Amyloid β-Induced Redistribution of Transcriptional Factor EB and Lysosomal Dysfunction in Primary Microglial Cells

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Impaired clearance of Amyloid β (Aβ) by microglia in the brain may be associated with the senile plaque formation, a pathological hallmark relevant to Alzheimer’s disease. Microglial cells in the brain are not able to efficiently degrade Aβ, suggesting that microglial lysosomal impairment may occur. However, the mechanism of Aβ-induced impairment of microglia remains poorly understood. We observed the effects of Aβ on the trafficking of nuclear transcriptional factor EB (TFEB), a master regulator of lysosome biogenesis, and the expression of a downstream osteoporosis-associated transmembrane protein 1 (OSTM1), a vital molecule involved in lysosome acidification in primary microglial cells. Aβ1-42 but not Aβ42-1 resulted in a significant release of tumor necrosis factor-α in primary microglia, but the total cellular TFEB was not changed. Further, Aβ induced a dose-dependent reduction of the TFEB in the nucleus of primary microglial cells, coincident with the increase in the plasma, as revealed by Western blot and confocal microscopy. In addition, a dramatic decrease of OSTM1 expression was observed in the Aβ-challenged microglial cells, along with the intracellular pH steady state, indicating the inadequate lysosomal acidification. These data suggest that Aβ might result in a lysosomal dysfunction via inhibiting nuclear TFEB translocation in microglial cells.

Keywords: microglia, amyloid, degradation, TFEB, lysosome

INTRODUCTION

Alzheimer’s disease (AD) is a neurodegenerative illness accounting for approximately 50–70% of late-onset dementia (Rademakers and Rovelet-Lecrux, 2009). A universal hallmark of Alzheimer’s disease is the presence of extracellular senile plaque consisting of aggregated fibrillar β-amyloid (fAβ) (Ittner and Götz, 2011). Studies have demonstrated that cerebral defects in the clearance of the misfolded Aβ contributed to the Aβ accumulation (Majumdar et al., 2007; Hickman et al., 2008; Mawuenyega et al., 2010). Lysosomes are the key digestive organelle of the cell and can degrade a wide variety of structurally diverse substances including misfolded Aβ. Moreover, microglial cell, as the native immune cell in the CNS, is crucially involved in phagocytosis and degradation of misfolded Aβ (Lee and Landreth, 2010; Fu et al., 2012). However, most studies found that microglial cells were incapable of digesting engulfed fAβ, since the lysosomal acidification is inadequate for the digesting process, thus strongly suggesting the involvement of lysosomal dysfunction in the
impaired fAβ degradation (Majumdar et al., 2007; Umeda et al., 2011). Evidences from both in vitro and in vivo showed that microglia could not effectively clear the fAβ in the brain, due to the lysosomal dysfunction (Hickman et al., 2008; Lee and Landreth, 2010; Perry et al., 2010). Furthermore, defective autophagy was a common phenomenon in AD mice, suggesting that the lysosomal dysfunction was involved in autophagy impairment (Wolfe et al., 2013). However, the mechanisms underlying the Aβ-induced lysosomal dysfunction remain unclear.

Transcription factor EB (TFEB), a transcription factor that coordinates the expression of lysosomal hydrolases, membrane proteins biogenesis involving in lysosomal function and autophagy (Sardiello et al., 2009), is a key regulator of lysosomal function (Sardiello and Ballabio, 2009). TFEB translocation from cytoplasm to nucleus has been shown to increase the number of lysosomes and promote protein clearance under various conditions of aliments involved in protein misfolding, including Huntington’s disease, Parkinson’s disease and Pompe’s disease (Dehay et al., 2010; Tsunemi et al., 2012). Moreover, osteopetrosis-associated transmembrane protein 1 (OSTM1), in cooperation with chloride channel 7 (CLCN7), is essential for regulating lysosomal pH and Aβ clearance (Lange et al., 2006; Majumdar et al., 2011; Spampanato et al., 2013).

Study in osteoclast showed that the expression of OSTM1 and CLCN7 was closely regulated by the nuclear translocation of TFEB (Lacombe et al., 2013). In this study, we observed the effects of Aβ stimulation on the activation of microglia and lysosomal function to elucidate the role of TFEB nuclear translocation and OSTM1 expression in the Aβ-induced lysosomal dysfunction, and to explore the possible mechanisms of compromised Aβ clearance in the microglial cells.

**METHODS**

**Materials**

Aβ1–42, Aβ1–42, Hoescht-32258, dimethyl sulfoxide (DMSO), and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO). DMEM-F12 and heat-inactivated endotoxin-free fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT). Cell viability was evaluated with a Cell Counting Kit-8 (CCK8) assay (Dojindo Molecular Technologies, Inc.). Nuclear Protein Extraction Kit (78833) was from Pierce (Thermo Fisher, USA). Antibody against LAMP1 (ab25630) and Iba1 (ab5076) were from Abcam (Cambridge, MA, USA). Rabbit polyclonal antibody against TFEB (catalog no. sc-48784) and goat polyclonal antibody against OSTM1 (catalog no. sc-168858) were from Santa Cruz. Antibody against PCNA (catalog no. 2586), rabbit polyclonal antibody against GAPDH (catalog no. 5174) and all the secondary antibodies used in the experiment were purchased from Cell Signaling Technology (Beverly, MA). Rat TNF-α ELISA kit was purchased from BD Bioscience (catalog no. 560479).

**Microglial Culture and Viability**

Purified primary microglial cells were obtained from cerebral cortices of neonatal Sprague-Dawley rats (1-day old), as previously described (Saura et al., 2003). All experimental protocols have been approved by the Institutional Animal Care and Use Committee of Xi’an Jiaotong University. After mechanical and chemical dissociation, cerebral cortical cells were suspended in DMEM-F12 medium supplemented with 10% FBS and seeded at a density of 5 × 10⁶ cells. The cells were cultured in 5% CO2 at the 37°C incubator, and the medium was changed every 4–5 days. After 2 weeks, when the mixed culture cells were grown to confluence, mild digestive method was used to harvest purified primary microglia, showing a 98% in purity. Cell viability was determined by the CCK-8 assay. Briefly, primary microglia cells were seeded in 96-well culture plates at a density of 5 × 10⁴ cells per well and cultured 24 h with 100 µl medium for stabilization. Cells were treated with different concentration of Aβ1–42 or Aβ1–42 as indicated concentration for 24 h. CCK8 (10 µl) solution was added to each well and incubated for further 2 h. The highly water-soluble tetrazolium salt (WST-8) from CCK8 kit was turn into a yellow-color formazan dye by microglial cells. Finally, the optical absorbance was measured at 450 nm with a microplate reader and high optical density indicated high cell viability. All the presented data were normalized to a control.

**Preparation of Fibril-Enriched Aβ**

Aβ1–42 peptides were dissolved in sterilized and distilled water to make a stock solution at a concentration of 0.1 mM and incubated at 37°C for 7 d to allow peptide aggregation and fibril formation (Lorenzo and Yankner, 1994; Reed-Geaghan et al., 2009). Amyloid fibril-enriched preparation was obtained by Aβ peptide incubated in water for at least 7 days. Fibrillar form of the peptide was confirmed by its ability to change thioflavine T fluorescence spectra, as described elsewhere (Hudson et al., 2009). Reverse peptides Aβ1–42 were prepared with the same method serving as the negative control.

**TNF-α and mRNA Measurement**

Levels of TNF-α secreted by microglia in the supernatants, a sensitive marker for microglia activation, were determined after the challenge of Aβ1–42 or Aβ1–42 for 24 h, by using an ELISA assay kit. Procedures were performed following the manufacturer’s instruction manual. TNF-α mRNA was detected by using real-time PCR.

**Western Blot Analysis**

Microglial cells were treated with Aβ (5 and 10 µM) or 10 µM Aβ1–42 as control and then lysed in RIPA lysis buffer, followed by the extraction of total cellular, nuclear, or cytoplasmic proteins by using protein extracting kits. After the protein concentrations were measured by the BCA protein assay reagents, equal amounts of proteins (15 or 20 µg) were subjected to SDS-PAGE by using 12% for TFEB and LAMP1 and 15% for OSTM1 polyacrylamide gels. Electrophoresed proteins were then...
transferred to polyvinylidene difluoride membranes and blocked with 5% (v/v) non-fat dry milk in PBS or rabbit serum for 1.5 h at ambient temperature before the incubation overnight at 4°C with various primary antibodies. After extensive washing, the appropriate HRP-conjugated secondary antibody (1: 2,000, CST) diluted in PBS containing 0.1% Tween 20 was added for 1 h. Blots were developed with the luminal reagent (Thermo Scientific). Integrated optical density (IOD) measurement and analysis were conducted with the software Gel-Pro Analyzer. The primary antibodies used were as follows: anti-TFEB (1:500), anti-OSTM1 (1:1,000), anti-LAMP1 (1 µg/ml), anti-Iba1 (1 µg/ml), anti-GAPDH (1:5,000) and anti-PCNA (1:1,000). GAPDH was used for internal control of total protein and PCNA for control of nuclear protein.

Confocal Microscopy Analysis
Purified microglia were rinsed with PBS and suspended with DMEM-F12. Cell suspension was subjected to centrifugation at 1,000 rpm for 8 min. The supernatant fluids were discarded and the cells were resuspended with pipettes in the remaining drops. Two small drops of the suspension were dripped on a cover slip in the plate to make it evenly distributed over the surface before adding DMEM-F12 containing 2% FBS into the plate 1 h later. After microglia was returned to the resting state, treatment was given as described above. The cover slips were rinsed three times and fixed for 20 min, permeabilized with 0.3% Triton X-100, blocked with donkey serum, and then incubated with polyclonal antibodies against TFEB and OSTM1 or LAMP1 for one night at 4°C. The cover slips were washed three times for over 15 min, then incubated for 1 h at room temperature in the darkroom with a FITC-conjugated donkey anti-rabbit IgG against TFEB antibody (1:100, CoWin Biotech, catalog no. cw-0219) and a Cy3-conjugated donkey anti-mouse IgG against LAMP1 antibody (1:100, CoWin Biotech, catalog no. cw-0224). Control plates were incubated with secondary antibodies only. The cover slips were rinsed three times for over 20 min and then incubated for 10 min with Hoescht-33258. Images under a laser scanning confocal microscopy were collected. All the antibodies used above were diluted with donkey serum.

Microglia Intracellular Lysosomal pH Detection
Intracellular pH of microglia was detected using fluorescent dye-based pHrodo Red AM intracellular pH indicator (Invitrogen, Carlsbad, CA). The excitation and emission wavelength of 560 nm and 580 nm was used in a Gemini EM microtiter plate reader (Molecular Devices). Microglial cells were harvested and maintained in the 96-well plate, with DMEM-F12 containing 2% FBS at least for 2 h for the return of a resting state. After treatment with Aβ of the indicated concentration and indicated time, microglia cells were rinsed with live cell imaging solution and dyed with pHrodo, according to the manufacturer’s protocol, followed by the intracellular pH measurement with a Gemini EM microtiter plate reader. Forskolin (100 µM), a protein kinase A (PKA) activator, capable of enhancing lysosomal acidification in microglial cells, was served as positive control. Microglia intracellular pH values were determined by the arbitrary pH ratios of light excited at 560 nm and emitted at 580 nm with pH calibration curve kit (Invitrogen).

RESULTS
The Effects of Aβ on Microglial Activation and TFEB
Purified microglial cells were collected after treatment with Aβ and cell extracts were run on SDS-PAGE for immunoblotting to detect total TFEB level. The cell viability of primary microglia was not altered by Aβ1−42 ranging from 2.5 to 10 and 10 µM Aβ1−42−1, as indicated by CCK-8 assay (Figure 1A). After being incubated with Aβ (5 or 10 µM) for 24 h, microglial cells were activated and transformed into amoeboid morphology (Figure S1). Meantime, both 5 and 10 µM Aβ1−42 induced significant increase of TNF-α in the supernatant, indicating a state of microglia activation (Figure 1B). The expression of interleukin 1 (IL-1), interleukin 6 (IL-6) and TNF-α mRNA was also increased after incubated with Aβ1−42 (Figure S2). Importantly, no difference of total cellular TFEB in microglia was found among control and those treated with Aβ1−42 or Aβ42−1 in 12 and 24 h (Figures 2A,C).

The Effects of Aβ on the Distribution of TFEB in Microglia
Since TFEB translocation into nucleus is a key process in the enhancement of lysosomal function (Sardiello et al., 2009), we thus explored the influence of Aβ on nuclear translocation of TFEB. After the addition of Aβ1−42 or Aβ42−1 for 12 or 24 h in purified primary microglia, the nucleus protein and cytoplasmic protein were extracted by using the nucleus protein extracting kit. Treatment with Aβ1−42 (5 or 10 µM) but not Aβ42−1 resulted in a transient increase of TFEB within nuclear part of microglia at 12 h (Figure 2B), followed by a sustained efflux, leading to a significant increase of plasma counterpart (Figure 2D). Western blot analysis showed that TFEB were predominantly located in the nucleus of resting microglia, while the level of TFEB in the cytoplasmic compartment was relatively low (Figures 3A, B). In addition, the challenge of Aβ significantly reduced the level of nuclear TFEB in a dose dependent manner with 20% inhibition for 5 µM and 80% inhibition for 10 µM (Figure 3A). In parallel, incubation with Aβ for 24 h strongly elevated the levels of cytoplasmic TFEB in microglia by approximately 270% at 5 µM and 360% at 10 µM (Figure 3B), respectively.
Confocal Microscopy Analysis for the Effects of Aβ on TFEB Distribution and OSTM1 Expression

To further confirm the finding that Aβ-induced changes of TFEB and OSTM1, purified microglial cells were incubated with Aβ₁₋₄₂ (5 or 10 µM) for 24 h. Antibodies against TFEB and OSTM1 were used to label the corresponding proteins. Confocal microscopy analysis revealed that Aβ addition resulted in a remarkable retention of TFEB from nuclear compartment, accompanied by a significant decrease of OSTM1 in microglia (Figures 3C–E).

Effects of Aβ on LAMP1 Expression in Microglia

After incubated with Aβ₁₋₄₂ (5 or 10 µM) and Aβ₄₂₋₁ for 12 h, total protein was extracted to analyze the expression of LAMP1. The results showed that both dose of Aβ dramatically increased the LAMP1 levels (Figure 4A), which was consistent to the Western blot results of OSTM1 and nucleus TFEB. Unexpectedly, treatment of microglia with Aβ for 24 h continued to raise the level of LAMP1 (Figure 4B). In addition, the results from cellular immunofluorescence also illustrated an increased expression of LAMP1 in microglial cytoplasm at 24 h (Figures 4C,D).

Inadequate Lysosomal Acidification Induced by Aβ

The pH value of resting microglia was around 5.96, while protein kinase A activator forskolin treatment induced a marked drop (0.71–0.86) of pH value (Figure 5). Challenge of Aβ (5 or 10 µM) induced a slight and transient drop (0.25 for 5 µM and 0.28 for 10 µM) of intracellular pH at the incubation-time of 4 h followed by sustaining pH recovery to the baseline, suggesting Aβ induced inadequate lysosomal acidification in primary microglial cells (Figure 5).

DISCUSSION

Abnormal protein aggregation is a universal pathogenesis of neurodegenerative diseases such as AD, Huntington’s disease (HD) and Parkinson’s disease (PD) (Hardy and Selkoe, 2002; Brundin et al., 2010; Zuccato et al., 2010; Jucker and Walker, 2011). Microglia is the main population in the central nervous system to phagocytize and degrade misfolded protein (Perry et al., 2010), and lysosome is the major organelle in microglia for the clearance of misfolded proteins (Nixon et al., 2008; Majumdar et al., 2011). Studies have shown that the function of lysosomal enzymes in the microglia of AD patients was potentially impaired, leading to the dysfunction in clearing misfolded Aβ and autophagy impairment (Krabbe et al., 2013), however, the underlying mechanism remained unknown (Majumdar et al., 2007). The enzyme activity in the lysosome depends on the acidification environment, impairment of which could lead to protein clearance deficiency and lysosomal storage diseases (Avrahami et al., 2013; Colacurcio and Nixon, 2016). Considering the key role in regulating lysosomal biogenesis and function, TFEB was regarded as a potential hub of misfolded protein processing (Sardiello et al., 2009; Settembre et al., 2011). Furthermore, a recent study indicated that TFEB could reduce the protein level of amyloid precursor protein (APP) and decrease the generation of Aβ (Xiao et al., 2015), suggesting a crucial role of TFEB in the generation and degradation of Aβ. In this study, we demonstrated that Aβ exerted a marked change of the cellular distribution of TFEB by enhancing its nuclear export. Moreover, OSTM1, an integral element of lysosome function, was potently inhibited by the administration of Aβ, indicating a likely cause of acidification impairment and lysosomal dysfunction. Indeed, overexpression of LAMP1 in AD brain and cerebrospinal fluid, accompanying by the dysfunction of autophagy in mice and human have been documented (Armstrong et al., 2014). Evidence from transgenic mice suggested that increased lysosomal biogenesis in activated microglia might exacerbate neuronal injury (Tanaka et al., 2013).
FIGURE 2 | Cellular distribution of TFEB and OSTM1 in Aβtreated microglia. After being incubated with Aβ of the indicated concentration for 24 h, primary microglial cells were lysed with RIPA buffer. Nuclear proteins were extracted to run SDS-PAGE for Western blot analysis. Treatment with Aβ (5 µM or 10 µM) and control reverse peptides Aβ_{42}−_{1} for 12 h did not alter the expression of total TFEB in microglia compared to that in resting microglia, but induce a significant increase of total OSTM1 (A). Nucleus TFEB in microglia was transiently increased after treated with 5 µM Aβ for 12 h (A), and then decreased to the baseline in 24 h (B). Further analysis for TFEB and OSTM1 in the cytosol and nucleus revealed that the distribution of TFEB in the nucleus was reduced in microglia after treatment with Aβ for 24 h in a dose-dependent manner (C,D), with a dramatical decrease of OSTM1 in the cytosol. TFEB or OSTM1 protein levels were normalized to that of GAPDH for cytoplasmic or PCNA for nuclear protein. Values were obtained from three independent experiments. *P < 0.05, (compared with resting microglia).
FIGURE 3 | TFEB redistribution induced by Aβ. Equal nuclear proteins (15 µg) were separated on 12% SDS-PAGE, and then analyzed by immunoblotting with polyclonal antibodies against TFEB. The result (A) showed a significant reduction of TFEB expression in nuclear compartment after being incubated with Aβ (5 and 10 µM) by approximately 20 and 80%, respectively, as compared with control (vehicle). Conversely, the cytoplasmic proteins analysis (B) showed that Aβ increased the level of TFEB located in cytoplasm. Controls of nuclear and cytoplasm extraction were performed by an analysis of nuclear marker PCNA and GAPDH respectively. Furthermore, a triple-staining confocal microscopy was applied to scan microglial nucleus (blue), TFEB (green) and OSTM1 (red) after microglia was treated with Aβ for 24 h. An even distribution of TFEB in both cytoplasm and nucleus was observed in resting microglial cells (C). Significant dose-dependent nuclear export of TFEB and inhibition of OSTM1 were observed in response to 5 µM (D) and 10 µM (E) Aβ. The high magnification image showed that TFEB was mainly sequestered in cytoplasm (arrowhead) and the expression of OSTM1 decreased in primary microglia. Values were obtained from three independent experiments. *P < 0.05 (compared with resting microglia). #P < 0.05 (compared with the indicated group). Scale bars = 25 µm.

These results supported that Aβ might lead to a decompensatory lysosomal dysfunction and resulted in the deficit of degradation by microglia in the brain. The phenomenon of Aβ-triggered TFEB efflux out of the microglial nucleus has a vital pathological implication in the impaired degradation of misfolded protein in the microglial cells.
It was reported that excessive neuronal levels of α-synuclein were closely linked to the progressive decline of lysosomal function and cytoplasmic retention of TFEB through mammalian target of rapamycin (mTOR) signaling pathway (Decressac et al., 2013). Both de-phosphorylated and phosphorylated forms of TFEB existed in nuclear compartment (Pe-a Llopis et al., 2011), and the migration of TFEB greatly influenced the function of TFEB, resulting in the facilitation of clearance of abnormal protein deposition in PD and HD (Tsunemi et al., 2012; Decressac and Björklund, 2013). Therefore, it is likely that misfolded protein in neurodegenerative diseases, like α-synuclein in PD and huntingtin in HD, might also possess the similar property in regulating TFEB expression and cellular distribution. Theoretically, the insult of exotic misfolded protein would cause a rigorous proteolysis reaction, which required the activation of lysosome biogenesis and assembly where the entrance of TFEB into nucleus was essentially needed. Unexpectedly, when microglial cells were incubated with Aβ, the distribution of TFEB appeared to be a dynamic change with the duration of incubation. There was a transient increase of TFEB in the nuclear of primary microglia, suggesting a compensatory reaction of microglia to Aβ, and then followed by a sustained efflux of TFEB from the nucleus, which was consistent to the finding in Parkinson’s disease rat model (Decressac and Björklund, 2013). It is not clear...
A chloride transporter CLCN7, an important molecule in regulating lysosomal pH, is necessarily required for the lysosomal function and $\beta$-amyloid degradation within microglia. Though we found $\beta$-amyloid could also significantly induced the expression of LAMP1 similar to OSTM1 at 12 h, unlike the decrease of OSTM1, the LAMP1 level continued to increase at 24 h. The aberrant increase of LAMP1 might suggest the accumulation of defective lysosome in microglia. This inconsistent result indicated that other potential transcriptional factors may be involved in LAMP1 expression. A study from Martina et al. demonstrated that TFE3 could regulate the expression of LAMP1 (Martina et al., 2014), which might be a potential compensatory pathway to increase LAMP1 expression. Further studies were critically needed to elucidate the reason of $\beta$-amyloid-induced excessive LAMP1 expression.

CONCLUSIONS

The challenge of $\beta$-amyloid export of TFEB, coupled with a marked inhibition of OSTM1 expression, excessive LAMP1 production and inadequate lysosomal acidification in primary microglia. Together with the finding that extracellular accumulated $\beta$-amyloid plaques with surrounding activated microglia develops the pathologic hallmark of AD, our study indicated that $\beta$-amyloid itself might lead to a disturbance of $\beta$-amyloid clearance in the AD brain through inhibition of lysosomal acidification, but not lysosome formation, thus forming a vicious cycle in $\beta$-amyloid clearance. To the best of our knowledge, since no specific agonist for TFEB nuclear translocation was developed yet, drug discovery based on this pathway would have a potential implication on the AD therapy.

AUTHOR CONTRIBUTIONS

XG, PT, LCHE, PL, and CH performed experiments; XZ, YL, and XL designed experiments; LCHO analyzed data; XG and RL...
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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnagi.2017.00228/full#supplementary-material
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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