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Mitoferrin-1 is Involved in the Progression of Alzheimer's Disease Through Targeting Mitochondrial Iron Metabolism in a *Caenorhabditis elegans* Model of Alzheimer's Disease

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Abstract—In mammals, mitoferrin-1 and mitoferrin-2, two homologous proteins of the mitochondrial solute carrier family are required for iron delivery into mitochondria. However, there is only one kind, called *W02B12* (*mitoferrin-1* or *mfn-1*), in *Caenorhabditis elegans* and its regulatory mechanism is unknown. In this study, we used *C. elegans* strains CL2006 and GMC101 as models to investigate what role mitoferrin-1 played in Alzheimer's disease (AD). We found that knockdown of mitoferrin-1 by feeding-RNAi treatment extended lifespans of both strains of *C. elegans*. In addition, it reduced the paralysis rate in the GMC101 strain. These results suggest that mitoferrin-1 may be involved in the progression of Alzheimer's disease. Knockdown of mitoferrin-1 was seen to disturb mitochondrial morphology in the CB5600 strain. We tested whether knockdown of mitoferrin-1 could influence mitochondrial metabolism. Analysis of mitochondrial iron metabolism and mitochondrial ROS showed that knockdown of mitoferrin-1 could reduce mitochondrial iron content and reduce the level of mitochondrial ROS in the CL2006 and GMC101 strains. These results confirm that knockdown of mitoferrin-1 can slow the progress of disease in Alzheimer model of *C. elegans* and suggest that mitoferrin-1 plays a major role in mediating mitochondrial iron metabolism in this process.

Key words: mitoferrin-1, mitochondrial iron metabolism, oxidative stress, β-amyloid, Alzheimer's disease, *Caenorhabditis elegans*.

INTRODUCTION

Alzheimer's disease (AD), a neurodegenerative disease associated with aging, was discovered by Bavarian neuropathologist Alzheimer in 1907 (Tanzi and Bertram, 2005). Its clinical manifestations were mainly cognitive impairment and memory loss (Mattsson et al., 2009; Aggleton et al., 2016). Currently, AD has become one of the diseases that threaten human health, and there is no effective cure. Mounting evidence suggests that AD is a neurodegenerative disease caused by a variety of factors which included iron overload (Weinreb et al., 2016), accumulation of β-amyloid (Aβ), neurofibrillary tangles comprising hyperphosphorylated tau protein (Savelieff et al., 2013; Jouanne et al., 2017), oxidative stress, autophagy dysfunction, and mitochondrial dysfunction (Colacurcio et al., 2018).

Although we still do not know enough about the pathogenesis and treatment of AD, AD was found to be associated with mitochondrial dysfunction which occurred not only in AD patient brains but also in AD patient platelets. These results led to the mitochondrial cascade hypothesis (Cadonic et al., 2016) that states that the presence of mitochondrial dysfunction in sporadic AD (sAD) increases with age, reaching a threshold with the pathological hallmarks of AD—hyperphosphorylation of Tau and Aβ oligomer-enriched senile plaques. Interestingly, mitochondrial dysfunction has also been widely characterized in subjects with mild cognitive impairment (MCI), a clinical syndrome that frequently appears prior to symptomatic AD. Moreover, when mitochondrial dysfunction occurred, the utilization of mitochondrial iron was limited and accompanied by increased generation of reactive oxygen species (ROS) through Fenton chemistry, resulting in the accumulation of ROS in the cell and causing oxidative damage to the body (Lin and Beal, 2006). The result is a vicious cycle of mitochondrial

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Abbreviations: mfn-1, mitoferrin-1; AD, Alzheimer's disease; ROS, reactive oxygen species; Aβ, β-amyloid; TMRE, tetramethylrhodamine ethyl ester; SLC25A28, solute carrier family 25 member 28; SLC25A37, solute carrier family 25 member 37; PCR, polymerase chain reaction; RT, reverse transcription; H_2O_2, hydrogen peroxide; NGM, nematode growth medium; SD, standard deviation.

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dysfunction, mitochondrial iron overload, oxidative stress, and the pathogenesis of AD (Onyango and Khan, 2006; Wang et al., 2014).

Mitoferrin acts like a transferrin in the mitochondrial membrane, mediating the transport of iron from cytoplasm to mitochondria (Hung et al., 2013). In mammals, mitoferrin-1 is highly expressed in developing erythrocytes, and mitoferrin-2, whose functions are less well understood, is expressed ubiquitously (Paradkar et al., 2009). *W02B12,* also called *mfn-1,* is an ortholog of human mitoferrin-1 and mitoferrin-2 in *Caenorhabditis elegans* (*C. elegans*). Due to the lack of blood system, there is only one mitoferrin in the *C. elegans*. The reduction in *mfn-1* expression was found to extend the lifespan of the worms (Ren et al., 2012). Studies showed that differential turnover of mitoferrin could affect the biosynthesis of iron-sulfur proteins and heme as well as the accumulation of mitochondrial iron and indicated that mitoferrin was involved in mitochondrial iron regulation (Paradkar et al., 2009). Further research confirmed that mitoferrin modulated iron toxicity in a *Drosophila* model of Friedreich's ataxia (Navarro et al., 2015), in which reducing the expression of mitoferrin could alleviate the progression of disease.

In this report, we used the transgenic strains CL2006 and GMC101, which express the human 42 amino acid sequence of Aβ under the control of the muscle-specific *unc-54* promoter and are considered to be a model of AD (Quan et al., 2015), to examine the effects of mitoferrin-1 *in vivo* in the *C. elegans* model. *C. elegans* is ideal for animal experimentation because of its high reproductive capacity, its rapid life cycle, and the fact that genetic resources (http://www.wormbase.org/) are readily available. Our experimental data show that down-regulation of mitoferrin-1 extends the lifespans of CL2006 and GMC101 and reduces the incidence of paralysis. Down-regulation of mitoferrin-1 also alters mitochondrial morphology, reduces mitochondrial iron content, up-regulates the transcription of mitochondrial iron-sulfur protein and ferritin-related genes and reduces mitochondrial ROS. These results suggest that mitoferrin-1 may play a role in AD by targeting mitochondrial iron metabolism.

EXPERIMENTAL PROCEDURES

Strains and maintenance

C. elegans strains were obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN, USA) and included CL2006 [*pCL12(unc-54/human Aβ peptide 1–42 minigene) + pRF4*], GMC101 [*unc-54p::Aβ-1–42::unc-54 3′-UTR + mtl-2p::GFP*], CB5600 [*(pSAK2) myo-3p::GFP::LacZ::NLS + (pSAK4) myo-3p:: mitochondrial GFP + dpy-20(+)],* BC14393 *[rCesW02 B12.9::GFP + pCeh361*] (Zuryn et al., 2008).

The GMC101 strains express full-length human Aβ1–42 peptide in body-wall muscle cells that aggregated *in vivo*, whereas shifting L4 or young adult animals from 20 °C to 25 °C could induce the expression of Aβ and cause paralysis. The CL2006 strains constitutively expressed Aβ when cultured at 20 °C. The BC14393 strains were labeled with the GFP fusion gene (*W02B12.9::GFP*).

We used the synchronization method to obtain the F1 generation of nematodes for the generation of young adult animals. Worms were cultured and maintained on nematode growth medium (NGM) plates at 20 °C unless otherwise stated and were fed with OP50 *Escherichia coli* bacteria but the RNAi experiments. When the nematodes developed to the L4 stage, the GMC101 strains were shifted from 20 °C to 25 °C for the induction of Aβ.

RNAi vector construction

The fragment used for RNAi constructs was obtained by polymerase chain reaction (PCR) of *C. elegans* genomic DNA. The fragment was located in the second exon of *W02B12.9*, according to blast searches. After we obtained the PCR product, we used the seamless cloning method to insert the fragment into the l4440 vector. Primers used for PCR were:

Forward primer: 5′-GTTAGATCTCTGAAACGAAAT GTCCA-3′ (Bgl Ⅱ enzyme site was underlined);

Reverse primer: 5′-TAAGGTACCAAGTCCTCCC GCAA-3′ (KpnⅠ enzyme site was underlined).

The l4440 vector was obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN, USA), which could be used to construct nematode RNAi vectors.

Feeding RNAi and synchronization method

L4-staged nematodes were transferred onto the bacterial lawn and grown at 20 °C for RNAi treatment. First, nematodes were synchronized to obtain a sufficient number, called the F0 generation of nematodes. When they developed into the adult stage, F0 generation nematodes were synchronized to obtain the F1 generation nematodes, and F1 generation nematodes were used for subsequent experiments. F1 generation nematodes were fed with l4440 bacteria (control group) or *W02B12.9 (mfn-1)* RNAi-containing bacteria (treatment group). Nematodes were fed with OP50 *Escherichia coli* bacteria unless otherwise stated (Kamath et al., 2000).

Swallowing frequency

GMC101/CL2006 nematodes were synchronized using the feeding-RNAi method described above to obtain F1 generation nematodes. When they developed to young adult stage, the F1 generation nematodes were transferred to food-rich NGM plates. After 1 min of adaptation, they were scored for swallowing frequency in a 60-s time interval. Swallowing behavior was defined as shaking along the long axis of the body. Assays were performed in triplicate.

Lifespan assay

GMC101/CL2006 nematodes were synchronized using the feeding-RNAi method described above to obtain more than sixty F1 generation nematodes for lifespan