IBRO Reports 1 (2016) 32–45

Contents lists available at ScienceDirect

IBRO Reports

journal homepage: http://www.journals.elsevier.com/ibro-reports

Presence of insoluble Tau following rotenone exposure ameliorates basic pathways associated with neurodegeneration

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ARTICLE INFO

Article history:
Received 7 June 2016
Received in revised form
23 September 2016
Accepted 24 September 2016

Keywords:
Hyperphosphorylated Tau
Hippocampus
Rotenone
Oxidative stress
Proteasome activity
Autophagy flux
Protein aggregation

ABSTRACT

Protein aggregation is an important feature of neurodegenerative disorders. In Alzheimer's disease (AD) protein aggregates are composed of hyperphosphorylated Tau and amyloid beta peptide (Aβ). Despite the involvement and identification of the molecular composition of these aggregates, their role in AD pathophysiology is not fully understood. However, depositions of these insoluble aggregates are typically reported as pathogenic and toxic for cell homeostasis. New evidences suggest that the deposition of these aggregates is a protective mechanism that preserves cell from toxic insults associated with the early stages of neurodegenerative diseases. To better understand the biological role of the protein aggregation with regard its effects in cellular homeostasis, the present study investigated the role of insoluble Tau and Tau aggregates on crucial cellular parameters such as redox homeostasis, proteasome activity and autophagy in hippocampal cell cultures and hippocampus of aged Lewis rats using a rotenone-induced aggregation model. Neurons were exposed to rotenone in different concentrations and exposure times aiming to determine the interval required for Tau aggregation. Our experimental design allowed us to demonstrate that rotenone exposure induces Tau hyperphosphorylation and aggregation in a concentration and time-dependent manner. Oxidative stress triggered by rotenone exposure was observed with the absence of Tau aggregates and was reduced or absent when Tau aggregates were present. This reduction of oxidative stress along with the presence of insoluble Tau was independent of alterations in antioxidant enzymes activities or cell death. In addition, rotenone induced oxidative stress was mainly associated with decrease in proteasome activity and autophagy flux. Conversely, when insoluble Tau appeared, autophagy turns to be overactivated while proteasome activity remained low. Our studies significantly advance the understanding that Tau aggregation might exert protective cellular effects, at least briefly, when neurons are facing neurodegeneration stimulus. We believe that our data add more complexity for the understanding of protein aggregation role in AD etiology.

1. Introduction

Neurodegeneration is often associated with the presence of extra and intracellular protein aggregates distributed throughout the central nervous system (CNS). Several neurodegenerative disorders, including Alzheimer's disease (AD) and frontotemporal dementia, are characterized by the presence of intracellular inclusions of neurofibrillary tangles (NTFs) and paired helical filaments (PHFs). These inclusions are mainly composed of hyperphosphorylated Tau (Ross and Poirier, 2005; Frost et al., 2015).

Tau is a microtubule-associated phosphoprotein expressed primarily in the CNS and mainly distributed at the axonal compartment promoting microtubules assembly and stabilization. Abnormal hyperphosphorylation of Tau at various sites negatively regulates its function and is implicated in degeneration and neuronal loss in AD and other tauopathies. Hyperphosphorylation not only reduces Tau housekeeping functions but also increases its cellular toxicity where in hyperphosphorylated Tau sequester the normal Tau (Kuret et al., 2005) to form Tau oligomers, being these

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http://dx.doi.org/10.1016/j.ibror.2016.09.001

oligomers described as precursors of PHFs and NTFs (Alonso et al., 1996).

The presence of intracellular inclusions of Tau is believed to disrupt neuron physiology, through alterations in microtubule dynamics (Kuret et al., 2005), axonal transport (Cuchillo-Ibanez et al., 2008; Shahpasand et al., 2012; Smith and Bennett, 1997), synaptic function (Scheff et al., 2013) and loss of redox homeostasis (Melo et al., 2011). Proteasome and autophagy pathways, mechanisms involved in clearing hyperphosphorylated Tau/Tau aggregates, are also suggested to be dysregulated during the etiology of tauopathies (Huang and Figueiredo-Pereira, 2010; Ben-Gedalya and Cohen, 2012).

To date, several reports from animal and cellular models that recapitulate the basic stages of disease progression suggested that neuronal loss occurs before NFTs are formed. Tau oligomers that are formed during the early stages of protein aggregation are considered to be more toxic to cells than protein inclusions themselves (Chung et al., 2001; Flach et al., 2012; Hoover et al., 2010; Lasagna-Reeves et al., 2010). Although Tau is implicated in the pathology of AD, the contribution of few Tau species (oligomers and insoluble Tau) in disease initiation are not fully appreciated. Studies also report protective effects associated with the formation of protein inclusions in AD (de Calignon et al., 2010; Sydow et al., 2011) and other neurodegenerative diseases models (Bodner et al., 2006).

These findings emphasize the importance of studying cellular events that occur prior and during the formation of protein aggregation to advance our understanding of neurodegeneration process in AD. Thus, although protein aggregation has been extensively investigated, it biological role remains contradictory. We hypothesize that this conundrum rises with the fact that most reported studies have relayed on Tau overexpression or transgenic systems expressing mutant Tau, lacking endogenous regulation and possibly inducing extreme toxic environments distinctly from the environment induced by constitutive protein aggregation.

We showed previously that low concentrations (0.5–1 nM) of rotenone, a mitochondrial complex I inhibitor widely employed in studies related to neurodegeneration (Radad et al., 2008; Ullrich and Humpel, 2009), were able to induce hyperphosphorylation and aggregation of constitutive Tau in the absence of cell death in primary hippocampus cell cultures (Chaves et al., 2010). Chronic administration of rotenone to rats also induces cerebral tauopathy (Höglinger et al., 2005; Almeida et al., 2016) revealing rotenone as a reliable method to induce constitutive Tau aggregation in *in vivo* and *in vitro* systems.

Based on this, rotenone exposure could be used, as a system to indirectly evaluate whether dysfunctions in neuronal homeostasis occur before the formation of Tau aggregation and whether Tau aggregation influences positively or negatively these early dysfunctions.

Oxidative stress induced by increase in reactive oxygen species (ROS) is considered an early event occurring in AD, possibly acting as an inductor of protein aggregation (Tabner et al., 2005; Hands et al., 2011). In addition to oxidative stress, decrease in protein degradation pathways, proteasome and autophagy, have been reported during the initial stages of AD (Cecarini et al., 2007; Resende et al., 2008).

Proteasome system plays a pivotal role in clearing oxidized and misfolded proteins and inhibition of proteasome activity led to increase in Tau accumulation (Tseng et al., 2008) and hyperphosphorylation (Agholme et al., 2014; Carrettiero et al., 2009). Moreover, oxidative stress might impair protein degradation pathways, such as the proteasome system (Cecarini et al., 2007; Lam et al., 2000), and inhibition of proteasome activity can induce oxidative stress (Maharjan et al., 2014) demonstrating a cross-talk between both pathways in early phases of AD.

Since exposure to high doses of rotenone (100 nM) disrupt proteasome activity and increase ROS synthesis (Chou et al., 2010), and rotenone exposure also induce Tau hyperphosphorylation, it is plausible postulate that rotenone reproduce some early aspects related with AD pathophysiology. Here, we propose investigate, using hippocampal cell cultures and hippocampus of aged Lewis rats, (1) whether dysfunctions in redox homeostasis, protein degradation machinery and Tau hyperphosphorylation, triggered by exposure to rotenone, occur before Tau aggregation; and (2) whether dysfunctions induced by rotenone exposure in the absence of aggregates are potentiated or mitigated by the presence of insoluble Tau and Tau aggregates.

2. Experimental procedures

All the procedures were performed in strict accordance with Institutional and International Guidelines for animal care and use (Demers et al., 2006), as well as respecting the Brazilian federal law 11794/08 for animal welfare.

2.1. Primary neuronal cell culture and rotenone exposure

Methodology employed for cell culture was a modification of the previously described protocol (Kivell et al., 2001). Briefly, 20 neonatal (1 day-old) Lewis rats had their brains dissected out to access the hippocampus, which was dissociated in sterile cold solution consisting of 120 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 13 mM glucose, pH 7.2. Cell solution was centrifuged at 300 × *g* for 5 min. The supernatant was discarded and cells were suspended in Neurobasal A medium (Gibco) supplemented with 0.25 mM Glutamax (Gibco), 2% B27 (Gibco), 0.25 mM L-Glutamine (Sigma) and 40 mg/L Gentamicin (Gibco).

Cells were plated on 12-well nunclon (Nunc), 96-well plate (Nunc) or confocal dishes (MatTek), coated with poli-$_D$-lysine, at the density of 1800 cells/mm^2. Cultures were kept in a humidified incubator with 5% CO_2 at 37 °C for nine days with the media changed every three days.

It was previously reported that exposure of primary cell cultures to rotenone in concentrations over 1 nM during 48 h induces massive cell death (Chaves et al., 2010). However, exposure to low concentration of rotenone (0.5 nM) during 48 h did not induce cell death and triggered Tau aggregation. Here, to further investigate rotenone effects in neuron homeostasis and in Tau aggregation, exposure to 0.5 and 0.3 nM of rotenone were used. Exposure to 0.3 nM was chosen to verify whether lowering rotenone concentration by 40% would impair its effects upon Tau aggregation.

Media were supplemented with rotenone (Sigma) 0.3 or 0.5 nM or with dimethyl sulfoxide (DMSO, Sigma), and applied to cell cultures during either 48 or 72 h. The concentration of DMSO was maintained at 0.002% in all treatment groups. Cells plated on confocal dishes were used for immunocytochemistry assays for MAP2 and Tau detection and to live cell analysis of ROS content and autophagy flux. Cells grown on 6 and 96 wells culture plates were used for western blot and biochemical assays.

2.2. Determination of cell viability by MTT reduction and trypan blue stain assays

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was carried on 96 wells plated neuronal cultures. After each treatment, 20 μl of MTT (5 mg/ml) was added into each well and the plate was further incubated for 1 h at 37 °C. Media in each well was discarded and the colored products of MTT in cells were solubilized with 200 μl of DMSO. Optical intensity at 570 nm