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Featured Article

Zfra restores memory deficits in Alzheimer's disease triple-transgenic mice by blocking aggregation of TRAPPC6AΔ, SH3GLB2, tau, and amyloid β, and inflammatory NF-κB activation

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Introduction: Zinc finger-like protein that regulates apoptosis (Zfra) is a naturally occurring 31-amino-acid protein. Synthetic peptides Zfra1–31 and Zfra4–10 are known to effectively block the growth of many types of cancer cells.

Methods: Ten-month-old triple-transgenic (3×Tg) mice for Alzheimer's disease (AD) received synthetic Zfra peptides via tail vein injections, followed by examining restoration of memory deficits.

Results: Zfra significantly downregulated TRAPPC6AΔ, SH3GLB2, tau, and amyloid β (β) aggregates in the brains of 3×Tg mice and effectively restored their memory capabilities. Zfra inhibited melanoma-induced neuronal death in the hippocampus and plaque formation in the cortex. Mechanistically, Zfra blocked the aggregation of amyloid β 42 and many serine-containing peptides in vitro, suppressed tumor necrosis factor–mediated NF-κB activation, and bound cytosolic proteins for accelerating their degradation in ubiquitin/proteasome-independent manner.

Discussion: Zfra peptides exhibit a strong efficacy in blocking tau aggregation and amyloid β formation and restore memory deficits in 3×Tg mice, suggesting its potential for treatment of AD.

Keywords: Zfra; TRAPPC6A; SH3GLB2; WWOX; Peptide polymerization; Protein degradation; Neurodegeneration

1. Introduction

Zinc finger-like protein that regulates apoptosis (Zfra) is a 31-amino-acid protein, possessing two cysteines and one histidine in the amino acid sequence [1–4]. Zfra is one of the binding proteins for tumor suppressor WW domain-containing oxidoreductase (WWOX, FOR, or WOX1) [5–9]. Substantial evidence shows that WWOX is involved in neural diseases. WWOX controls the functional activities of glycogen synthase kinase 3 beta (GSK-3β) and other enzymes in hyperphosphorylating tau and thereby prevents neurodegeneration [9–11]. Downregulation of WWOX protein is associated with neurodegeneration such as Alzheimer's disease (AD) [9–12]. Alteration of *WWOX/Wwox* gene, for example, null mutations and missense mutations, results in severe neural diseases and metabolic disorders, including ataxia, epilepsy, dementia, neurodegeneration, growth retardation,

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abnormal HDL (high density lipoprotein) lipid metabolism, and early death [9,13–16]. Under WWOX deficiency or dysfunction, a cascade of protein aggregation, including TRAPPC6AΔ (trafficking protein particle complex 6A delta, TPC6AΔ), TIAF1 (TGFβ1-induced anti-apoptotic factor 1), tau, and amyloid β (Aβ), occurs in the mitochondria and leads to apoptosis [17–20]. Both TPC6AΔ and TIAF1 tend to aggregate in the brain extracellular matrix when WWOX is deficient or dysfunctional. However, transiently overexpressed WWOX induces mitochondrial apoptosis in vitro [21,22]. Transgenic mutant WWOX proteins cause mitochondrial dysfunction by affecting the respiratory complex in *Drosophila* [23].

Zfra counteracts the function of WWOX by blocking Tyr33 phosphorylation [2–4]. Transiently overexpressed WWOX induces mitochondrial apoptosis through cytochrome c release [21,22], whereas Zfra binds and blocks WWOX-mediated apoptosis without causing cytochrome c release [3,4]. Recently, we demonstrated that when nude mice and BALB/c mice receive a synthetic full-length Zfra1–31 or a truncated Zfra4–10 peptide via tail veins, these mice resist the growth, metastasis, and stemness of melanoma xenografts and 11 other malignant cancer cells [24]. The injected Zfra peptide mainly goes to the spleen but not other organs and activates a novel non-T/non-B lymphocyte, designated Hyal-2^+ $CD3^-$ $CD19^-$ Z cell, to block cancer growth and metastasis [24,25]. Z cell possesses a memory function in blocking cancer growth.

Here, we determined that Zfra restores memory deficits in triple-transgenic mice for AD. Zfra works by accelerating protein degradation and blocking TRAPPC6AΔ, tau, and Aβ aggregation. Under WWOX deficiency or dysfunction or sustaining stimulation of cells with transforming growth factor beta (TGF-β), a cascade of protein aggregation occurs [17–20]. TRAPPC6AΔ starts to undergo Ser35 phosphorylation, then polymerizes, and initiates TIAF1 aggregation with Ser37 phosphorylation. The TRAPPC6AΔ/TIAF1 complex induces caspase 3 activation, dephosphorylation of membrane amyloid precursor protein (APP) at Thr688 and degradation for Aβ formation, and pT181-tau aggregation [17–20]. Notably, Zfra suppressed WWOX phosphorylation at Ser14. pSer14-WWOX appears to be associated with disease progression and severity.

2. Methods

2.1. Peptides

Zfra and serine-containing peptides were synthesized by Genemed Synthesis (San Antonio, TX, USA): (1) Zfra1–31, MSSRRSSSCKYCEQDFRAHTQKNAATPFLAN; (2) Zfra4–10, RRSSSCK; (3) WWOX7-21, AGLDDTDSEDELPPG; (4) pS14-WWOX7-21, AGLDDTD**pS**EDELPPG; (5) WWOX286-299, DYWAMLAYNRSKLC; (6) pY287-WWOX286-299, D**pY**WAMLAYNRSKLC; (7) TPC6A24-38, DPGPGGQKMSLSVLE; (8) pS35-TPC6A24-38, DPGPGGQKMSL**pS**VLE; (9) TPC6A84-100, KDLWVAVFQKQMDSLR; (10) ANKRD40266-281, RIQNPSLRENDFIEIE; (11) pS271-ANKRD40266-281, RIQNP**pS**LRENDFIEIE; (12) tetramethylrhodamine-labeled Zfra (TMR-Zfra), the full-length Zfra1–31 was labeled with a red-fluorescent Texas Red maleimide fluorescent probe tetramethylrhodamine. The peptide stocks were made as 10 mM in degassed sterile Milli-Q water. Each tube was flushed with nitrogen and stored in −80°C freezer. For tail vein injections, peptides were freshly prepared in degassed Milli-Q water at 1–4 mM in 100-μL Milli-Q. GenBank accession for ANKRD40 is EU164539. Aβ peptides were from AnaSpec: (1) Aβ42: DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA; (2) Aβ40: DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV. Where indicated, Zfra peptide was mixed and incubated with an aforementioned peptide for 12–24 hours at room temperature, followed by determining peptide aggregation using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining.

2.2. Antibodies

Homemade antibodies used in the experiments were against Zfra, pS8-Zfra, and WWOX (WOX1) [10,17,18,20,21]. Commercial antibodies used were as follows: (1) monoclonal Aβ antibody (AbD/Serotec), (2) monoclonal paired helical filament (PHF)–tau antibody (Pierce/Invitrogen), (3) EGFP (Santa Cruz Laboratory), (4) His tag antibody (Sigma), (5) pT181-tau antibody (Biosource) [17,21]. An approved protocol for rabbit use in antibody production was from the IACUC of the National Cheng Kung University Medical College. Antibodies were produced using the following synthetic peptides: (1) WWOX7-21, CAGLDDTDSEDELPPG; (2) pS14-WWOX7-21, CAGLDDTD**pS**EDELPPG; (3) TPC6A, CKDLWVAVFQKQMDSLR, amino acid #84-100 for pan-specific antibody production. These peptides were conjugated with keyhole limpet hemocyanin (KLH) via the *N*-terminal cysteine for antibody production in rabbits (using an Antibody Production and Purification kit from Pierce) [21,26,27]. The *N*-terminal cysteine in each peptide sequence was added for covalently conjugating with KLH. The specific pS14-WWOX antibody was purified as described [27]. The specificity of the antisera was tested using the synthetic peptides to block immunoblots. Where indicated, Western blotting was carried out as described [1,8,10,14]. In addition, we generated antibody against self-polymerizing SH3GLB2 (SH3 domain-containing GRB2-like endophilin B2), using the synthetic peptide NH-CDACKARLKKAKAAEAK-COOH (amino acid 170–185).

2.3. Animals

All experiments were carried out in accordance with the National Institutes of Health Guidelines for animal research