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Induction of sestrin2 as an endogenous protective mechanism against amyloid beta-peptide neurotoxicity in primary cortical culture

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ABSTRACT

Accumulation of amyloid β-peptide (Aβ) in senile plaques, a pathological hallmark of Alzheimer's disease (AD), has been implicated in neurodegeneration. Recent studies suggested sestrin2 as a crucial mediator for reactive oxygen species (ROS) scavenging and autophagy regulation that both play a pivotal role in age-dependent neurodegenerative diseases. However, the potential link between sestrin2 and Aβ neurotoxicity has never been explored. The present study was therefore undertaken to test whether sestrin2 may be induced by Aβ and its possible role in modulating Aβ neurotoxicity. We showed that sestrin2 expression was elevated in primary rat cortical neurons upon Aβ exposure; a heightened extent of sestrin2 expression was also detected in the cortices of 12-month-old APPswe/PSEN1dE9 transgenic mice. Exposure of cortical neurons to Aβ led to formation of LC3B-II, an autophagic marker; an increased LC3B-II level was also observed in the cortices of 12-month-old AD transgenic mice. More importantly, downregulation of sestrin2 by siRNA abolished LC3B-II formation caused by Aβ that was accompanied by more severe neuronal death. Inhibition of autophagy by bafilomycin A1 also enhanced Aβ neurotoxicity. Together, these results indicate that sestrin2 induced by Aβ plays a protective role against Aβ neurotoxicity through, at least in part, regulation of autophagy.

Introduction

Amyloid deposition is a prominent pathological feature in the brains of patients suffering from Alzheimer's disease (AD); the major component of amyloid deposits is amyloid beta-peptide (Aβ), a peptide fragment of 39–43 amino acids derived from sequential cleavage of amyloid precursor protein (APP) by β- and γ-secretases (for a recent review, see O'Brien and Wong, 2011). Aβ-induced cytotoxicity is mediated by multiple mechanisms including enhanced production of hydrogen peroxide (Behl et al., 1994) causing oxidative stress (Ju et al., 2005). In addition, Aβ is also a potent modulator of gene transcription (Caldeira et al., 2013), and hence protein expression, that may regulate neuronal survival/death in AD-related pathophysiology.

Sestrins belong to a family of highly conserved antioxidant proteins that were initially discovered as p53-inducible proteins. As stress-inducible proteins, sestrins are believed to protect cells against various insults (Budanov et al., 2002). Mammalian cells express three isoforms of sestrins referred to as sestrin1 (sesn1; also known as PA26), sestrin2 (sesn2; also known as Hi95), and sestrin3 (sesn3) (Budanov et al., 2004; Velasco-Miguel et al., 1999). Among these three isoforms, sestrin2 has recently gained more attention. At least two molecular mechanisms have been proposed to underlie the protective roles of sestrin2. The first one is that sestrin2 can function as an antioxidant to scavenge excessive reactive oxygen species (ROS) (Budanov et al., 2004). The second mechanism is that, under stressful condition, p53-induced sestrin2 may activate AMP-dependent protein kinase (AMPK) leading to suppression of mammalian target of rapamycin (mTOR) and ultimately autophagy induction (for a recent review see Budanov, 2011). Both antioxidant action and autophagy induction are important for age-related neurodegenerative diseases like AD.

Autophagy is a highly regulated process that breaks down organelles and macromolecules through lysosomal degradation and is essential for maintenance of intracellular homeostasis under starvation, differentiation, and normal growth control (Komatsu et al., 2007a,b). The integrity of postmitotic neurons is heavily dependent on functional autophagy compared to non-neuronal cells as misfolded proteins and damaged organelles cannot be diluted through cell division; defects in autophagy may thus affect the intercellular communication and subsequently,

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contributing to neurodegeneration (Son et al., 2012). Indeed, the role of autophagy in AD is just beginning to be elucidated (Salminen et al., 2013). An overload of Aβ in brain tissue may cause oxidative stress that damages multiple intracellular targets. Efficient clearance of damaged organelles and macromolecules is expected to play a protective role whereas uncontrolled autophagy may lead to progressive digestion of affected neurons resulting in neuronal demise during the progression of AD.

Despite the critical roles of sestrin2 in antioxidation and autophagy induction, however, study of sestrin2 in AD is quite limited. In the present report, we examined whether sestrin2 may be involved in the regulation of autophagic pathway and its potential roles in Aβ-induced neuronal injury using primary cortical cultures as a model system.

Materials and methods

Preparations of Aβs, primary fetal rat cortical culture, and AD transgenic mice

Aβ25–35 and Aβ1–42 were aggregated according to our published protocols (Chen et al., 2012). Primary neuronal cultures were prepared from cortices of Sprague–Dawley fetal rat brains at embryonic day 18 as previously described (Ju et al., 2004). APPswe/PSEN1dE9 transgenic mice were from the Jackson Laboratory (Bar Harbor, ME, USA). All the procedures involving experimentation on animal subjects, including primary cortical cultures, were reviewed and approved by the Institutional Animal Care and Use Committee of National Yang-Ming University.

Western blotting

Preparations of cellular protein extracts and cortical lysates for Western analyses were performed as described in details in our recent study (Chen et al., 2012). All the primary and secondary antibodies were diluted in the fresh TTBS buffer (0.05% Tween-20, 0.2 M NaCl in 20 mM Tris–HCl, pH 7.5) containing 5% non-fat milk. The following primary antibodies were used in the present study: rabbit sestrin2 antibody (1:1000; Cat. No. 10795-1-AP, Proteintech Group Inc., Chicago, IL, USA), rabbit LC3B antibody (1:1000; Cat. No. 2775, Cell Signaling Technology, Danvers, MA, USA), and mouse β-actin antibody (1:7000; Cat. No. MAB1501, Chemicon, Billerica, MA, USA). The horseradish peroxidase (HRP)-conjugated anti-mouse (1:5000; Cat. No. AP124P) and anti-rabbit (1:5000; Cat. No. AP132P) antibodies were from Millipore, Billerica, MA, USA). Hybridizations with the primary and secondary antibodies were performed at 4 °C overnight and room temperature for 1 h, respectively. Post-hybridization washes as well as detection and quantification of immunoreactive signals on the blots were conducted as described (Chen et al., 2012).

Immunocytochemistry by confocal microscopy

Cortical cells seeded on the coverslips were fixed for 3 min with the methanol pre-cooled to −20 °C after rinsing twice with 1× phosphate-buffered saline (PBS). The coverslips were transferred into −20 °C acetone for 30 s for further dehydration and air-dried. This was followed by incubation in 1× PBS containing 2% normal goat serum to block non-specific protein binding sites after rehydration of the cells in 1× PBS for at least 5 min. The rabbit sestrin2 antibody used in Western blotting was diluted at 1:50 in 1× PBS containing 2% normal goat serum. The mouse antibody for microtubule-associated protein-2 (MAP-2, 1:100, Cat. No. AB5622, Millipore) was used to label neurons. The Alexa Fluor® 488-conjugated goat anti-rabbit IgG (1:600; Cat. No. 111-545-003) and the Cys5-conjugated goat anti-mouse IgG secondary antibody (1:600; Cat. No. 115-175-146), both from Jackson ImmunoResearch (West Grove, PA, USA), were applied to recognize the primary antibodies for sestrin2 and MAP-2, respectively. The coverslips were then examined under a laser scanning confocal microscope (Zeiss LSM700, Oberkochen, Germany) equipped with filter sets to detect Cys5 (excitation/emission: 633-nm/650 ± 20-nm) and Alexa Fluor® 488 (excitation/emission: 488-nm/520 ± 20-nm) fluorescence signals.

Immunohistochemistry by confocal microscopy

Preparations of frozen coronal sections (20-μm thickness) and post-hybridization washes for immunohistochemistry to detect sestrin2 in AD transgenic mice cortices were conducted as described in our previous publication (Chen et al., 2012). Briefly, the brain sections were immunostained with the primary sestrin2 antibody (the same antibody used in Western blotting and immunocytochemistry) diluted at 1:25 in fresh blocking buffer for 1 h. After washes, the Alexa Fluor® 488-conjugated secondary antibody (1:600 in fresh blocking buffer) was then applied at room temperature for 1 h to recognize the primary sestrin2 antibody. The coverslips were examined under a laser scanning confocal microscope as described above.

Transfection of siRNAs

The detailed protocols for transfection of siRNAs into primary cortical neurons have been described in our earlier publication (Wu et al., 2012). The siRNA targeted at *sestrin2* and the scrambled negative control (NC) siRNA were all purchased from Dharmacon Inc. (Lafayette, CO, USA). The target sequences for the *sestrin2* Accell SMARTpool siRNA mixture were as follows: UCUUUGGCAUCAGAUACGA (A-052642-16), GCAGCCUGUUCUUUGGUUA (A-052642-15), CCAUCAAUGUGAAAGUUGG (A-052642-14), GUUUUGAGCUGGAGAAGUC (A-052642-13). A non-targeting Accell siRNA (D-001910-01) was used as a negative control in all siRNA transfection experiments. Primary cortical neurons grown on 6-well plates were transfected with the *sestrin2* siRNA or the NC siRNA, 1 μM each, for 3 days (DIV4-7) in neurobasal medium supplemented with B27 (NB/B27). At the end of transfection, cells were washed once in fresh NB/B27 medium before further experimentation.

MTT reduction assay

MTT reduction assay, which measures mitochondrial function as an index for cell survival, was performed according to the protocols described in our published paper (Yang et al., 2005).

Statistical analysis

Results are presented as mean ± SEM. Multiple groups were analyzed by one-way analysis of variance (ANOVA) followed by a *post-hoc* Student–Newman–Keul test. P-values less than 0.05 were considered significant.

Results

Effects of Aβs on sestrin2 expression in primary rat cortical neurons

We first examined whether expression of sestrin2 may be affected by Aβs in primary rat cortical cultures. Western blotting showed a time-dependent elevation of sestrin2 levels in the cortical cultures treated with 10 μM Aβ25–35; quantitative analyses of immunoblots confirmed that sestrin2 expression was increased beginning at 24 h upon exposure to Aβ25–35 and sustained until 40 h (Fig. 1A). Because the co-culture system used in the present study contained mainly neurons and a minor population of astrocytes (Ju et al., 2004), immunofluorescence confocal microscopy was conducted to confirm neuronal expression of sestrin2. Results demonstrated an increased expression of sestrin2 that can be co-localized to the cells positively stained with MAP-2, the neuronal marker protein, following an episode of 32-h exposure to Aβ25–35 (Fig. 1B). While Aβ25–35 is an active peptide