Molecular and Cellular Neuroscience 58 (2014) 29–39

Contents lists available at ScienceDirect

Molecular and Cellular Neuroscience

journal homepage: www.elsevier.com/locate/ymcne

Proteasome inhibition induces stress kinase dependent transport deficits — Implications for Alzheimer's disease

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ARTICLE INFO

Article history:
Received 20 August 2013
Revised 10 October 2013
Accepted 14 November 2013
Available online 21 November 2013

Keywords:
Alzheimer's disease
Proteasome dysfunction
Tau phosphorylation
Axonal transport
ERK 1/2
c-Jun

ABSTRACT

Alzheimer's disease (AD) is characterized by accumulation of two misfolded and aggregated proteins, β-amyloid and hyperphosphorylated tau. Both cellular systems responsible for clearance of misfolded and aggregated proteins, the lysosomal and the proteasomal, have been shown to be malfunctioning in the aged brain and more so in patients with neurodegenerative diseases, including AD. This malfunction could be contributing to β-amyloid and tau accumulation, eventually aggregating in plaques and tangles. We have investigated the impact of decreased proteasome activity on tau phosphorylation as well as on microtubule stability and transport. To do this, we used our recently developed neuronal model where human SH-SY5Y cells obtain neuronal morphology and function through differentiation. We found that exposure to low doses of the proteasome inhibitor MG-115 caused tau phosphorylation, microtubule destabilization and disturbed neuritic transport. Furthermore, reduced proteasome activity activated several proteins implicated in tau phosphorylation and AD pathology, including c-Jun N-terminal kinase, c-Jun and extracellular signal-regulated protein kinase (ERK) 1/2. Restoration of the microtubule transport was achieved by inhibiting ERK 1/2 activation, and simultaneous inhibition of both ERK 1/2 and c-Jun reversed the proteasome inhibition-induced tau phosphorylation. Taken together, this study suggests that a decrease in proteasome activity can, through activation of c-Jun and ERK 1/2, result in several events related to neurodegenerative diseases. Restoration of proteasome activity or modulation of ERK 1/2 and c-Jun function can open new treatment possibilities against neurodegenerative diseases such as AD.

Introduction

Alzheimer's disease (AD) is pathologically characterized by accumulation of amyloid-β (Aβ) in extracellular plaques, and tau in intracellular neurofibrillary tangles (Blennow et al., 2006). The familial forms of AD, representing about 1% of all cases, are caused by a number of mutations resulting in increased Aβ production. This in turn is thought to alter tau into a hyperphosphorylated form, more prone to aggregate into neurofibrillary tangles (Götz et al., 2011). The sporadic form is similarly characterized by Aβ and tau aggregates, but the cause of this has so far not been found. However, the progressive nature of AD has been explained by a possible transmission of Aβ oligomers (Hallbeck et al., 2013; Nath et al., 2012) or tau (Clavaguera et al., 2009).

Plaques and tangles were for many years the focus of AD research, but are now thought to appear late in the disease process. Instead, microtubule de-stabilization, obstruction of axonal transport and defects in synaptic function are believed to be early pathological events in AD (Stokin et al., 2005; Takahashi et al., 2002; Terry et al., 1991), and these events have all been shown to cause neuronal death (Perlson et al., 2010). The microtubule stability and subsequent transport along neurites are regulated by binding of tau to microtubules (Drewes et al., 1998). Tau regulates its binding to microtubules by phosphorylation and de-phosphorylation, where hyperphosphorylation of tau causes it to completely detach from microtubules, resulting in its destabilization (Biernat and Mandelkow, 1999; Lindwall and Cole, 1984).

More recently, it was also discovered that hyperphosphorylated tau interferes with proteins connecting cargo vesicles to microtubules, thus resulting in disturbance of axonal transport (Ittner et al., 2009). Glycogen synthase kinase (GSK) 3β and cyclin dependent kinase (CDK) 5 are implicated to be important for phosphorylation of tau in AD. In addition, several other kinases, such as those belonging to the MAP kinase pathway have also been implicated in AD pathology (Churcher, 2006).

All cells, including neurons, have two major systems for clearance of worn out, misfolded and aggregated proteins; the autophagy/lysosomal

Abbreviations: Aβ, β-amyloid; AD, Alzheimer's disease; APP, amyloid precursor protein; CDK, cyclin dependent kinase; ECM, extracellular matrix; ERK, extracellular signal-regulated protein kinase; GSK, glycogen synthase kinase; MSD, Meso Scale Discovery.

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http://dx.doi.org/10.1016/j.mcn.2013.11.001

and the proteasomal systems. The lysosome, an acidic, protease-containing organelle, is generally responsible for degradation of long-lived proteins and organelles, such as worn out mitochondria. The proteasome is a cytosolic, barrel shaped, protein complex. It is a key player in rapid degradation of partially folded or misfolded proteins, being tagged for degradation through ubiquitination (Knecht et al., 2009). As AD is pathologically characterized by deposits of misfolded and aggregated proteins, disturbances of lysosomal and/or proteasomal function have been suggested to be involved in the pathogenesis. Indeed, several studies suggest that disruption of both lysosomal and proteasomal functions is present in AD brain (Nixon and Cataldo, 2006; Oddo, 2008), and there is also evidence of defective ubiquitination, as well as accumulation of ubiquitinated proteins (López Salon et al., 2000; Perry et al., 1987). Degradation of tau has been shown to take place both in lysosomes and proteasomes (Grune et al., 2010; Wang et al., 2009), and aggregated tau and Aβ have been shown to inhibit proteasome function (Keck et al., 2003; Tseng et al., 2008). A decreased proteasome activity has also been shown to cause increased Aβ generation (Agholme et al., 2012; Marambaud et al., 1997), possibly due to degradation of the C-terminal fragment of amyloid precursor protein (APP) by the proteasome (Nunan et al., 2001) or by increased levels of presenilin-1 upon proteasome inhibition (Marambaud et al., 1998).

Not only AD, but also other neurodegenerative diseases such as Frontotemporal Lobar Degeneration (FTLD) display tau pathology (Seltman and Matthews, 2012). Proteasome dysfunction has also been related to other neurodegenerative diseases than AD, such as Parkinson's disease and Huntington's disease (Ross and Pickart, 2004; Valera et al., 2007). Taken together, there is much evidence pointing at the involvement of proteasome disturbance in AD and other neurodegenerative diseases. However, whether it contributes directly to disease progression, and if so how, is not known. Earlier studies investigating the effects of proteasome inhibition on tau levels and phosphorylation have resulted in conflicting results (Brown et al., 2005; Delobel et al., 2005; Liu et al., 2009). In addition, it is not known if decreased proteasome function can affect microtubule stability and axonal transport.

In this study, the effects on tau phosphorylation, microtubule stability and axonal transport upon proteasome inhibition were investigated. We used our recently developed neuronal model, enabling studies of tau phosphorylation and microtubule function in human cells (Agholme et al., 2010). We found that proteasome inhibition caused an increase in tau phosphorylation and disruption of neuritic transport. This was, at least partly, due to activation of two kinases earlier implicated in AD; c-Jun and extracellular signal-regulated protein kinase (ERK) 1/2.

Results

Proteasome inhibition causes cell death

As reduced proteasome function has been implicated in AD (Keller et al., 2000), proteasome inhibition was induced using low concentrations of MG-115, a reversible inhibitor of 20 and 26S proteasome subunits. Exposure of ECM gel differentiated SH-SY5Y neuroblastoma cells (Agholme et al., 2010) to 0.1, 0.25 and 0.5 μM MG-115 for 24 and 48 h resulted in a dose- and time dependent reduction in proteasome activity (Fig. 1A). Exposure to 0.1 μM MG-115 resulted in a small, but significant decrease in cell viability after 24 h (Fig. 1B–C). Exposure to 0.25 and 0.5 μM MG-115 resulted in a dose- and time dependent decrease in cell viability (Fig. 1B–C). However, as the 24 hour incubation time did not result in toxicity to the same extent as 48 hour incubation, the shorter time point was chosen for further experiments.

Proteasome inhibition disturbs vesicle transport along neurites

Disturbed axonal transport is believed to be a key event in early AD pathogenesis (Morfini et al., 2009). Therefore, vesicle transport in neurites, upon proteasome inhibition, was investigated using time-lapse imaging. In this setting all vesicles, regardless of their origin, were included in the analysis. Exposure to 0.1 μM MG-115 significantly decreased vesicle velocity compared to control, and the transport was further decreased using 0.5 μM MG-115 (Fig. 2A–B). Vesicles in control cells moved rapidly within neurites (Movie 1) whereas exposure to 0.5 μM MG-115 resulted in almost complete halt of vesicles (Movie 2). Decrease in vesicle velocity was not due to increase in vesicle size, as the changes in vesicle size did not correlate to the pattern of impaired transport (Fig. 2C). Another sign of disturbed axonal transport is the retraction of mitochondria from neurites (Ebneth et al., 1998). Localization of mitochondria was investigated using fluorescent probe MitoTracker® for mitochondrial staining. MG-115 exposure decreased the number of mitochondria in neurite dose dependently, being almost abolished upon exposure to 0.25 or 0.5 μM MG-115 (Fig. 2D, arrowheads). The decrease in mitochondria was not due to loss of MitoTracker® staining, as the staining intensity in the cell soma remained unchanged, although the high density of mitochondria there made quantification impossible. When quantifying the number of mitochondria in neurites, a significant decrease was seen upon exposure to 0.1 or 0.25 μM MG-115, and was further decreased using 0.5 μM MG-115 (Fig. 2E). These results indicate that a small reduction in proteasome activity is enough to disturb neuritic transport.

Proteasome inhibition affects microtubule stability

The above detected disturbance in neuritic transport could be a result of destabilized microtubules. Therefore, we went further to investigate if proteasome inhibition also caused a destabilization of microtubule in our system. The appearance of “beaded” tubulin is one sign of microtubule destabilization (Stokin et al., 2005). By immunochemistry we found that control cells displayed healthy, elongated tubulin-positive neurites (Fig. 3A, most left panel), and the majority of neurites in cells exposed to 0.1 μM MG-115 appeared healthy, with occasional beaded tubulin (Fig. 3A, second left panel; arrowheads). Higher concentrations of MG-115 caused disruption and beading of tubulin, as well as loss of tubulin positive neurites, indicating that microtubules were destabilized upon proteasome inhibition (Fig. 3A, right panels; arrowheads). Destabilization of microtubules is accompanied by two post-translational modifications, de-acetylation and tyrosination. A decrease in acetylated tubulin indicates de-stabilization of microtubules, as does increase in tubulin tyrosination (Hempen and Brion, 1996; Wehland and Weber, 1987). Western blot using acetylated- and tyrosinated tubulin-specific antibodies showed no change in the ratio of acetylated to total α-tubulin, irrespective of MG-115 concentration (Fig. 3B–C). Surprisingly, we detected a dose-dependent decrease in tubulin tyrosination upon MG-115 exposure, reaching significance using 0.25 and 0.5 μM (Fig. 3B–D). Thus, proteasome inhibition seems to induce microtubule destabilization without classical post-translational changes of tubulin.

Proteasome inhibition increases tau phosphorylation at several disease-relevant epitopes

Hyperphosphorylation of tau, causing detachment from microtubules has, for a long time, been suggested to cause microtubule destabilization in AD and other neurodegenerative diseases (Li et al., 2007; Lindwall and Cole, 1984). Furthermore, hyperphosphorylation of tau is thought to impede axonal transport (Mudher et al., 2004). Phosphorylation at the PHF1, AT8, and S422 epitope was investigated using western blot (Fig. 4A). Quantification using densitometry revealed no change in phosphorylation at the PHF1 epitope (Fig. 4B). However, 0.25 and 0.5 μM MG-115 significantly increased phosphorylation at the AT8 epitope (Fig. 4C) and as low concentrations as 0.1 μM MG-115 was enough to significantly increase phosphorylation at S422 (Fig. 4D). As western blot is a semi quantitative method, we also used the Meso Scale Discovery (MSD) system, a quantifiable antibody-based electrochemiluminescence method, to investigate tau phosphorylation. This system enabled simultaneous