Suppressive Effects of SuHeXiang Wan on Amyloid-β42-Induced Extracellular Signal-Regulated Kinase Hyperactivation and Glial Cell Proliferation in a Transgenic Drosophila Model of Alzheimer’s Disease

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SuHeXiang Wan (SHXW), a Chinese traditional medicine, has been used to treat infantile convulsions, seizures and strokes. Previously, we reported that modified SHXW, called KSOP1009, suppressed the hyper-activation of c-Jun N-terminal kinase (JNK) and Alzheimer’s disease (AD)-like phenotypes in amyloid-β42 (Aβ42)-expressing Drosophila AD models. In the present study, we, further, investigated the detailed mechanism by which KSOP1009 suppresses the AD-like phenotypes of the model flies. As seen in the brains of AD patients, pan-neuronal expression of Aβ42 in Drosophila increased activation of extracellular signal-regulated kinase (ERK), which was monitored by its phosphorylation level, and the number of glial cells in the brain. Suppression of caspase activity did not affect these phenomena, suggesting that Aβ42 induces ERK activation and glial cell proliferation independently of apoptotic processes. KSOP1009 intake significantly reduced the level of ERK activation and the number of glial cells. Moreover, KSOP1009 intake also effectively decreased the defects in the wing vein formation induced by Epidermal growth factor receptor (Egfr) overexpression in fly wings, suggesting that it may contain an inhibitory substance that inhibits the EGFR/ERK signaling pathway. In addition, the Aβ42-induced locomotive defect was partially rescued by inhibition of the elevated ERK activity through its antagonistic drug treatment. Taken together, these results suggest that KSOP1009 exerts its therapeutic effect by inhibiting the EGFR/ERK pathway and glial cell proliferation and by suppressing the JNK pathway and apoptosis.

Key words amyloid-β42; Alzheimer’s disease; Drosophila; extracellular signal-regulated kinase; glial cell; SuHeXiang Wan

Alzheimer’s disease (AD), the most well known type of dementia, is characterized by extracellular senile plaques and intracellular neurofibrillary tangles. The extracellular plaques contain amyloid-β42 (Aβ42) protein, which is produced from amyloid precursor protein (APP) through the action of beta and gamma secretases. Aβ42 accumulation is observed in most AD patients, and it induces cell death and memory loss in AD animal models. Accumulated Aβ42 exerts its neurotoxicity via complex and widely-distributed pathological mechanisms. Hyperactivation of mitogen-activated protein kinases (MAPKs), which include c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38MAPK, has been observed in the brains of AD patients and animal models chronically expressing Aβ42. In particular, the JNK signaling pathway is activated in the brains of humans with AD, and many studies have shown that Aβ42 activates JNK, which leads to cell death via caspase-dependent apoptosis. Similar to JNK, ERK has been shown to be activated in degenerating AD neurons and APP transgenic mouse models. ERK hyperactivation has also been implicated in AD pathology. In general, ERK activation induces cell growth, differentiation, and survival. However, other reports suggest that the ERK pathway is crucial to the pathology of AD and contributes to death signaling. For example, chronic ERK activation in pathological conditions, such as potassium withdrawal, increased cell death. Moreover, ERK activation affects neuronal symptoms, such as neuronal plasticity and central nervous system activity, which have been implicated in memory and pain hypersensitivity.

In addition, the brains of AD patients are characterized by the presence of Aβ42 senile plaques that are surrounded by activated glial cells, such as astrocytes and microglia. Neurinflammatory processes have been strongly implicated in the pathophysiology of AD. Activated microglia, the inflammatory cells of the brain, are found in AD patients, and characterized by morphological changes, proliferation, and increased expression of cell surface receptors. Aβ42-activated astrocytes and microglia secrete proinflammatory cytokines, free radicals, and chemokines (e.g., interleukin-1, reactive oxygen species, and nuclear factor-κB), which play a crucial role in the cause of AD. The resulting inflammation induces further neurodegeneration, neuronal dysfunction, and cell death in the AD brain. Furthermore, inflammation has been also implicated in the pathogenesis of neurodegenerative disease animal models, including mice and Drosophila. These findings have led to the postulation that neuronal inflammation is the cause of neurodegenerative diseases, such as AD, and that anti-inflammatory drugs may act as protective agents for the diseases.

Several drug candidates have been developed to treat AD. For example, acetylcholinesterase inhibitors (e.g., donepezil) and N-methyl-D-aspartate (NMDA)-receptor antagonists (e.g.,...
memantine) have been used to improve the symptoms of AD.\(^{36}\) However, in terms of the pharmacology, MAPK regulation and glial activation have not been targeted in AD therapy. Moreover, multi-faceted approaches will likely be required to effectively treat or prevent AD due to its complexity and diverse pathology.

SuHeXiang Wan (SHXW) is a Chinese-traditional drug that consists of 15 crude herbs, which is widely prescribed to treat infantile convulsions, seizures, and strokes.\(^{37}\) A previous study showed that SHXW exerts an inhibitory effect on convulsion through its agonistic action on the \(\gamma\)-aminobutyric acid (GABA)/benzodiazepine receptor.\(^{38}\) Inhalation of SHXW is effective for the prevention of stress, and it acts by regulating stress hormones.\(^{39}\) In addition, we reported that intake of KSOP1009 improved \(\text{A}^\beta_{42}\)-induced memory impairment and suppressed \(\text{A}^\beta_{42}\) levels and plaque deposition in the brain of \(T^\beta\)-APPswe/PS1dE9 mice to the same extent as donepezil treatment.\(^{40}\)

**Fig. 1.** KSOP1009 Intake Reduced \(\text{A}^\beta_{42}\)-Induced ERK Phosphorylation

(A, B) The level of ERK phosphorylation in \(\text{A}^\beta_{42}\) and \(\text{DIAP1}\)-expressing fly heads. (C) Representative images of acridine orange (AO)-stained \(\text{A}^\beta_{42}\) and \(\text{DIAP1}\) in head extracts from flies expressing the indicated transgenes. (D, E) Intake of 5 \(\mu\)g/mL KSOP1009 reduced \(\text{A}^\beta_{42}\)-induced ERK phosphorylation. (G) Dose-dependent effect of KSOP1009 on \(\text{A}^\beta_{42}\)-expressing flies. (A, E, G) Representative images of Western blot analyses of phosphorylated ERK (pERK) and ERK in head extracts from flies expressing the indicated transgenes. The bottom panel was probed for ERK, which served as a loading control. (B, F) Graphs showing the relative levels of pERK to ERK. All data are expressed as the means±S.E. (*** \(p<0.001\), * \(p<0.05\), Student’s t-test, \(n=5\)). The genotypes of the samples are \(\text{elav-GAL4} [\text{UAS-A}^\beta_{42}]/\text{UAS-DIAP1} {\text{elav-GAL4}}, \text{elav}\rangle\text{A}^\beta_{42} [\text{UAS-A}^\beta_{42}], \text{elav}\rangle\text{DIAP1} [\text{UAS-DIAP1}/\text{elav-GAL4}].\)
Since KSOP1009 is a mixture of herbs, it might contain various beneficial components that act on the diverse pathological pathways of AD. However, the detailed mechanism by which KSOP1009 suppresses the AD-like phenotypes of model animals is not clear. In the present study, we evaluated the effects of KSOP1009 intake on ERK activation and glial cell proliferation in Drosophila AD models. KSOP1009 intake significantly reduced ERK activation and glial cell proliferation in Aβ42-expressing fly neurons, indicating that KSOP1009 exerts its therapeutic effects by inhibiting the epidermal growth factor receptor (EGFR)/ERK pathway and glial cell proliferation. Together with our previous findings in the suppressive functions on the JNK pathway and apoptosis, these results suggest that KSOP1009 provides a good example of the advantages of combination therapy.

MATERIALS AND METHODS

Preparation of KSOP1009 Extract and Treatment with KSOP1009 or ERK Inhibitor The modified SHXW extract KSOP1009 was prepared as previously described. All medicinal herbs used in this study were purchased from Dong Yang Herb Pharm. Co. (Seoul, Korea). Fifty embryos of each genotype were reared at 25°C in standard plastic vials containing cornmeal-soybean standard media with DMSO (control), 50 µg/mL PD98059 (Calbiochem), or 0.1, 0.5, 2, 5, 50 µg/mL KSOP1009.

Fly Strains Elav-GAL4 (pan-neuronal driver), UAS-Drosophila inhibitor of apoptosis protein 1 (DIAP1), and UAS-Epidermal growth factor receptor (Egfr) were obtained from the Bloomington Drosophila Stock Center. UAS-Aβ42 and MS1096-GAL4 (developing wing driver) were provided by Dr. Damian C. Crowther (University of Cambridge, U.K.) and Dr. M. Freeman (MRC Laboratory of Molecular Biology, U.K.), respectively.

Western Blot Analysis Antibodies against ERK (1:2000 in TBST, Cell Signaling) and phospho-ERK (1:2000 in TBST, Cell Signaling) were used to detect ERK activation. Western blot analyses were performed using standard procedures and horseradish peroxidase-conjugated secondary antibodies.
Acridine Orange Staining  The brains of *Drosophila* larvae were dissected in phosphate-buffered saline (PBS). The brains were incubated for 5 min in $1.6 \times 10^{-6}$ M solution of acridine orange (Sigma-Aldrich), and rinsed in PBS. The samples were observed under an Axiophot2 fluorescence microscope (Carl Zeiss).

Primers  To determine the existence of the transgenes, *UAS-Aβ42* and *UAS-DIAP1*, in the indicated lines, polymerase chain reactions were conducted using the following primer pair, which are correspondent to the 5′ upstream and 3′ downstream of multi-cloning site of pUAS vector, respectively: 5′-TACTGA AAT CTC CAA GAA G-3′ and 5′-TCT CGT TAG GTA GTT TGTC CC-3′. The expected sizes of the PCR products are 364 bp for *UAS-Aβ42* and 1792 bp for *UAS-DIAP1* transgene.

Climbing Assay  The climbing assay was performed as described previously, with some modifications. Ten male flies were incubated for 1 h at 25°C in climbing ability test vials for environmental acclimation. After tapping the flies

Fig. 3. Inhibition of ERK Activity Ameliorated the Locomotive and Vein Formation Defects of Aβ42-Expressing Flies

(A) The effect of ERK inhibition on the locomotive defect of Aβ42-expressing flies. The climbing ability of Aβ42-expressing flies fed media containing 50 μM PD98059 (P+) was restored to control levels (elav-GAL4), similar to flies fed media containing 5 μg/ml KSOP1009 (K+) (n=100). (B) Representative wing images showing the effect of ERK inhibition on the defective vein formation (arrows) in flies expressing Egfr or Aβ42. (C) Graph showing the number of defective veins (n=20). (D, E) Representative image (D) and graph (E) of Western blot analyses showing the ERK phosphorylation level in the heads of Aβ42-expressing flies fed media with or without 50 μM PD98059 (n=5). All data are expressed as the means±S.E. (**p<0.01, *p<0.05, Student’s t-test). K- and P-, control media for KSOP1009 media and PD98059 media, respectively. The genotypes of the samples are elav-GAL4 (elav-GAL4/elav-GAL4), elav>Aβ42 (UAS-Aβ42/UAS-Aβ42; elav-GAL4/elav-GAL4), MS1096>Egfr (MS1096-GAL4/MS1096-GAL4; UAS-Egfr/+), and MS1096>Aβ42 (MS1096-GAL4/MS1096-GAL4; UAS-Aβ42/+).
down to the bottom, we counted the number of flies that climbed to the top of the vial within 7 s. Ten trials were performed for each group.

**Immunohistochemistry** Larval brains and imaginal discs were fixed in 4% paraformaldehyde and then washed with PBST (PBS + 0.1% Triton X-100). The samples were blocked with 2% normal goat serum (NGS) and 20% bovine serum albumin (BSA) in PBT (PBS + 0.1% Tween 20) and incubated overnight with mouse anti-\( \text{Aβ42} \) (1:200 in PBT; Santa Cruz) and mouse anti-repo (1:10 in PBT; Developmental Studies Hybridoma Bank) antibodies overnight at 4°C. They were then washed with PBST and incubated for 1 h at 25°C in PBST with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse immunoglobulin G (IgG) secondary antibody (1:200 in PBST; Invitrogen).

**RESULTS**

**Aβ42 Expression Increased the Level of ERK Phosphorylation Regardless of Apoptosis** ERK activation has been implicated in AD progression. Therefore, we investigated whether ectopic expression of \( \text{Aβ42} \) in fly neurons augmented ERK activation. When \( \text{Aβ42} \) was expressed in all neurons, the levels of phosphorylated ERK (pERK), the active form of ERK, were higher than those in the control (Figs. 1A, B, lanes 1, 2). We previously showed that ectopic expression of \( \text{Aβ42} \) induced apoptosis via the JNK pathway in \textit{Drosophila} neurons. Therefore, we tested if the elevation in ERK phosphorylation levels is the result of \( \text{Aβ42} \)-induced apoptosis by assessing pERK levels in flies co-expressing \( \text{Aβ42} \) and \textit{Drosophila inhibitor of apoptosis protein 1} (DIAP1), which strongly suppresses \( \text{Aβ42} \)-induced apoptosis (Fig. 1C). If increased apoptosis is the cause of ERK activation, then \( \text{DIAP1} \) overexpression should reduce pERK levels in \( \text{Aβ42} \)-expressing flies. However, \( \text{Aβ42} \)-induced ERK phosphorylation in AD fly brains was not affected by co-expression of \( \text{DIAP1} \) (Figs. 1A, B, lanes 3, 4), indicating that the elevated ERK activity in neurons is independent of JNK-induced apoptosis. The existence of transgenes, \( \text{Aβ42} \) and \( \text{DIAP1} \), in the indicated lines were confirmed by genomic DNA polymerase chain reaction (PCR) and sequencing of the PCR products (Fig. 1D).

**KSOP1009 Intake Inhibited Aβ42-Induced Activation**
To determine if KSOP1009 affects the Aβ42-induced activation of the EGFR/ERK signaling pathway, we investigated the effect of KSOP1009 intake on ERK phosphorylation. KSOP1009 intake significantly reduced ERK phosphorylation levels in Aβ42-expressing fly heads in a dose-dependent manner (Figs. 1E–G). The effect of KSOP1009 on the Aβ42-induced ERK phosphorylation was prominent at a dose more than 5 µg/mL (Fig. 1G). Because ERK activation is independent of increased apoptosis in Aβ42-expressing flies, this result suggests that KSOP1009 has diverse effects on the regulation of Aβ42-related signaling pathways.

KSOP1009 Intake Ameliorated Wing Vein Formation Defect in Egfr-Overexpressing Fly Since KSOP1009 is a mixture of various compounds that has no effect on Aβ42 accumulation, we reasoned that KSOP1009 exerts its inhibitory effects through component(s) that inhibit the EGFR/ERK signaling pathway itself, rather than acting directly on the function of Aβ42 in ERK activation.

To test this hypothesis, we examined the effect of KSOP1009 on EGFR-induced vein formation defect (Fig. 2A arrows), which is a prominent phenotype of flies with elevated EGFR/ERK signaling. KSOP1009 intake ameliorated the vein formation defect in the fly wing induced by overexpression of Egfr (middle panels of Figs. 2A, B), which suggests that KSOP1009 contains some compound(s) that inhibit the EGFR/ERK signaling pathway. Moreover, Aβ42 expression in developing wings also induced defective vein formation, indicating that the accumulation of Aβ42 increased ERK activity in developing wings (Figs. 2A, B). KSOP1009 intake also suppressed the defective vein formation induced by Aβ42 (right panels of Figs. 2A, B). The accumulation of Aβ42 in larval wing discs was confirmed by immunohistochemistry using anti-Aβ42 antibody (Fig. 2C).

Inhibition of the EGFR/ERK Signaling Pathway Ameliorated the Aβ42-Induced Defective Phenotypes Next, we investigated the consequence of ERK hyperactivation in Aβ42-expressing flies by examining the effect of ERK down-regulation on the defects in Aβ42-expressing flies. As previously reported, the Aβ42-induced locomotive defect was completely rescued by KSOP1009 intake (Fig. 3A). Interestingly, this defect was also restored by treatment with PD98059, a specific ERK inhibitor (Fig. 3A). Moreover, PD98059 intake significantly reduced both Egfr- and Aβ42-induced defective phenotypes.
Increased glial cell number induced by Aβ42 was not associated with ERK proliferation caused by glial cell proliferation, suggesting that the increased glial cell number in the larval brain (Figs. 4C, D).

Since glial cells are the main cleaners of the central nervous system (CNS), and function through engulfment and degradation of dying neurons and degenerating neuronal branches in *Drosophila*, the increased glial cell number in *Aβ42*-expressing larval brains could be a result of neuronal apoptosis. To test this, we counted the number of glial cells in the brain of larvae co-expressing *Aβ42* and *Drosophila inhibitor of apoptosis protein 1* (DIAP1). However, unexpectedly, the increased glial cell number induced by *Aβ42* overexpression was only slightly reduced by co-expression of DIAP1, indicating that elevated glial cell proliferation in the *Aβ42*-expressing fly is not a consequence of increased apoptosis (Figs. 4C, D).

Next, we examined the effect KSOP1009 intake on the hyperproliferation of glial cells in the brains of AD model flies. KSOP1009 intake almost completely restored the number of glial cells in the larval brains of AD model flies to that of control levels (Figs. 5A, B), suggesting that KSOP1009 has beneficial effects in glial cells as well as in neurons. Furthermore, we examined whether EGFR/ERK signaling pathway is also involved in the *Aβ42*-induced glial cells proliferation. As shown in Figs. 5C and D, feeding PD98059 did not affect glial cell proliferation, suggesting that the increased glial cell proliferation caused by *Aβ42* was not associated with ERK hyperactivation.

**DISCUSSION**

We previously showed that KSOP1009 intake ameliorated the neuronal degeneration, reduced survival, and locomotive dysfunction of *Aβ42*-expressing *Drosophila* AD models. The observed neurological phenotypes were associated with JNK hyperactivation and increased apoptosis. Here, we show that expression of *Aβ42* in fly neurons increased ERK activation and glial cell proliferation, which are not a consequence of *Aβ42*-induced apoptosis. These results suggest that *Aβ42* exerts its toxicity in *Drosophila* neurons via various pathways, including activation of the JNK and ERK signaling pathways and inflammation as is observed in the brains of human AD patients. Since KSOP1009 is a prescription containing several herbs, the beneficial effect of KSOP1009 on *Aβ42* cytotoxicity might be achieved by diverse pathways. Therefore, in the present study, we investigated the effect of KSOP1009 on ERK phosphorylation and glial cell proliferation in *Drosophila* AD models. Studies on the detailed mechanisms by which KSOP1009 acts as a suppressant of *Aβ42* cytotoxicity are important for understanding the anti-AD functions of KSOP1009.

**KSOP1009 Intake Reduced Aβ42-Induced Glial Cell Proliferation**

Increased inflammation due to activated microglia has been implicated in AD progression. Therefore, we also assessed glial cell proliferation in *Aβ42*-expressing larval brains by counting the cells that expressed reversed polarity (repo), a glia-specific marker, in areas of Aβ42 accumulation (Figs. 4A, B white box). As expected, *Aβ42* expression increased the number of glial cells in the larval brain (Figs. 4C, D).

KSOP1009 intake ameliorates *Aβ42*-induced toxicity by inhibiting JNK and ERK activity and glial cell proliferation.

Here we show that KSOP1009 intake suppressed ERK phosphorylation and glial cell proliferation. *Aβ42*-induced chronic activation of ERK in neurons reportedly results in apoptosis and alteration of synaptic plasticity. Consistent with the mammalian data, our study showed that inhibition of ERK hyperactivation by intake of a specific-ERK inhibitor improved the locomotive activity of *Aβ42*-expressing flies (Fig. 3A), indicating that ERK hyperactivation has a harmful effect on these animals. Therefore, the inhibitory activity of KSOP1009 on ERK signaling might contribute to the suppression of *Aβ42* cytotoxicity. In addition, increased inflammation has also been shown to have detrimental effects on neuronal cell survival in both the brains of AD patients and the *Drosophila* nervous system. Therefore, inhibition of glial cell hyperproliferation by KSOP1009 might decrease the detrimental effect of increased inflammation in the nervous system of AD patients. Collectively, our results suggest that KSOP1009 exerts its beneficial effect on neuronal health by inhibiting ERK hyperactivation, glial cell proliferation, and JNK hyperactivation (Fig. 6).

Since KSOP1009 is a mixture of herbs, it might contain numerous beneficial components that act on the diverse pathological pathways of AD. Given the complexity and widespread distribution of the pathology, combination drug treatment will likely be more effective than monotherapy for the treatment or prevention of AD. Indeed, recent studies on the efficacy of AD drugs showed that combination drug therapy might be an effective treatment for AD. Oriental medicines, which are usually based on a combination of beneficial components,
have an increased probability of treating diseases that have complex pathological pathways. The data presented here support this idea, and KSOP1009 provides a good example of the advantages of combination therapy.

In conclusion, our results indicate that KSOP1009 exerts its neuroprotective effect against Aβ42 cytotoxicity by inhibiting ERK hyperactivation, glial cell proliferation, and JNK hyperactivation. This suggests that KSOP1009, a mixture of various herbs, may play a role as a good combination drug therapy candidate for AD.

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