Experimental Neurology 275 (2016) 84–98

Contents lists available at ScienceDirect

Experimental Neurology

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

Amyloid-β suppresses AMP-activated protein kinase (AMPK) signaling and contributes to α-synuclein-induced cytotoxicity

Chih-Li Lin [a,b], Yu-Shih Cheng [a], Hsin-Hua Li [a], Pai-Yi Chiu [a,c], Yen-Ting Chang [a], Ying-Jui Ho [d,e], Te-Jen Lai [a,e,*]

[a] Institute of Medicine, Chung Shan Medical University, Taichung City, Taiwan
[b] Department of Medical Research, Chung Shan Medical University Hospital, Taichung City, Taiwan
[c] Department of Neurology, Show Chwan Memorial Hospital, Changhua, Taiwan
[d] School of Psychology, Chung Shan Medical University, Taichung City, Taiwan
[e] Department of Psychiatry, Chung Shan Medical University Hospital, Taichung City, Taiwan

ARTICLE INFO

Article history:
Received 7 May 2015
Received in revised form 11 October 2015
Accepted 24 October 2015
Available online 26 October 2015

Keywords:
α-Synuclein
Amyloid-β
Dementia with Lewy bodies
AMP-activated protein kinase
Sirtuin 1

ABSTRACT

Dementia with Lewy bodies (DLB) is a neurodegenerative disorder caused by abnormal accumulation of Lewy bodies, which are intracellular deposits composed primarily of aggregated α-synuclein (αSyn). Although αSyn has been strongly implicated to induce neurotoxicity, overexpression of wild-type αSyn is shown to be insufficient to trigger formation of protein aggregates by itself. Therefore, investigating the possible mechanism underlying αSyn aggregation is essential to understand the pathogenesis of DLB. Previous studies have demonstrated that amyloid β (Aβ), the primary cause of Alzheimer's disease (AD), may promote the formation of αSyn inclusion bodies. However, it remains unclear how Aβ contributes to the deposition and neurotoxicity of αSyn. In the present study, we investigated the cytotoxic effects of Aβ in αSyn-overexpressed neuronal cells. Our results showed that Aβ inhibits autophagy and enhances αSyn aggregation in αSyn-overexpressed cells. Moreover, Aβ also reduced sirtuin 1 (Sirt1) and its downstream signaling, resulting in increased intracellular ROS accumulation and mitochondrial dysfunction. Our in vitro and in vivo studies support that Aβ-inhibition of AMP-activated protein kinase (AMPK) signaling is involved in the neurotoxic effects of αSyn. Taken together, our findings suggest that Aβ plays a synergistic role in αSyn aggregation and cytotoxicity, which may provide a novel understanding for exploring the underlying molecular mechanism of DLB.

1. Introduction

Dementia with Lewy bodies (DLB) is the second most common progressive dementia after Alzheimer's disease (AD) (Mayo and Bordelon, 2014). The main pathological features of DLB are abnormal protein deposits inside brain neurons. These deposits, known as Lewy bodies, cause cytotoxic effects to the neuronal cells. Structurally, Lewy bodies are composed primarily of α-synuclein (αSyn), a 140-amino soluble protein encoded by the *SNCA* gene abundantly expressed in the central nervous system (Kim et al., 2014). αSyn is likely to play a role in membrane stability and neuronal plasticity; however, its exact function remains largely unknown (Schulz-Schaeffer, 2010). Although some studies have implicated that αSyn is associated with cytotoxicity in DLB pathology, overexpression of wild-type αSyn is hard to generate αSyn protein aggregates (Ko et al., 2008). Therefore, investigating the mechanism underlying αSyn aggregation is of great importance in understanding DLB. To date, research has shown three missense mutations (A30P, E46K, A53T) present in the *SNCA* gene in causing familial synucleinopathies (Ozansoy and Basak, 2013). However, mutations in *SNCA* gene are responsible for only a very small fraction of familial cases, indicating mutated αSyn cannot be the primary cause of most DLB patients (Meeus et al., 2012). In fact, high levels of αSyn alone do not readily aggregate or form fibrils even with prolonged incubation (Hong et al., 2011). This indicates that some other pathological attributes may contribute to the process of αSyn aggregation. In addition, it is interesting that oxidative stress appears to accelerate abnormal aggregation of αSyn, supporting a cause–effect relationship between oxidative stress and αSyn aggregation in the pathogenesis of DLB (Pukass and Richter-Landsberg, 2014). However, the molecular mechanisms underlying wild-type αSyn aggregation are still largely unknown.

Although αSyn is the main component of Lewy bodies in DLB or Parkinson's disease (PD), it is originally found in senile plaques from AD patients' brains (Ueda et al., 1993). As we know, DLB shares symptoms and sometimes overlaps with AD. Actually, patients who develop DLB often have behavioral and memory symptoms of dementia like those in AD. Furthermore, most DLB patients also have concomitant AD pathology, making it difficult to distinguish between AD and DLB (Fujishiro et al., 2013). In fact, up to one-fourth of clinically-diagnosed DLB patients also develop cortical deposition of amyloid-β (Aβ), the

* Corresponding author at: Institute of Medicine, Chung Shan Medical University, No. 110, Sec. 1, Jianguo N. Rd., Taichung City 402, Taiwan.
E-mail address: ltj3123@ms2.hinet.net (T.-J. Lai).

http://dx.doi.org/10.1016/j.expneurol.2015.10.009
0014-4886/

most characteristic hallmark of AD (Hishikawa et al., 2003). In addition, imaging studies also show a significant increase of αSyn aggregates in human brains coexisting with Aβ deposits (Maetzler et al., 2009). Similarly, animal studies demonstrate Aβ brain deposits are highly associated with local aggregation of αSyn in Tg2576 transgenic mice (Emmer et al., 2012). These findings indicate that the presence of Aβ in neurons may contribute to αSyn aggregation and neurotoxicity (Pletnikova et al., 2005), but the exact nature of Aβ in this process is still unclear.

In addition, αSyn neurotoxicity is also linked to oxidative stress (Scarlata and Golebiewska, 2014). Likewise, Aβ-induced oxidative stress has also been shown to interfere in protein folding and degradation in neuronal cells, and these pathologic features are very similar to those found in DLB (Kotzbauer et al., 2001). In fact, emerging evidence has implicated Aβ can suppress neuronal autophagy (Silva et al., 2011), suggesting that a reduced autophagic clearance may deteriorate the accumulation of some aggregation-prone proteins such as αSyn (Nilsson and Saido, 2014). Particularly, Aβ has been reported to impair the activity of AMP-activated protein kinase (AMPK) (Park et al., 2012), a kinase which plays a key role in the activation of autophagy by inhibiting activity of mammalian target of rapamycin (mTOR) (Perez-Revuelta et al., 2014). Moreover, AMPK also stimulates sirtuin 1 (Sirt1) signaling to attenuate oxidative stress (Salminen and Kaarniranta, 2012). For example, FoxO3a has been characterized as the downstream substrate of Sirt1 which forms a complex that induces ROS detoxifying enzymes such as superoxide dismutase (SOD) in response to oxidative stress (Hori et al., 2013). This Sirt1–FoxO3a axis is an evolutionarily conserved pathway that protects against oxidative stress (Salminen et al., 2013). Since αSyn neurotoxicity is reported to be associated with reduced AMPK activation (Dulovic et al., 2014), these findings raise a possibility that Aβ may contribute to αSyn-induced toxicity by suppression of AMPK. Particularly, increased levels of reactive oxygen species (ROS) has been suggested to induce progressive oxidative damage and aging, which ultimately leads to mitochondrial dysfunction and trigger several apoptotic pathways in the brain (Navarro et al., 2009).However, the molecular basis that links Aβ and αSyn-induced cytotoxicity remains unclear. Since αSyn aggregation plays a key role in DLB pathogenesis, it is crucial to understand how Aβ induces aggregation and cytotoxicity in αSyn.

2. Materials and methods

2.1. Materials

Chemicals such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 4′,6-diamidino-2-phenylindole (DAPI), acridine orange (AO), 5-aminoimidazole-4-carboxamideribonucleoside (AICAR), JC-1 and EX-527 were purchased from Sigma (München, Germany). Amyloid-β (Aβ) 1–42 was purchased from AnaSpec Inc. (San Jose, CA, USA). We purchased α-Synuclein (*SNCA*, GeneID: 6622) coding sequence from transOMIC (Huntsville, AL, USA). We purchased antibodies against antibodies against αSyn (#sc-7011-R), AMPK (#sc-25792), p-AMPK (#sc-33524), mTOR (#sc-8319), p-mTOR (#sc-101738), LAMP1 (#sc-17768), caspase 3 (#sc-7148), and poly(ADP-ribose) polymerase (PARP) (#sc-7150) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and antibodies against SOD1 (#GTX13498), FoxO3a (#EP1949Y) and Sirt1 (#E104) were purchased from GeneTex (Irvine, CA, USA). Antibodies against Atg7 (#8558) and Atg12-Atg5 (#4180) were purchased from Cell Signaling Technology (Danvers, MA, USA). The β-actin (#NB600-501) and LC3 (#NB100-2220) antibody was obtained from Novus Biologicals. (Littleton, CO, USA). Primary antibodies were used at a dilution of 1:1000 in 0.1% Tween 20 and secondary antibodies were used at 1:5000 dilutions. All the chemicals were prepared by dissolving phosphate buffer saline solutions stored at −20 °C until needed for use in experiments.

2.2. Vector construction and transfection

In αSyn overexpression studies, the human wild-type αSyn coding sequence was amplified and cloned into the SPORT6-pCMV or recombinant adeno-associated viral (rAAV) vectors. For in vitro transfection, cells were transiently transfected with the SPORT6-pCMV-SNCA or control SPORT6-pCMV vector by using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) with Opti-MEM (Gibco, Carlsbad, CA, USA). Cells are typically harvested 24 h post-transfection for studies designed for exposure to Aβ. For in vivo transfection, rats were stereotaxically injected by adeno-associated viral rAAV-pCMV-SNCA or control rAAV-pCMV vector (viral titer: 1.5E12 genome copies per mL) (Aldrin-Kirk et al., 2014).

2.3. Cell culture and viability assay

Human neuroblastoma SK-N-MC cells were obtained from the American Type Culture Collection (Bethesda, MD, USA). Cells were maintained in Minimal Eagle's medium (MEM; Gibco), supplemented with 10% fetal calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine at 37 °C, 5% CO_2. The Aβ solutions were prepared as described previously (Ono and Yamada, 2006). Briefly, $Aβ_{1-42}$ lyophilizates were dissolved at 10 mM in 10% 60 mMNaOH and 90% 10 mM phosphate buffer (pH 7.4) as a stock reagent, and stored at −78 °C until use. For the aggregation protocols, the $Aβ_{1-42}$ peptide was dissolved in dimethyl sulfoxide to a concentration of 5 mM. Based on the amyloid-derived diffusible ligand protocols, MEM culture medium was added to bring the peptide to a final concentration of 100 μM and incubated at 4 °C for 24 h for oligomeric conditions, whereas 10 mM HCl was added to bring the peptide to a final concentration of 100 μM and incubated for 24 h at 37 °C for fibrillar conditions (Dahlgren et al., 2002). For viability assay, cells were seeded in 96-well plates at a density of 1×10^4 cells/well overnight and then treated as indicated. After 24 h, the tetrazolium salt MTT was added to the medium following the manufacturer's instructions. Only viable cells could metabolize MTT into a purple formazan product, of which the color density (OD) was further quantified by an EZ Read 400 microplate reader (Biochrom, Holliston, MA, USA) at 550 nm. Cell viability was determined by the percentage of OD of the treated cells divided by that of the untreated controls. For trypan blue dye exclusion assay, the number of viable cells was counted by 0.4% trypan blue stain using a hemocytometer. The result was expressed as a percentage relative to control groups.

2.4. Western blot analysis

After treatment, cells were harvested and homogenized in a protein extraction lysis buffer (50 mM Tris–HCl, pH 8.0; 5 mM EDTA; 150 mM NaCl; 0.5% Nonidet P-40; 0.5 mM phenylmethylsulfonyl fluoride; and 0.5 mM dithiothreitol), and centrifuged at 12,000 *g* for 30 min at 4 °C. The supernatants were used as cell extracts for immunoblotting analysis. To determine the SDS soluble and insoluble fractions of αSyn, samples were homogenized in RIPA buffer (Tris buffer containing 1% Triton X-100; 0.1% SDS; 2 mM $MgCl_2$ and 0.5% deoxycholate). The solubilized pellet was centrifuged at 15,000 *g* at 4 °C for 30 min, and the supernatant was termed the SDS-soluble fraction and the final pellet the SDS-insoluble fraction (Ihara et al., 2007). Equal protein amounts of total cell lysates were resolved by 10% SDS-PAGE, transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA), and then probed with a primary antibody followed by a secondary antibody conjugated with horseradish peroxidase. The immunocomplexes were visualized with enhanced chemiluminescence kits (Millipore). The relative expression of proteins was quantified densitometrically by using the software Quantity One (BioRad, Hercules, CA, USA), and was calculated according to the reference bands of β-actin. Each blot represents at least three independent experiments.