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Annonacin, a natural lipophilic mitochondrial complex I inhibitor, increases phosphorylation of tau in the brain of FTDP-17 transgenic mice

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ABSTRACT

Both genetic and environmental factors likely contribute to the neuropathology of tauopathies, but it remains unclear how specific genetic backgrounds affect the susceptibility towards environmental toxins. Mutations in the tau gene have been associated with familial tauopathies, while annonacin, a plant-derived mitochondrial inhibitor, has been implicated in an environmental form of tauopathy. We therefore determined whether there was a pathogenic synergy between annonacin exposure and the expression of the R406W-tau mutation in transgenic mice. We found that annonacin exposure caused an increase in the number of neurons with phosphorylated tau in the somatodendritic compartment in several brain areas in R406W$^{+/+}$ mice as opposed to mice that had only the endogenous mouse tau (R406W$^{-/-}$). Western blot analysis demonstrated a concomitant increase in total tau protein without increase in tau mRNA, but reduced proteasomal proteolytic activity in R406W$^{+/+}$, but not R406W$^{-/-}$ mice, upon annonacin-treatment. Phosphorylated tau levels exceeded the increase in total tau protein, along with increased levels of different tau kinases, foremost a striking increase in the p25/p35 ratio, known to activate the tau kinase Cdk5. In summary, we observed a synergistic interaction between annonacin exposure and the presence of the R406W-tau mutation, which resulted in reduced degradation, increased phosphorylation and redistribution of neuronal tau.

Introduction

The microtubule-associated protein tau (MAPT), normally found in axons, is involved in microtubule polymerization and stabilization (Weingarten et al., 1975). From one MAPT gene on chromosome 17q21.31 encoding 16 exons, six different tau protein isoforms are generated by alternative splicing in the adult human brain (Spillantini and Goedert, 2013). Tauopathies are neurodegenerative diseases defined by the presence of filamentous inclusions of hyperphosphorylated tau in neurons or glial cells (Ludolph et al., 2009). Present evidence strongly suggests that the etiological spectrum of tauopathies ranges from monogenetic variants to predominantly environmental forms, with a rather large group of "sporadic" forms occupying the middle spectrum, where a genetic predisposition and hypothetical environmental triggers are probably jointly involved.

An example of an environmentally caused tauopathy is the atypical Parkinson syndrome endemic in Guadeloupe, linked to high consumption of nutritional products of Annonaceae plants (Caparros-Lefèbvre and Elbaz, 1999; Champy et al., 2005, 2009; Lannuzel et al., 2007). The implicated neurotoxins are Annonaceous acetogenins, a unique family of potent and lipophilic inhibitors of complex I of the respiratory chain (Bermejo et al., 2005) showing tropism towards mitochondria (Derbré et al., 2005; Kojima et al., 2010). Annonacin, a prototypical representative of this class, causes neuronal loss and somatodendritic accumulation of phospho-tau in vivo and in vitro (Champy et al., 2004; Escobar-Khondiker et al., 2007; Lannuzel et al., 2003). A systematic investigation of 24 globally distributed lipophilic complex I inhibitors has demonstrated a correlation between their potency to inhibit mitochondrial

Abbreviations: Cdk5, cyclin-dependent kinase 5; FTDP-17, frontal temporal dementias with Parkinsonism linked to chromosome 17; GSK-3β, glycogen synthase kinase 3β; MAPK (Erk1/2), mitogen-activated protein kinase; SAPK, stress-activated protein kinase.

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complex I with their potency to induce somatodendritic redistribution of phosphorylated tau protein in cultured rodent striatal neurons (Höllerhage et al., 2009). Finally, a number of studies in patients with progressive supranuclear palsy, a prototypical tauopathy, using different methodological approaches, point to a primary impairment of mitochondrial energy metabolism (Albers et al., 2001; Martinelli et al., 2000; Stamelou et al., 2009; Swerdlow et al., 2000). Together, these clinical and experimental observations suggest that complex I inhibition could contribute to pathogenic mechanisms in some sporadic tauopathies.

At the other end of the spectrum, 51 pathogenic MAPT mutations have been associated with a wide range of autosomal dominantly inherited tauopathies, as the frontotemporal dementias with Parkinsonism linked to chromosome 17 (FTDP-17-Tau; Spillantini and Goedert, 2013). Typically, the neuropathological findings consist of frontotemporal neuronal loss and gliosis with intraneuronal neurofibrillary tangles (NFTs) and, occasionally, glial tau deposits (Lindquist et al., 2008; van Swieten et al., 1999). Interestingly, distinct clinical presentations may occur in association with the same mutation (Baba et al., 2005; Ishizuka et al., 2011; Reed et al., 2001), even within the same family (Bird et al., 1999), suggesting that additional genetic or environmental modifiers contribute to the determination of the final clinical and neuropathological phenotype.

The R406W mutation is a particularly interesting MAPT missense mutation in exon 13 (Arg^{406} to Trp, numbered according to the longest tau isoform), first described in North America (Hutton et al., 1998; Reed et al., 1997), later in the Netherlands (Rizzu et al., 1999; van Swieten et al., 1999), Belgium (Rademakers et al., 2003), Sweden (Ostojic et al., 2004; Passant et al., 2004), Denmark (Lindquist et al., 2008, 2009) and Japan (Ikeuchi et al., 2008). High variability in the clinical manifestation has been described between and within R406W kindreds. Genetic testing in two siblings fulfilling diagnostic criteria for familial Alzheimer's disease (AD) revealed the R406W tau mutation in both individuals (Tolboom et al., 2010). Age of onset varied from 45 to 75 years and most studied cases initially resemble Alzheimer's disease (AD) with early memory impairment, with frontal lobe symptoms and language problems appearing later (Lindquist et al., 2008; Reed et al., 1997). However, some patients showed frontal personality changes as the earliest and most striking feature with the presence of Parkinsonian symptoms being variable (Reed et al., 1997; van Swieten et al., 1999).

The phenotypic variability of inherited tauopathies suggests the presence of environmental modifiers. Potential candidates are toxins already implicated in environmental variants of tauopathies. Searching for gene–environment interactions leading to pathogenic synergy, we investigated therefore the effects of annonacin in mice overexpressing the longest human tau isoform with the R406W mutation (Zhang et al., 2004).

Methods

Animals

$R406W^{+/+}$ transgenic mice breeder pairs were donated by Dr. Virginia M.-Y. Lee, University of Pennsylvania School of Medicine (Philadelphia, USA). Their full description is given at Zhang et al. (2004). Briefly, the longest human tau isoform containing the R406W mutation was cloned into the mouse prion promoter (MoPrP.Xho) expression vector at the XhoI site, and fertilized in a mixed C57BL6 × C3H (B6C3/F1) strain background. Homozygous $R406W^{+/+}$ and non-transgenic wild-type ($R406W^{-/-}$) mice used were kept in the same B6C3 background. Only males were used in the experiments. The genotype was confirmed by genomic DNA samples isolated from the tails using a peqGold tissue DNA extraction kit (PEQLAB, Erlangen, Germany). Cerebral expression of the mutated human protein was confirmed by Western blots.

Complex I inhibitor

Annonacin was obtained from fruit pulp of *Annona muricata*, as described (Champy et al., 2005). The purity of this compound was confirmed by analytical reversed-phase HPLC-DAD/ESI-Q-MS.

Annonacin exposure

The animal work was approved by the appropriate governmental authority (Regierungspräsidium Giessen, V54-19c20/15(1)MR20/15-Nr03/2005) and conducted in accordance with the European Community Council Directive 86/609/EEC. Male $R406W^{-/-}$ and $R406W^{+/+}$ mice were 16–18 weeks old, weighting 30–40 g at the time of operation. Three to six animals were housed per cage with free access to food and water under a 12-h/12-h light/dark cycle at 26 °C. Osmotic minipumps (Alzet 1003, 100 μl, 1.04 μl/h; Charles River Laboratory, Sulzfeld, Germany) were filled with solutions containing annonacin or vehicle in equal volumes of dimethylsulfoxide (DMSO) and polyethyleneglycol 400 (PEG) and incubated in sterile 0.9% (wt/vol) NaCl for 4 h prior to implantation. Mice were anesthetized by an intraperitoneal (i.p.) injection of 10 ml/kg of ketamine:xylazine:saline (1:1:8), corresponding to a dose of 10 mg/kg of ketamine and 20 mg/kg of xylazine. Pumps were implanted under the skin on the back of the mice, at approximately the level of the cervical column. Forty-one $R406W^{-/-}$ and 42 $R406W^{+/+}$ mice were infused with annonacin at a rate of 0, 6 or 9 mg/kg/day, calculated with respect to their body weight. A higher dose of 12 mg/kg/day provoked 100% mortality within 3 days of exposure and was not further pursued. Animals were weighted before and after the 3-day infusion period.

Tissue preparation

After the 3-day infusion period, mice received a lethal dose of 200 mg/kg of sodium pentobarbital i.p. and were perfused transcardially with ice-cold 0.1 M phosphate-buffered saline (PBS) for 2 min. Thereafter, the brains were quickly removed. The hemispheres were separated by a mid-sagittal cut. One hemisphere was post-fixed in 4% (wt/vol) paraformaldehyde in 0.1 M PBS for 24 h, cryoprotected in 10% (wt/vol) sucrose in 0.1 M PBS for 48 h, frozen in isopentane at −30 °C for 2 min and stored at −80 °C for histology. The other hemisphere was immediately dissected, frozen in −30 °C isopentane and stored at −80 °C for Western blotting, measurement of proteasomal proteolytic activities and quantitative PCR. A minimum of three samples per treatment group was analyzed in each assay.

Immunohistochemistry

The brains were cut into 40-μm sections using a cryostat (Leica, Wetzlar, Germany), collected in 10 regularly spaced series, and stored in 0.1 M PBS containing 0.01% (wt/vol) sodium azide at 4 °C. Free-floating sections were incubated successively for 15 min with 0.1% H_2O_2 in 0.1 mol/l PBS to block endogenous peroxidase activity, for 1 h with 5% (vol/vol) normal donkey serum (NDS) in 0.1 M PBS and 0.2% Triton X-100 to inhibit non-specific binding sites, and for 24 h at 4 °C with the mouse monoclonal primary antibodies raised against the following phospho-tau epitopes: AT270 antibody against pT181, AT8 antibody against pS202/pT205, AT100 antibody against pT212/pS214, AT180 antibody against pT231, (all from Thermo Scientific, Rockford, IL, USA; used 1:100), and AD2 antibody against pS396/pS404 (Bio-Rad, Hercules, CA; 1:1000). We used the anti-NeuN mouse monoclonal antibody (Millipore, Billerica, MA, USA; 1:1000) for labeling neurons, and the anti-Iba1 rabbit polyclonal antibody (Wako, Osaka, Japan; 1:1000) for microglia. All antibodies were diluted in 0.1 M PBS with 5% NDS and 0.2% Triton X-100. Sections were then incubated for 2 h at room temperature with the appropriate biotinylated secondary antibody (anti-rabbit or anti-mouse IgG; Jackson ImmunoResearch, West