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Amyloid β peptide promotes lysosomal degradation of clusterin via sortilin in hippocampal primary neurons

WangYunling [a], QinXike [a], Hemant K. Paudel [a,b,*]

[a] Lady Davis Institute for Medical Research, Jewish General Hospital, Montreal, Quebec H4H 1R3, Canada
[b] Department of Neurology and Neurosurgery, McGill University, Montreal, Quebec H4H 1R3, Canada

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ABSTRACT

Progressive accumulation of amyloid-β peptide (Aβ) in the brain is implicated as the central event in the development of Alzheimer's disease (AD). It is thought that extracellular Aβ triggers toxic signals leading to neurodegeneration. The events downstream of Aβ however are not entirely clear. Clusterin (Apo J) is one of the major risk factors for sporadic form of AD. Clusterin binds to Aβ and prevents Aβ aggregation. In addition, clusterin promotes Aβ degradation and accelerates Aβ clearance from the brain. Clusterin thus protects neurons from Aβ and loss of clusterin level in the brain is implicated as promoting AD pathology. In this study, we found that the level of clusterin protein but not mRNA is reduced in the brains of 3xTg-AD mice. When rat hippocampal primary neurons were treated with Aβ1-42, level of clusterin protein but not mRNA was downregulated. Aβ1-42-induced downregulation of clusterin was blocked by lysosome inhibitors bafilomycin A1 and ammonium chloride. In neurons, Aβ1-42 induced expression of sortilin, a lysosomal sorting protein that targets proteins to lysosome for degradation. In BE(2) M17 human neuroblastoma cells, clusterin bound to sortilin and when sortilin expression was silenced, Aβ1-42-induced clusterin downregulation was almost completely blocked. Our data demonstrate that in neurons, Aβ1-42 promotes lysosomal degradation of clusterin by inducing expression of sortilin and provide a novel mechanism by which Aβ promotes AD pathogenesis.

1. Introduction

Alzheimer's disease (AD) is the major cause of dementia in the elderly population. Progressive accumulation of Aβ peptide derived from the proteolytic cleavage of a large transmembrane amyloid precursor protein (APP), is one of the characteristic neuropathological features of AD (Hardy et al., 2014). Genetic and biochemical studies indicate that Aβ plays a central role in the pathogenesis of AD. It is suggested that extracellular Aβ deposition triggers pathological events leading to neurodegeneration and perhaps dementia (Hardy et al., 2014). Many pathological events downstream of Aβ however are still not clear.

Two independent genome–wide association studies have identified clusterin (also known as Apo-J) as one of the major risk genes for late onset type of AD (LOAD) (Harold et al., 2009; Lambert et al., 2009). Subsequent meta analyses have substantiated these finding (Seshadri et al., 2010; Corneveaux et al., 2010). Clusterin is a member of the heat shock family (Humphreys et al., 1999). The *clusterin* gene is highly conserved and is widely expressed in many organs and tissues where it participates in physiological processes including cell cycle, inflammation, lipid transport, membrane recycling, apoptosis and cell adhesion (Nuutinen et al., 2009). Clusterin inhibits activation of the complement system and NF-κB pathway and suppresses inflammation (Santilli et al., 2003). Clusterin binds to Bax and inhibits apoptosis (Zhang et al., 2005) and potentiates neuronal survival through TGFβ-mediated signalling (Lee et al., 2008). More importantly, clusterin binds to Aβ and prevents Aβ aggregation (Oda et al., 1995; Matsubara et al., 1995). In addition, clusterin enhances lysosomal Aβ degradation (Bell et al., 2007; DeMattos et al., 2004) and promotes Aβ clearance through the blood brain barrier (Bell et al., 2007). Clusterin is considered to be a multi-potent guardian of the brain (Giannakopoulos et al., 1998; Nuutinen et al., 2009). Earlier studies showed that the protein level of clusterin is elevated in the AD brain (May et al., 1990; Lidstrom et al., 1998; Calero et al., 2000). However, a recent report, by using a modern isobaric tag for relative and absolute quantitation (iTRAQ)-based two dimensional liquid chromatography coupled with tandem mass spectrometry brain site specific proteomic strategy determined that the clusterin level was more that 50% less in AD hippocampus when compared to normal controls (Manavalan et al., 2013). Moreover, the C allele at the rs11136000 locus in the *clusterin* gene is the third strongest known

* Corresponding author at: Lady Davis Institute for Medical Research, Jewish General Hospital, 3755 Cote Ste Catherine, Montreal, Quebec H3T 1E2, Canada.
E-mail address: hemant.paudel@mcgill.ca (H.K. Paudel).
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genetic risk factor for LOAD after the APOE4 allele and TREM2(Rajagopalan et al., 2013). rs11136000 is associated with lower clusterin expression resulting in decreased soluble secreted clusterin protein throughout the life (Allen et al., 2012; Ling et al., 2012). Likewise, ApoE4/4 allele significantly decreases the amount of clusterin in the frontal lobe of AD patients (Harr et al., 1996). Furthermore, very recently three patient specific clusterin mutations were reported to promote clusterin degradation before subsequent targeting to Golgi and cause reduction in secreted clusterin in neurons (Bettens et al., 2015). Reduced level of clusterin in the brain was suggested to contribute neurodegenerative process because of the reduced chaperone function and decreased anti-apoptotic activity (Manavalan et al., 2013; Roussotte et al., 2014). Understanding the mechanism by which clusterin level is decreased in AD brain can provide better understanding of AD pathology and help to develop novel therapies.

Sortilin, a member of Vps10p-domain receptors is widely expressed in various tissues including brain and spinal cord. It is a receptor for pronerve growth factor (NGF) and pro-brain derived neurotropic factor and is required for neuronal apoptosis induced by any of the two pro-apoptotic factors (Nykjaer et al., 2004). In addition, sortilin is a lysosomal sorting protein. It binds to a variety of proteins and targets them to lysosome from Golgi for degradation (Nielsen et al., 2001). Sortilin has a strong genetic association with lipoproteins (Strong and Rader, 2012). In hepatocytes, sortilin interacts with apolipoprotein B100 and controls hepatic release of lipoprotein into the circulation (Musunuru et al., 2010; Kjolby et al., 2010). Likewise, hepatic sortilin regulates apolipoprotein B secretion and LDL catabolism (Strong et al., 2012). In neurons, sortilin binds to apolipoprotein E for catabolism of Aβ peptide (Carlo et al., 2013). The interaction of sortilin with clusterin however has never been analyzed.

3xTg-AD mice harbour mutated APP, presenilin 1 and tau and display many salient features of AD pathology (Oddo et al., 2003). We found that the level of clusterin in the brains of these mice is significantly reduced when compared to the age-matched wild type (WT) controls. 3xTg-AD mice display elevated levels of both Aβ and neurofibrillary tangles (NFTs) (Oddo et al., 2003). However, increasing evidence suggest that clusterin interacts with Aβ in AD brain (Giannakopoulos et al., 1998; Nuutinen et al., 2009). Therefore, to evaluate if Aβ plays any role in the observed reduced clusterin level in 3xTg-AD mice brain, we have exposed rat hippocampal primary neurons with Aβ. Herein we show that Aβ promotes lysosomal degradation of clusterin by inducing expression of sortilin. Our results have revealed a novel mechanism by which Aβ downregulates clusterin in neurons.

2. Materials and methods

2.1. Animals

All experiments involving animals were performed following the Guidelines of the Canadian Council of Animal Care and the Lady Davis Institute for Medical Research. 3xTg-AD mice were kindly provided by Dr. Hyman Schipper of Lady Davis Institute. These mice harbour a knock-in mutation of presenilin 1 ($PS1_{M146V}$), the Swedish double mutation of APP ($APP_{KM670/671NL}$), and a frontotemporal dementia mutation in tau (tau_{P301L}) on a 129/C57BL/6 background (Oddo et al., 2003). Control hybrid 129/C57BL6 WT mice were generated by in house breeding. Five-month old female mice (10 in each group) were euthanized and the cerebral cortex of each animal was immediately removed and either homogenized in extraction RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8.0) supplemented with protease inhibitor cocktail from Sigma-Aldrich containing AEBSF (2 mM), aprotinin (0.3 μM), bestatin (130 μM), leupeptin (1 μM), EDTA (1 mM) and E-64 (14 μM) for Western or used for RNA extraction (see below).

2.2. Cell culture, exposure to Aβ1-42, drug treatment and transfection

Hippocampal neurons were prepared from rat pups P0 (Charles River) as described previously (Qin et al., 2015). Neurons maintained in neurobasal medium supplemented with B27 (Invitrogen), streptomycin, penicillin, glutamate, and glutamine were treated with cytosine arabinoside (5 mg/ml) to inhibit glia proliferation after 3 days of plating. After 2 weeks, neurons were treated with Aβ1-42 or Aβ1-42 scrambled (5 μM each). BE(2)-M17 human neuroblastoma cells were cultured in Ham's F-12 high glucose Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum, L-glutamine, non-essential amino acids, sodium pyruvate and penicillin–streptomycin (Song et al., 2009; Li and Paudel, 2016). After overnight seeding of cells in 6-well plates (10^6/well) cells were treated with Aβ1-42 or Aβ1-42 scrambled (5 μM each) for 4 h. Treated cells were either homogenized in extraction buffer for Western blotting or used for RNA extraction. To inhibit lysosome, rat primary hippocampal neurons or M17 cells were treated with bafilomycin A1 (10 nM) or NH_4Cl (10 mM) for 24 h. Likewise, to inhibit proteasome cells were treated with MG132 (15 μM) for 24 h (Qureshi et al., 2013).

2.3. siRNAs, microRNA mimics and transfections

The siRNAs and microRNA (miRNA) mimics were purchased from Dharmacon Inc., including: SiGENOME™ Smartpool human SORT1(M-010620-01), Si GENOME™ control pool non-targeting #1 (D-001206-13), miRIDIAN microRNA mimic negative control #1 (CN-001000-01), miRIDIAN microRNA mmu-miR-325-3 mimic (C-310990-01) and miRIDIAN microRNA mmu-miR-370 mimic (C-310619-07). HEK-293 cells were maintained in Dulbecco's modified Eagle's (high-glucose) medium supplemented with 10% fetal bovine serum (Invitrogen). Cells were plated in 100 mm culture dishes. At ~70% confluency, 0.4 μg of miRNA mimic was transfected into each well with Lipofectamine RNAiMAX (Invitrogen) following manufacturer's instruction manual. For siRNA transfection, M17 cells were plated on 12-well plates and transfected using Lipofectamine RNAiMAX. The final concentrations of each siRNA used was 10 nM. Cells were harvested 48 h after transfection.

2.4. Western blotting and immunoprecipitation

For Western blotting, protein concentrations of brain and cells extracts were measured by Bio-Rad protein assay using BSA as the standard. Each extract was mixed with 10 x SDS-PAGE sample buffer. Each sample (50 μg protein) was Western blotted. Each protein band of the blot was normalized against respective tubulin band. To analyze condition medium, 30 μg of protein (determined by Bio-Rad protein assay) of each sample was loaded on a SDS gel and proteins on the gel were transferred to a membrane. The membrane was first stained with Ponceau S and then Western blotted for secreted clusterin (s-clusterin). To quantify s-clusterin of a sample, s-clusterin band on the Western blot of a sample was normalized against Ponceau S stained protein band (indicated by arrow) of that sample. Immunoprecipitation was carried out as described previously with some modifications (Li and Paudel, 2016). M17 cell lysate (300 μl) was precleared with 50 μl of protein G agarose beads (Sigma-Aldrich) pre-equilibrated with lysis buffer. To 100 μl of precleared lysate, either rabbit anti-clusterin antibody (10 μl) or non-specific rabbit serum (10 μl) was added. Samples were incubated at 4 °C with end-over-end shaking. After 12 h of shaking, 20 μl of protein G agarose beads pre-equilibrated in lysis buffer were added to each sample and shaking continued. After 2 h, samples were centrifuged and the supernatant of each sample was removed. The pellet containing beds was washed three times with lysis buffer and bead-bound proteins were eluted with 40 μl of SDS-PAGE sample buffer. Each sample (20 μl) was Western blotted using monoclonal antibody against clusterin or sortilin.