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Full-length Article

Toll-interacting protein deficiency promotes neurodegeneration via impeding autophagy completion in high-fat diet-fed ApoE$^{-/-}$ mouse model

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ABSTRACT

The excessive accumulation of specific cellular proteins or autophagic vacuoles (AVs) within neurons is a pathologic hallmark of neurodegenerative diseases. Constitutive autophagy in neurons prevents abnormal intracellular protein aggregation and is critical for maintaining cell survival. Since our previous study showed that Toll-interacting protein (Tollip)-deficient macrophages had constitutive disruption of endosome-lysosome fusion, we hypothesize that Tollip deficiency may also promote neuron death via blockage of autophagy completion. Indeed, we observed significantly higher levels of neuron death in the brain regions of cerebral cortex, hippocampus, and cerebellum from ApoE$^{-/-}$/Tollip$^{-/-}$ mice as compared to ApoE$^{-/-}$ mice fed with high fat diet (HFD). We further documented diminished density of neurons and increased ratios of TUNEL positive cells in the hippocampus of ApoE$^{-/-}$/Tollip$^{-/-}$ mice. The ultrastructural electron microscopy analyses revealed neuron cell shrinkage as well as loss of intracellular structure in brain tissues from ApoE$^{-/-}$/Tollip$^{-/-}$ mice. There was dramatic accumulation of autophagosomes in the cytoplasm, elevated accumulation of β-amyloid and α-synuclein, and increased levels of p62 and Parkin in the brain tissues from ApoE$^{-/-}$/Tollip$^{-/-}$ mice as compared to ApoE$^{-/-}$ mice. Our data suggest that Tollip may play a crucial role in sustaining neuron health by facilitating the completion of autophagy, and that Tollip-deficiency may accelerate neuron death related to neurological disease such as Alzheimer's disease.

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1. Introduction

Neurological disorders such as Alzheimer's disease have significant impacts on human health (Winblad et al., 2016). Among probable causes, the defective clearance of cellular debris and/or aggregated proteins such as β-amyloid and α-synuclein may exacerbate neurodegeneration (Marsh and Blurton-Jones, 2012; Nilsson et al., 2013; Stefanis, 2012). In neurons, there are two intracellular proteolytic pathways involved in clearing abnormal or obsolete cellular proteins, the ubiquitin-proteasome system (UPS) and the autophagic-lysosomal system (Boland et al., 2008). With particular interest, autophagy has been recognized to play a critical role in removing aggregated proteins as well as damaged cellular organelles that are too large for ubiquitin-proteasome system mediated clearance (Rideout et al., 2004). Thus, disruption of autophagy may closely correlate with the pathogenesis of neurodegenerative diseases (Alirezaei et al., 2010).

Autophagy is a complex cellular process that involves the initiation, maturation, and completion (Boland et al., 2008; Klionsky et al., 2008), and the proper degradation of the autophagic substrates is mediated by the fusion of autophagosome with degradative compartments of the endosomal–lysosomal system (Berg et al., 1998). Basal autophagy in neurons assists the clearance of ubiquitinated proteins and damaged organelles such as mitochondria and is critical for maintaining healthy neuron survival (Hara et al., 2006; Komatsu et al., 2006). In contrast, dysfunctional autophagy may lead to neuronal cell death in various disease states

Abbreviations: ApoE, apolipoprotein E; Tollip, Toll-interacting protein; HFD, high fat diet; TEM, transmission electron microscopy; AVs, autophagic vacuoles.

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such as Alzheimer's disease (Nixon, 2006). Indeed, the accumulation of autophagosomes and other AVs within neurons has been recognized as a pathologic hallmark of many neurodegenerative diseases including Alzheimer's disease (Anglade et al., 1997; Boellaard et al., 1989; Chu, 2006; Kegel et al., 2000; Ko et al., 2005; Nixon et al., 2005; Rubinsztein et al., 2005; Zhu et al., 2007). Despite the emerging clinical significance of defective autophagy completion in neurological disease, molecular mechanisms responsible for proper completion of autophagy are not well understood.

Tollip is a ubiquitously expressed and conserved protein from vertebrate (human, mouse, rat, zebrafish, Xenopus) to worm (TLI-1 in *Caenorhabditis elegans*), and serves as an adaptor molecule within the Toll-like receptor (TLR) signaling pathway (Bulut et al., 2001). Recent data including studies from our group reveal that Tollip is critically involved in the completion of autophagosome fusion with lysosome in macrophages/monocytes (Baker et al., 2015). Intriguingly, Tollip has also been implicated in the clearance of toxic misfolded protein aggregates in neuronal cells and preventing neuron toxicity (Oguro et al., 2011; Shimizu et al., 2014). Misfolded Protein aggregates accumulate in various neurodegenerative diseases such as polyQ disease (Huntington disease), Alzheimer's disease and Parkinson's disease (Shimizu et al., 2014). Tollip was shown to facilitate the transport of polyQ protein aggregates to late endosome and protect neuronal cells from death (Oguro et al., 2011). Further supporting the role of Tollip in the pathogenesis of human Alzheimer's disease, a recent study reported that Tollip expression was significantly reduced in human brain samples collected from aged and Alzheimer's individuals as compared to young individuals (Cribbs et al., 2012). Based on these findings, we aim to examine the causal connection between Tollip deficiency and neurodegeneration *in vivo* by employing the Tollip deficient mouse model.

ApoE deficient mice offer a unique model to study the pathogenesis of Alzheimer's disease (Avdesh et al., 2011). Under the regimen of high fat diet (HFD) feeding, ApoE deficient mice develop Alzheimer's disease-like symptoms, potentially due to elevated oxidative modifications of proteins, lipids and DNAs resembling AD patients (Galloway et al., 2008; To et al., 2011). A recent study suggest that ApoE deficiency may facilitate the build-up of b-amyloid plaque (Yeh et al., 2016). However, there has been no previous mechanistic study with regard to the regulation of brain cell autophagy due to ApoE deficiency. Intriguingly, HFD has also been shown to potentially reduce the levels of Tollip in muscle tissues (Kim et al., 2010). To further test the role of Tollip in neurodegeneration in a pathologically relevance setting, we have developed the ApoE and Tollip double deficient mice. Utilizing these mice, we aim to examine the role of Tollip during HFD mediated neurodegeneration. We found significantly elevated neuron death in the cerebral cortex, hippocampus, cerebellum and pons in ApoE$^{-/-}$/Tollip$^{-/-}$ mice as compared to that in ApoE$^{-/-}$ mice fed with HFD. With particular relevance to Alzheimer's disease, the hippocampus of ApoE$^{-/-}$/Tollip$^{-/-}$ mice had significantly less neuron cells. At the molecular level, we observed significant accumulation of autophagosomes in the cytoplasm, elevated levels of p62, Parkin, as well as β-amyloid and α-synuclein aggregation in the neurons of ApoE$^{-/-}$/Tollip$^{-/-}$ mice. Our data suggest that Tollip may play a crucial role in modulating neurodegeneration by facilitating the completion of autophagy.

2. Materials and methods

2.1. Mice

ApoE$^{-/-}$ mice and ApoE$^{-/-}$/Tollip$^{-/-}$ mice were bred and maintained in the animal facility at Virginia Tech with the approved Animal Care and Use Committee protocol. All littermate mice were 8 weeks of age and 25–30 g weight when experiments were initiated.

2.2. Experimental design

ApoE$^{-/-}$ and ApoE$^{-/-}$/Tollip$^{-/-}$ mice (male, 8 weeks old) were fed with Western Diet (TD.88137, Harlan) for 8 weeks. Western Diet includes cholesterol (0.2% total cholesterol), total fat (21% by weight; 42% kcal from fat), saturated fatty acids (>60% of total fatty acids), and sucrose (34% by weight).

2.3. Histology

Histological analyses of brain regions including cerebral cortex, Hippocampus, Cerebellum and Pons were performed on freshly frozen, OCT (Optimal-Cutting-Temperature compound)-embedded and sectioned slides (5 μm). Slides were fixed in 4% neutral buffered formalin for 5 min. Haematoxylin and eosin (H&E) staining were performed.

2.4. Immunofluorescence

Immunofluorescence analyses were performed on freshly frozen, OCT-embedded and sectioned slides (10 μm). 6 mice from ApoE$^{-/-}$ and ApoE$^{-/-}$/Tollip$^{-/-}$ mice were used for the study. TUNEL stainings were performed with *in situ* BrdU-Red DNA Fragmentation (TUNEL) assay Kit according as the product protocol (Abcam). The average numbers of positive staining cells per viewing field were quantified from 2 to 4 slides collected from each of the six mice.

For the measurement of β-amyloid, α-synuclein, 2–4 Slides from each mouse brain were fixed in 4% neutrally buffered formalin for 5 min, and stained with anti-mouse primary antibodies (1:100, anti-mouse β-amyloid, α-synuclein antibodies, as well as isotype control antibodies) followed by a biotinylated anti-Ig secondary Ab (BD Biosciences) and streptavidin-PE or FITC. DAPI was used to stain nucleus. 2–4 viewing fields from each slide were captured under fluorescent microscope. Pixel values reflecting the fluorescent intensities of each viewing field were quantitated with the NIH ImageJ software.

2.5. Transmission electron microscopy

Hippocampus area isolated (2 mm × 2 mm × 1 mm) were fixed in 2.5% gluteraldehyde in 100 mM sodium cacodylate, pH7.4, and post-fixed in 1% osmium tetroxide in sodium cacodylate followed by 1% uranyl acetate. After ethanol dehydration and embedding in LX112 resin (LADD Research Industries), ultrathin sections were stained with uranyl acetate followed by lead citrate. All grids were viewed on a JEOL 100CX II transmission electron microscope at 80 kV. Samples were prepared from the hippocampus regions of 6 ApoE$^{-/-}$ mice and 6 ApoE$^{-/-}$/Tollip$^{-/-}$ mice fed with HFD for 8 weeks. Two separate hippocampus regions were sampled from each mouse. 5 sections were observed in TEM for each sample. Average numbers of apoptotic cells from three 3 ApoE$^{-/-}$ mice and 3 ApoE$^{-/-}$/Tollip$^{-/-}$ mice were quantified and presented in Fig. 3. Average autophagosome numbers/cell from observations obtained from 6 mice each were quantified and presented in Fig. 3.

2.6. Immunoblotting

Hippocampus blocks (~100 mg) were harvested, immediately soaked in liquid nitrogen, smashed into power, dissolved in SDS lysis buffer (50 mM Tris-HCl, pH7.0, 2% SDS, 6% glycerol, 100 mM DTT) containing a protease inhibitor mixture, and subjected to SDS-

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