identified with an enhanced chemiluminescence kit (ECL, Millipore, USA). The band intensity was measured, normalized by β-actin and calculated as the ratio of the optical density (OD). Reproducibility of the results was ensured by repeating all experiments at least three times.

Immunofluorescence staining

For immunofluorescence staining, cultured cells were fixed with 4% paraformaldehyde at 4 °C for 20 min and treated with 0.1% Triton X-100 for 15 min. After being blocked with 10% serum at 4 °C for 30 min, cells were incubated with primary antibodies overnight at 4 °C: mouse anti-MAP2 monoclonal antibody (Santa Cruz Biotechnology, USA, 1:50) to label neurons, and rabbit