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Axon degeneration is key component of neuronal death in amyloid-β toxicity

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ABSTRACT

Depending upon the stimulus, neuronal cell death can either be triggered from the cell body (soma) or the axon. We investigated the origin of the degeneration signal in amyloid β (Aβ) induced neuronal cell death in cultured *in vitro* hippocampal neurons. We discovered that $A\beta_{1-42}$ toxicity-induced axon degeneration precedes cell death in hippocampal neurons. Overexpression of Bcl-xl inhibited both axonal and cell body degeneration in the Aβ-42 treated neurons. Nicotinamide mononucleotide adenylyltransferase 1 (Nmnat1) blocks axon degeneration in a variety of paradigms, but it cannot block neuronal cell body death. Therefore, if the neuronal death signals in $A\beta_{1-42}$ toxicity originate from degenerating axons, we should be able to block neuronal death by inhibiting axon degeneration. To explore this possibility we over-expressed Nmnat1 in hippocampal neurons. We found that inhibition of axon degeneration in $A\beta_{1-42}$ treated neurons prevented neuronal cell death. Thus, we conclude that axon degeneration is the key component of $A\beta_{1-42}$ induced neuronal degeneration, and therapies targeting axonal protection can be important in finding a treatment for Alzheimer's disease.

1. Introduction

The pathological hallmarks of Alzheimer's disease (AD) include the presence of extracellular senile plaques primarily composed of amyloid-β (Aβ) peptide, and intracellular neurofibrillary tangles constituted by hyper-phosphorylated aggregates of the microtubule-associated protein, tau (Cavallucci et al., 2012). The Aβ protein fragments (39–43 amino acids) which are deposited as amyloid in the brains of patients with Alzheimer's disease are derived from the proteolytic processing/cleavage of Amyloid Precursor Protein (APP) (Shoji et al., 1992). APP is cleaved by β and γ secretase to produce N-terminus Aβ cleavage products in the extracellular spaces (Zhang et al., 2011). $A\beta_{1-42}$ (the most common isoform of Aβ) is implicated in the development and progression of AD (Younkin, 1998; Lippa et al., 1998). In the trophic factor deprivation paradigm, APP cleavage by Beta-site APP cleavage enzyme (BACE1) activates caspase-6 and caspase-3 pathways in axons and neuronal cell body, respectively (Vohra et al., 2010; Nikolaev et al., 2009). The accumulation of Aβ causes neuronal damage in Alzheimer's disease (O'Brien and Wong, 2011; Zhou et al., 2011) and Aβ also contributes to the formation of intracellular neurofibrillary tangles, which further accelerates the process of neuronal loss and causes the symptoms of dementia (Takata and Kitamura, 2012).

Oxidative stress, altered calcium homeostasis, mitochondrial dysfunction, and activation of caspases are implicated in the origination and progression of AD (Cai et al., 2011; Ferreira et al., 2012; Devi and Ohno, 2012; Graham et al., 2011). However, depending upon the stimulus, neuronal cell death can either be triggered from the cell body (soma) or axon. Degeneration of neuronal soma and axons is regulated by different sets of executioner molecules (Nikolaev et al., 2009; Vohra et al., 2010), but the sequence of these events (what dies first; soma or axon) is not known in AD.

Axonal destruction shares morphological features with apoptosis, but it is a caspase-independent process as manipulation of the mitochondrial apoptotic machinery or caspase inhibitors fail to block axonal degeneration in the case of axotomy and vincristine treatment (Sagot et al., 1995; Finn et al., 2000; Watts et al., 2003; Whitmore et al., 2003). However, in the paradigms of trophic factor deprivation, apoptotic activation in the form of caspase-6 activity indeed takes place in axons (Nikolaev et al., 2009; Vohra et al., 2010). Further, different caspases are compartmentalized in different components of neurons; for example caspase-3 is activated only in neuronal cell body and caspase-6 activation is restricted to the axons. Moreover, inhibition of caspase-6 can protect axons, while caspase-3 inhibition can only block neuronal cell body degeneration (Nikolaev et al., 2009; Vohra et al., 2010). Caspase-6 activation has also been reported in hippocampus and cerebral cortex of mild, moderate, severe and very severe sporadic Alzheimer's disease (Albrecht et al., 2009). Caspases have been

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directly implicated in the cleavage of amyloid-beta precursor protein during apoptosis, resulting in elevated Aβ peptide formation (Stone et al., 2002). Caspase-6 deficient neurons are protected against excitotoxicity, nerve growth factor deprivation and myelin-induced axonal degeneration (Uribe et al., 2012). Caspase-6 is also activated in familial AD brains (Albrecht et al., 2009), and caspase-6 mediates axon degeneration in the primary human neurons expressing familial Alzheimer disease-associated autosomal dominant mutants (Sivananthan et al., 2010). Thus, caspases appear to be involved in both the processing of APP, and hence Aβ peptide formation, as well as in neuronal death.

Many members of the Bcl-2 family, including Bcl-xl, are potent inhibitors of programmed cell death and inhibit the activation of caspases in cells (Garcia-Saez, 2012). Bcl-xl blocks the opening of the mitochondrial permeability pore, thereby blocking activation of caspases (Sharpe et al., 2004). Bcl-xl also blocks Aβ-evoked cell death in differentiated PC12 cells (Song et al., 2004). In human primary neurons, $A\beta_{1-42}$ treatment down-regulates anti-apoptotic Bcl-2 and upregulates pro-apoptotic Bax (Paradis et al., 1996). Thus, we hypothesize that Bcl-xl overexpression might protect against $A\beta_{1-42}$-induced degeneration in hippocampal neurons.

Although Bcl-xl can effectively block cell death in a mouse model of motor neuron disease, overexpression of Bcl-xl could only save the neuronal cell body. Surprisingly, overexpression of "slow Wallerian degeneration" (Wlds) could save both axons and cell body and prolonged the life span of these mice (Ferri et al., 2003). Wlds cannot inhibit apoptosis but it can effectively block axon degeneration in various paradigms (Beirowski et al., 2008; Meyer zu Horste et al., 2011). Therefore if the degeneration signal is initiated in the axons, axonal protection can be the key to the neuronal protection. Therefore, we performed experiments to explore whether the initial degeneration signals emanate from the axon or neuronal cell body in $A\beta_{1-42}$ toxicity.

Nmnat1 is responsible for the axon protective properties of Wlds fusion protein (Araki et al., 2004). Nmnat1 prevents axonal degeneration in various paradigms (Sasaki et al., 2009a,b, Vohra et al., 2010; Yan et al., 2010). The ability of Nmnat1 to protect the axon from various perturbations suggests that Nmnat1 controls the central mechanism of axonal degeneration (Vohra et al., 2010). Nmnat1 protects axon degeneration in various kinds of insults, but it cannot thwart cell body death (Vohra et al., 2010). Therefore, we investigated whether Nmnat1 can prevent axonal degeneration caused by $A\beta_{1-42}$ and also whether we can prevent neuronal cell death by blocking axonal degeneration in Aβ-treated neurons. During the course of this study we found that Aβ-induced neurodegeneration begins in the axons, and by protecting the axon we were able to prevent neuronal death.

2. Materials and methods

2.1. Materials

CD1 mice were obtained from Charles River Laboratories (Wilmington, MA), neuronal tubulin specific antibody (Tuj1) and anti-Aβ antibody 6E10 was purchased from Covance (Madison, WI), Hoechst 33342 and other reagents were procured from Sigma (Saint Louis, MO).

2.2. Culture of hippocampal neurons

Hippocampi were dissected from CD1 (Charles River) mice at postnatal day 0 (P0). Animals were anesthetized on ice and sacrificed by decapitation. Hippocampi were removed and incubated in 40 μL/ml of papain. Hippocampi were triturated by passing repeatedly through a 1 ml pipette tip and filtered through 40 μm nylon mesh. Neurons were washed three times in 500 μl of culture medium consisting of neurobasal medium (Invitrogen) supplemented with B-27, penicillin, streptomycin and L-glutamine. Neurons were plated (5×10^4) in 24-well chambered poly-D lysine and laminin coated culture plates in 500 μl of culture medium containing anti-mitotic 1 μM 5-fluoro-2-deoxyuridine and 1 μM uridine to inhibit proliferation of astrocytes and other dividing cells. Neuronal cultures were incubated at 37 °C with 5% CO_2 for the duration of experiments.

2.3. Treatment of neurons with fibrillar and oligomeric Aβ

Fibrillar $A\beta_{1-42}$ and control reverse peptide $A\beta_{42-1}$ (American Peptides) were prepared by incubating freshly solubilized peptide at 25 μM in sterile ammonia (0.5%) at 37 °C for 5 day (Pike et al., 1993). After incubation period Aβ preparations were centrifuged at room temperature (10 min at 15,000*g*), and the pellet containing fibrillar Aβ was resuspended in Hepes buffer (pH 7.5). The formation of fibrillar Aβ was evaluated by a Thioflavin T (ThT) fluorometric assay (Dahlgren et al., 2002). 1 ml of ThT (3 mM in 50 mM sodium phosphate buffer, pH 6.0) was added to fibrillar Aβ preparations and fluorescence was measured at excitation and emission wavelengths of 450 nm and 482 nm. We observed that β-sheet content of fibrillar Aβ, prepared after incubation of the peptide solution for 5 days at 37° C, were significantly higher in comparison with the fresh Aβ, used immediately after peptide solubilization (Supplemental Fig. 1A).

To generate oligomeric we dissolved Aβ peptide in cold hexafluoro-2-propanol (HFIP) to a final concentration of 1 mM (Chromy et al., 2003). The peptide was aliquoted and dried under vacuum and stored at −20° C. The peptide was resuspended in DMSO then diluted to 100 μM in Ham's/F12 media and incubated overnight at 4 °C. The preparation was centrifuged (15,000*g* for 10 min at 4 °C) to remove insoluble aggregates. Supernatant containing soluble oligomers was transferred to clean tubes and stored at 4 °C.

Gel electrophoresis and Western blot analyses were performed to determine the presence and purity of oligomers and fibrils used in our system. To confirm that the $A\beta_{1-42}$ peptide maintained the appropriate fibrillar or oligomeric conformation throughout the period of treatment, we incubated the peptide in B27 media alone at 37 °C for 72 h. $A\beta_{1-42}$ peptide (fibrillar or oligomeric) samples were diluted (1:2) with SDS–PAGE sample buffer (62.5 mM Tris (pH 6.8) containing 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol and 0.001% (w/v) bromophenol blue) and were separated by electrophoresis on a 4–16% Tris–Tricine SDS gel. Samples were not boiled to minimize disaggregation prior to electrophoresis. Protein immunoblots were probed using 6E10 mouse monoclonal anti Aβ antibody (1:1000). In accordance with previous studies (Pike et al., 1993; Resende et al., 2008; Sondag et al., 2009; Itkin et al., 2011) the fibrillar peptide did not resolve into the separating gel and remained in the stacking portion of the gel (Supplemental Fig. 1B). Oligomeric peptide forms high molecular weight SDS-stable oligomers with incubation (Gong et al., 2003). The bulk of the peptide in the oligomeric preparation migrated in the molecular weight range of dimer/trimers (Supplemental Fig. 1C).

At 7 days *in vitro* (DIV), neuronal cultures were treated with 5 μM of fibrillar or oligomeric $A\beta_{1-42}$ for different time periods. Phase-contrast microscopy was performed to monitor neurodegeneration. Neurons were imaged using phase contrast microscopy (Olympus IX71; cell solutions software) with a 20× objective lens. Healthy neurons were recognized by their morphology in phase contrast images, axonal segments were considered degenerated if they showed evidence of fragmentation. Images of 6 random field (50–100 cells) per well were typically used. The observer was blind to the condition ($n \geqslant 5$ wells per condition from six experiments).