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Unfolded protein response activates glycogen synthase kinase-3 via selective lysosomal degradation

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ABSTRACT

The unfolded protein response (UPR) is a stress response that is activated upon disturbed homeostasis in the endoplasmic reticulum. In Alzheimer's disease, as well as in other tauopathies, the UPR is activated in neurons that contain early tau pathology. A recent genome-wide association study identified genetic variation in a UPR transducer as a risk factor for tauopathy, supporting a functional connection between UPR activation and tau pathology. Here we show that UPR activation increases the activity of the major tau kinase glycogen synthase kinase (GSK)-3 in vitro via a selective removal of inactive GSK-3 phosphorylated at $Ser^{21/9}$. We demonstrate that this is mediated by the autophagy/lysosomal pathway. In brain tissue from patients with different tauopathies, lysosomal accumulations of $pSer^{21/9}$ GSK-3 are found in neurons with markers for UPR activation. Our data indicate that UPR activation increases the activity of GSK-3 by a novel mechanism, the lysosomal degradation of the inactive $pSer^{21/9}$ GSK-3. This may provide a functional explanation for the close association between UPR activation and early tau pathology in neurodegenerative diseases.

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1. Introduction

Aggregates of the microtubule associated protein tau are found in several neurodegenerative disorders, commonly referred to as tauopathies. This includes Alzheimer's disease (AD), Pick's disease (PiD), progressive supranuclear palsy (PSP), and frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) (Ballatore et al., 2007; Gendron and Petrucelli, 2009), a familial tauopathy that is associated with mutations in the gene encoding tau. Tau is predominantly involved in facilitating the structure and function of microtubules. Under physiological conditions, tau phosphorylation is a rapid and reversible process, mediated by the opposing actions of several protein kinases and phosphatases (Cowan et al., 2010; Cuchillo-Ibanez et al., 2008). In contrast, hyperphosphorylation of tau is thought to precede the formation of tau aggregates and is therefore associated with pathology (Alonso et al., 2001; Alonso et al., 2010; Bancher et al., 1989; Rankin et al., 2008).

The unfolded protein response (UPR), a stress response pathway that is activated upon a disturbance in endoplasmic reticulum (ER) homeostasis, is strongly associated with the pathology of tauopathies (Hoozemans et al., 2009; Nijholt et al., 2011c). Three ER transmembrane proteins function as stress sensors and mediate the effects of the UPR: PERK, IRE1, and ATF6. During ER stress, these proteins are activated by phosphorylation (PERK and IRE1) or cleavage (ATF6), and activate responses aimed at restoring homeostasis in the ER (Schroder and Kaufman, 2005). Activation of the UPR leads to the following: an overall reduction in translation to reduce the amount of novel polypeptides in the ER, and the transcriptional and translational upregulation of factors that facilitate protein folding and degradation. We and others previously reported UPR activation markers in close spatio-temporal connection with phosphorylated tau in AD brain (Hoozemans et al., 2005; Hoozemans et al., 2009; Unterberger et al., 2006). Our group observed the same association of the UPR and phosphorylated tau protein in non-AD tauopathies, including cases of sporadic PiD, PSP, and FTDP-17 (Nijholt et al., 2011c). In this cohort, the involvement of amyloid β (Aβ) pathology and overall ageing effects could be excluded,

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strengthening the connection between the UPR and early tau pathology. Interestingly, a recent genome-wide association study has identified genetic variation in the gene encoding the ER stress transducer PERK as a risk factor for PSP, suggesting that perturbation of the UPR can influence the risk for developing tau pathology (Hoglinger et al., 2011).

A large number of kinases phosphorylate tau in vitro (Hanger et al., 2009), but the precise mechanisms of physiological regulation have not yet been elucidated. A major kinase implicated in physiological and pathological phosphorylation of tau is glycogen synthase kinase 3 (GSK-3). Tau is phosphorylated by GSK-3 in vitro (Hanger et al., 1992; Ishiguro et al., 1992), in cultured cells (Lovestone et al., 1994; Lovestone et al., 1996), and in transgenic mice overexpressing GSK-3 (Brownlees et al., 1997; Gomez-Sintes et al., 2007; Lucas et al., 2001). GSK-3 phosphorylates tau at multiple sites, and most of these sites are also phosphorylated in disease (Hanger et al., 1992; Hanger and Noble, 2011; Wagner et al., 1996). GSK-3 plays a role in a wide variety of signaling pathways including glycogen metabolism, protein synthesis, mitosis, apoptosis, and microtubule dynamics (Forde and Dale, 2007). GSK-3 exists as 2 highly homologous isoforms, GSK-3α and GSK-3β, which are encoded by different genes located on chromosomes 19 and 3, respectively (Woodgett, 1990). Both isoforms are expressed in the brain (Lau et al., 1999; Yao et al., 2002), however GSK-3β appears to be the predominantly expressed isoform (Lau et al., 1999). Some, but not all, substrates require priming by phosphorylation at a nearby residue by another kinase that enables phosphorylation by GSK-3 (Bax et al., 2001; Fiol et al., 1987). GSK-3β phosphorylates tau at primed and unprimed sites; however, phosphorylation at primed sites was shown to play a key role in regulating in vitro tau microtubule binding (Cho and Johnson, 2003). GSK-3 activity is inhibited by phosphorylation of the Ser^{21} and Ser^{9} residue in GSK-3α/β, respectively. This residue is located in the N-terminus of GSK-3; when phosphorylated, this acts as a primed pseudo-substrate that loops back in the active site (Forde and Dale, 2007). Inhibitory phosphorylation of GSK-3 at this site occurs in response to several signaling pathways. Protein kinase A phosphorylates GSK-3 upon an increase in levels of the second messenger cyclic AMP, caused by binding of ligands to G-protein–coupled receptors on the cell membrane (Fang et al., 2000). The insulin signaling pathway leads to increased activity of phosphatidyl inositol kinase 3 (PI3K) and protein kinase B (PKB)/Akt. The commonly used activator of GSK-3, wortmannin (Wm), inhibits PI3K and in this manner prevents inhibitory phosphorylation by PKB/Akt (Grimes and Jope, 2001; Li et al., 2006). Phosphorylation of GSK-3α/β at Tyr^{216} and Tyr^{279}, respectively, is associated with increased GSK-3 activity (Bax et al., 2001). Inhibition of this phosphorylation event either by enzymatic dephosphorylation or mutation of Tyr into Phe, decreases the kinase activity (Hagen et al., 2002). Phosphorylation at the Tyr residue is suggested to be an autophosphorylation event; however, some kinases have been shown to target GSK-3 $Tyr^{216/279}$ in vitro (Hartigan et al., 2001; Lesort et al., 1999; Medina and Wandosell, 2011). The precise mechanism remains unknown but the position of the $Tyr^{216/279}$ residue in the GSK-3 activation loop suggests it induces a conformational change that results in more efficient substrate binding (Bax et al., 2001; Hagen et al., 2002).

The role of GSK-3 in tau pathology has been the subject of several studies; however, its regulation and involvement in the pathogenesis of tauopathies is not yet fully elucidated. GSK-3β co-isolates with brain-derived microtubules (Ishiguro et al., 1993) and, in cells, phosphorylates tau at epitopes also found in AD (Hanger et al., 1992; Lovestone et al., 1994). Inhibition by lithium or more specific GSK-3 inhibitors have prevented tau phosphorylation and aggregation in several murine models (Leroy et al., 2010; Noble et al., 2005; Sereno et al., 2009). The association of the UPR with early tau pathology prompted the investigation of effects of in vitro UPR induction on GSK-3 activity. Chemical induction of the UPR results in decreased phosphorylation at the inhibitory $Ser^{21/9}$ epitope and increased phosphorylation at the activating $Tyr^{279/216}$ epitope (Fu et al., 2010). Another study identified only decreased $Ser^{21/9}$ phosphorylation (Song et al., 2002). Although the mechanism remains elusive, these studies suggest that activation of the UPR leads to increased activity of GSK-3.

In this study, we further investigated the connection between activation of the UPR and GSK-3 activity in vitro. Furthermore, we analyzed the presence of GSK-3 in connection to UPR activation and tau pathology in post-mortem brain material from patients with different types of tauopathies. Our data provide evidence for UPR-induced regulation of GSK-3 activity via lysosomal degradation.

2. Methods

2.1. Cell culture, differentiation, and treatment

Human SK-N-SH neuroblastoma cells were cultured in Dulbecco's modified Eagle's medium with GlutaMax (Gibco BRL, Carlsbad, CA) supplemented with 10% fetal calf serum (Lonza, Basel, Switzerland) and 100 U/mL penicillin (Yamanouchi Pharma BV, Leiderdorp, the Netherlands). Cells were differentiated in culture medium supplemented with all trans-retinoic acid (Sigma, St Louis, MO) in a final concentration of 10 μmol/L for 5 days. Differentiated cells were subsequently treated with tunicamycin (Tm), lithium chloride (LiCl), and Bafilomycin (BAF) at indicated concentrations for 16 hours. Treatment with wortmannin (Wm) was performed at 100 nmol/L for 1 hour before harvest. For starvation, medium was removed and cells were washed twice in phosphate-buffered saline (PBS) to remove excess nutrients. Cells were subsequently cultured for 2 hours in Earle's balanced salt solution (Sigma), which lacks essential amino acids.

Dissociated cortical neurons were prepared from embryonic day 18 mice as described elsewhere (De Wit et al., 2009). Cerebral cortices were dissected in Hanks Buffered Salts Solution (HBSS, Sigma) and digested with 0.25% trypsin (Invitrogen Life Technologies, Carlsbad, CA, USA) for 20 minutes at 37 °C. Tissue was washed and triturated with fire-polished Pasteur pipettes, counted, and plated in 6-well plates (200,000 neurons per well) in Neurobasal medium supplemented with 2% B-27, 1.8% HEPES, 1% glutamax, and 1% Pen-Strep (all from Invitrogen). For immunocytochemistry, high-density cultures (25,000 neurons per well) were plated on pre-grown cultures of rat glia cells (37,500 cells per well) on 18-mm glass coverslips in 12-well plates. Neurons were treated with Tm at DIV7.

2.2. ELISA colorimetric assay

The InCell ELISA colorimetric assay (Thermo Scientific, Waltham, MA) was performed according to the manufacturer's recommendations. In short, SK-N-SH cells were plated in 96-well plates (15,000 cells per well) and differentiated as described above. All conditions were assayed in triplicate. Unless otherwise stated, all incubations were performed at room temperature (RT) with gentle rocking. Cells were fixed in 4% formaldehyde for 15 minutes. Formaldehyde was aspirated; cells were washed twice in 1x Tris buffered saline (TBS) and incubated with permeabilization buffer for 15 min. After permeabilization, cells were washed with 1x TBS and incubated with quenching solution (containing H_2O_2) for 20 minutes. Cells were washed with 1x TBS and subsequently blocked for 30 minutes in blocking solution. After blocking, cells were incubated with the primary antibody solution for 16 hours at 4 °C. Antibodies and their dilutions are described in Table 1. Cells were washed 3 times with wash buffer and subsequently incubated with

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