Suppressive Effects of SuHeXiang Wan on Amyloid- β 42-Induced Extracellular Signal-Regulated Kinase Hyperactivation and Glial Cell Proliferation in a Transgenic *Drosophila* Model of Alzheimer's Disease

Seung Hwan Park,^{a,#} Soojin Lee,^{a,#} Yoon Ki Hong,^a Soojin Hwang,^a Jang Ho Lee,^a Se Min Bang,^a Young-Kyoon Kim,^b Byung-Soo Koo,^c Im-Soon Lee,^a and Kyoung Sang Cho^{*,a}

^aDepartment of Biological Sciences, Konkuk University; Seoul 143–701, Republic of Korea; ^bDepartment of Forest Products & Biotechnology, Kookmin University; Seoul 136–702, Republic of Korea; and ^cDepartment of Neuropsychiatry, Graduate School of Oriental Medicine, Dongguk University; Seoul 100–715, Republic of Korea. Received September 10, 2012; accepted December 6, 2012; advance publication released online December 14, 2012

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 hyperactivation of c-Jun N-terminal kinase (JNK) and Alzheimer's disease (AD)-like phenotypes in amyloid- β 42 (A β 42)-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 A β 42 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 A β 42 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 A β 42-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- β 42; 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.¹⁾ The extracellular plaques contain amyloid- β 42 (A β 42) protein, which is produced from amyloid precursor protein (APP) through the action of beta and gamma secretases.^{2,3)} A β 42 accumulation is observed in most AD patients, and it induces cell death and memory loss in AD animal models.^{4,5)} Accumulated A β 42 exerts its neurotoxicity via complex and widely-distributed pathological mechanisms.⁶⁾

Hyperactivation of mitogen-activated protein kinases (MAPKs), which include c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38MAPK,⁷⁾ has been observed in the brains of AD patients and animal models chronically expressing A β 42.^{8,9)} In particular, the JNK signaling pathway is activated in the brains of humans with AD,^{10,11)} and many studies have shown that A β 42 activates JNK.^{12–15)} We previously showed that overexpression of A β 42 in *Drosophila* neurons induced JNK activation, which leads to cell death via caspase-dependent apoptosis.^{15,16)} Similar to JNK, ERK has been shown to be activated in degenerating AD neurons^{17,18)} and APP transgenic mouse models.¹⁹⁾ ERK hyperactivation has also been implicated in AD pathology.⁸⁾ In general, ERK activation induces cell growth, differentiation, and survival.^{20,21)} However, other reports suggest that the ERK pathway is crucial to the pathology of AD and contributes to

death signaling.^{8,22,23)} For example, chronic ERK activation in pathological conditions, such as potassium withdrawal, increased cell death.^{24,25)} Moreover, ERK activation affects neurological symptoms, such as neuronal plasticity and central nervous system activity, which have been implicated in memory and pain hypersensitivity.^{26,27)}

In addition, the brains of AD patients are characterized by the presence of A β 42 senile plaques that are surrounded by activated glial cells, such as astrocytes and microglia.¹⁾ Neuroinflammatory processes have been strongly implicated in the pathophysiology of AD.²⁸⁾ 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.^{28,29)} A β 42-activated astrocytes and microglia secrete proinflammatory cytokines, free radicals, and chemokines (e.g., interleukin-1, reactive oxygen species, and nuclear factor- κ B), which play a crucial role in the cause of AD.³⁰⁾ The resulting inflammation induces further neurodegeneration, neuronal dysfunction, and cell death in the AD brain.³¹⁾ Furthermore, inflammation has been also implicated in the pathogenesis of neurodegenerative disease animal models, including mice and *Drosophila*.^{32,33)} These findings have led to the postulation that neuronal inflammation is the cause of neurodegenerative diseases, such as AD, and that anti-inflammation drugs may act as protective agents for the diseases.^{34,35)}

Several drug candidates have been developed to treat AD.³⁶⁾ For example, acetylcholinesterase inhibitors (e.g., donepezil) and N-methyl-D-aspartate (NMDA)-receptor antagonists (e.g.,

The authors declare no conflict of interest.

[#]These authors contributed equally to this work.

*To whom correspondence should be addressed. e-mail: kscho@konkuk.ac.kr

© 2013 The Pharmaceutical Society of Japan

Fig. 1. KSOP1009 Intake Reduced $A\beta42$ -Induced ERK Phosphorylation

(A,B) The level of ERK phosphorylation in $A\beta42$ - and *DIAP1*-expressing fly heads. (C) Representative images of acridine orange (AO)-stained $A\beta42$ - and *DIAP1*-expressing larval brains. (D) Confirmation of the genotypes by genomic DNA PCR using primers for the *UAS-DIAP1* and *UAS-Aβ42* transgenes. (E,F) Intake of 5 μ g/mL KSOP1009 reduced $A\beta42$ -induced ERK phosphorylation. (G) Dose-dependent effect of KSOP1009 on $A\beta42$ -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 $\pm$ S.E. (*** p < 0.001, * p < 0.05, Student's *t*-test, n = 5). The genotypes of the samples are *elav-GAL4* (*elav-GAL4/elav-GAL4*), *elav>Aβ42* (*UAS-Aβ42/UAS-Aβ42*; *elav-GAL4/elav-GAL4*), *elav>DIAP1* (*elav-GAL4*, *UAS-DIAP1/elav-GAL4*, *UAS-DIAP1*), and *elav>DIAP1*, $A\beta42$ (*UAS-Aβ42/UAS-Aβ42*; *elav-GAL4*, *UAS-DIAP1/elav-GAL4*, *UAS-DIAP1*).

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 γ -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 a modified version of SHXW, called KSOP1009, ameliorated neurological defects by suppressing the JNK pathway and apoptosis in a *Drosophila* AD model.¹⁵ Moreover, one study showed that intake of KSOP1009 improved $A\beta42$ -induced memory impairment and suppressed $A\beta42$ levels and plaque deposition in the brain of *Tg-APPswe/PS1dE9* mice to the same extent as donepezil treatment.⁴⁰

Given the complexity and widespread distribution of AD pathology, combinational drug treatment will likely be more helpful than monotherapy for the treatment or prevention of

Fig. 2. KSOP1009 Intake Suppressed the EGFR/ERK Signaling Pathway

(A) Representative wing images showing the effects of KSOP1009 intake on defective wing vein formation (arrows) in flies expressing *Egfr* or *Aβ42* ($n \geq 40$). (B) Graph showing the number of defective veins in flies overexpressing the indicated transgenes reared on control media or media containing $5 \mu\text{g/mL}$ KSOP1009. (C) Confocal images of larval wing disc showing Aβ42 accumulation. All data are expressed as the means $\pm$ S.E. (** $p < 0.01$, Student's *t*-test). The genotypes of the samples are *MS1096-GAL4* (*MS1096-GAL4/Y*), *MS1096>Egfr* (*MS1096-GAL4/Y; UAS-Egfr/+*) and *MS1096>Aβ42* (*MS1096-GAL4/Y; UAS-Aβ42/+*).

AD. 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.¹⁵⁾ 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), $50 \mu\text{M}$ PD98059 (Calbiochem), or 0.1, 0.5, 2, 5, $50 \mu\text{g/mL}$ 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

Fig. 3. Inhibition of ERK Activity Ameliorated the Locomotive and Vein Formation Defects of $A\beta 42$ -Expressing Flies

(A) The effect of ERK inhibition on the locomotive defect of $A\beta 42$ -expressing flies. The climbing ability of $A\beta 42$ -expressing flies fed media containing $50\mu\text{M}$ PD98059 (P+) was restored to control levels (*elav-GAL4*), similar to flies fed media containing $5\mu\text{g/mL}$ 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 $A\beta 42$. (C) Graph showing the number of defective veins ($n\geq 20$). (D,E) Representative image (D) and graph (E) of Western blot analyses showing the ERK phosphorylation level in the heads of $A\beta 42$ -expressing flies fed media with or without $50\mu\text{M}$ PD98059 ($n=5$). All data are expressed as the means $\pm$ S.E. (*** $p<0.001$, ** $p<0.01$, * $p<0.05$, 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/Y*; *UAS-Egfr/+*) and *MS1096>Aβ42* (*MS1096-GAL4/Y*; *UAS-Aβ42/+*).

(1:2000 in TBST; Cell Signaling).

Acridine Orange Staining The brains of *Drosophila* larvae were dissected in phosphate-buffered saline (PBS). The brains were incubated for 5 min in $1.6\times 10^{-6}\text{M}$ 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'-TACTGAAATCTCCAAGAA G-3' and 5'-TCTCTGTAG GTA GTTTGTG C-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^{16,41} 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. 4. *Aβ42* Expression Increased the Number of Glial Cells in the Larval Brain Independent of Apoptosis

(A,B) Confocal images of larval brains showing Aβ42 accumulation (A) and repo-positive glial cells (B). (C,D) *Aβ42* expression increased the number of glial cells in the larval brain regardless of *DIAP1* overexpression. (C) Representative confocal images of immunohistochemistry with an anti-repo antibody showing repo-positive glial cell-containing larva brain areas, corresponding to the white box in (B). (D) Graph showing the relative number of glial cells. All data are expressed as the means ± S.E. (***) $p < 0.001$, (**) $p < 0.01$; Student's *t*-test; $n \geq 20$). The genotypes of the samples are *elav-GAL4* (*elav-GAL4/elav-GAL4*), *elav>Aβ42* (*UAS-Aβ42/UAS-Aβ42*; *elav-GAL4/elav-GAL4*), *elav>DIAP1* (*elav-GAL4, UAS-DIAP1/elav-GAL4, UAS-DIAP1*), and *elav>DIAP1, Aβ42* (*UAS-Aβ42/UAS-Aβ42; elav-GAL4, UAS-DIAP1/elav-GAL4, UAS-DIAP1*).

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-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.^{8,24} Therefore, we investigated

whether ectopic expression of *Aβ42* in fly neurons augmented ERK activation. When *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 *Aβ42* induced apoptosis via the JNK pathway in *Drosophila* neurons.¹⁶ Therefore, we tested if the elevation in ERK phosphorylation levels is the result of Aβ42-induced apoptosis by assessing pERK levels in flies co-expressing *Aβ42* and *Drosophila inhibitor of apoptosis protein 1 (DIAP1)*, which strongly suppresses Aβ42-induced apoptosis (Fig. 1C). If increased apoptosis is the cause of ERK activation, then *DIAP1* overexpression should reduce pERK levels in *Aβ42*-expressing flies. However, Aβ42-induced ERK phosphorylation in AD fly brains was not affected by co-expression of *DIAP1* (Figs. 1A,B, lanes 3, 4), indicating that the elevated ERK activity in neurons is independent of JNK-induced apoptosis. The existences of transgenes, *Aβ42* and *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

Fig. 5. KSOP1009 Intake Decreased the Number of Glial Cells in the Larval Brains of AD Model Flies

(A,B) The increased number of glial cells due to *Aβ42* expression was reduced by KSOP1009 intake. (C,D) The glial cell number was not affected by intake of PD98059 in *Aβ42*-expressing larval brain. (A,C) Representative confocal images of glial cells in larvae raised with or without 5 μg/mL KSOP1009 or 50 μM PD98059. (B,D) Graph showing relative glial cell numbers. All data are expressed as the means ± S.E. (***p* < 0.01, Student's *t*-test; *n* ≥ 45). The genotypes of the samples are *elav-GAL4* (*elav-GAL4/elav-GAL4*) and *elav>Aβ42* (*UAS-Aβ42/UAS-Aβ42; elav-GAL4/elav-GAL4*).

of the EGFR/ERK Signaling Pathway 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,¹⁵ 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,¹⁵ 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

vein formation in *Drosophila* wings (Figs. 3B,C), and ERK phosphorylation in *Aβ42*-expressing brain (Figs. 3D,E). These results suggest that the ERK hyperactivation induced by *Aβ42* expression is harmful to the fly, and that KSOP1009 exerts its neuroprotective activity in *Aβ42*-expressing flies, in part, by suppressing ERK hyperactivation.

KSOP1009 Intake Reduced *Aβ42*-Induced Glial Cell Proliferation Increased inflammation due to activated microglia has been implicated in AD progression.^{6,42} 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).

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*,⁴³ 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.¹⁵ The observed neurological phenotypes were associated with JNK hyperactivation and increased apoptosis.¹⁶ 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.⁴⁴ 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

Fig. 6. Illustration of the Therapeutic Pathways of KSOP1009 in *Aβ42*-Induced Neuronal Toxicity

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

KSOP1009.

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.^{24,26} 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.^{6,42,45} 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.^{46–48} 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 A β 42 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.

Acknowledgement This study was supported by the faculty research fund of Konkuk University in 2010.

REFERENCES

- Walsh DM, Selkoe DJ. Deciphering the molecular basis of memory failure in Alzheimer's disease. *Neuron*, **44**, 181–193 (2004).
- Glenner GG, Wong CW. Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem. Biophys. Res. Commun.*, **120**, 885–890 (1984).
- Wirths O, Multhaup G, Bayer TA. A modified beta-amyloid hypothesis: intraneuronal accumulation of the beta-amyloid peptide—the first step of a fatal cascade. *J. Neurochem.*, **91**, 513–520 (2004).
- Yankner BA, Duffy LK, Kirschner DA. Neurotrophic and neurotoxic effects of amyloid beta protein: reversal by tachykinin neuropeptides. *Science*, **250**, 279–282 (1990).
- Calhoun ME, Wiederhold KH, Abramowski D, Phinney AL, Probst A, Sturchler-Pierrat C, Staufenbiel M, Sommer B, Jucker M. Neuron loss in APP transgenic mice. *Nature*, **395**, 755–756 (1998).
- Mattson MP. Pathways towards and away from Alzheimer's disease. *Nature*, **430**, 631–639 (2004).
- Chang L, Karin M. Mammalian MAP kinase signalling cascades. *Nature*, **410**, 37–40 (2001).
- Zhu X, Lee HG, Raina AK, Perry G, Smith MA. The role of mitogen-activated protein kinase pathways in Alzheimer's disease. *Neurosignals*, **11**, 270–281 (2002).
- Ma QL, Harris-White ME, Ubeda OM, Simmons M, Beech W, Lim GP, Teter B, Frautschy SA, Cole GM. Evidence of Abeta- and transgene-dependent defects in ERK-CREB signaling in Alzheimer's models. *J. Neurochem.*, **103**, 1594–1607 (2007).
- Pearson AG, Byrne UT, MacGibbon GA, Faull RL, Dragunow M. Activated c-Jun is present in neurofibrillary tangles in Alzheimer's disease brains. *Neurosci. Lett.*, **398**, 246–250 (2006).
- Thakur A, Wang X, Siedlak SL, Perry G, Smith MA, Zhu X. c-Jun phosphorylation in Alzheimer disease. *J. Neurosci. Res.*, **85**, 1668–1673 (2007).
- Bozyczko-Coyne D, O'Kane TM, Wu ZL, Dobrzanski P, Murthy S, Vaught JL, Scott RW. CEP-1347/KT-7515, an inhibitor of SAPK/JNK pathway activation, promotes survival and blocks multiple events associated with Abeta-induced cortical neuron apoptosis. *J. Neurochem.*, **77**, 849–863 (2001).
- Borsello T, Forloni G. JNK signalling: a possible target to prevent neurodegeneration. *Curr. Pharm. Des.*, **13**, 1875–1886 (2007).
- Braithwaite SP, Schmid RS, He DN, Sung ML, Cho S, Resnick L, Monaghan MM, Hirst WD, Essrich C, Reinhart PH, Lo DC. Inhibition of c-Jun kinase provides neuroprotection in a model of Alzheimer's disease. *Neurobiol. Dis.*, **39**, 311–317 (2010).
- Hong YK, Park SH, Lee S, Hwang S, Lee MJ, Kim D, Lee JH, Han SY, Kim ST, Kim YK, Jeon S, Koo BS, Cho KS. Neuroprotective effect of SuHeXiang Wan in *Drosophila* models of Alzheimer's disease. *J. Ethnopharmacol.*, **134**, 1028–1032 (2011).
- Hong YK, Lee S, Park SH, Lee JH, Han SY, Kim ST, Kim YK, Jeon S, Koo BS, Cho KS. Inhibition of JNK/dFOXO pathway and caspases rescues neurological impairments in *Drosophila* Alzheimer's disease model. *Biochem. Biophys. Res. Commun.*, **419**, 49–53 (2012).
- Perry G, Roder H, Nunomura A, Takeda A, Friedlich AL, Zhu X, Raina AK, Holbrook N, Siedlak SL, Harris PL, Smith MA. Activation of neuronal extracellular receptor kinase (ERK) in Alzheimer disease links oxidative stress to abnormal phosphorylation. *Neuroreport*, **10**, 2411–2415 (1999).
- Zhu X, Castellani RJ, Takeda A, Nunomura A, Atwood CS, Perry G, Smith MA. Differential activation of neuronal ERK, JNK/SAPK and p38 in Alzheimer disease: the “two hit” hypothesis. *Mech. Ageing Dev.*, **123**, 39–46 (2001).
- Dineley KT, Westerman M, Bui D, Bell K, Ashe KH, Sweatt JD. Beta-amyloid activates the mitogen-activated protein kinase cascade via hippocampal alpha7 nicotinic acetylcholine receptors: *In vitro* and *in vivo* mechanisms related to Alzheimer's disease. *J. Neurosci.*, **21**, 4125–4133 (2001).
- Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science*, **270**, 1326–1331 (1995).
- Cobb MH. MAP kinase pathways. *Prog. Biophys. Mol. Biol.*, **71**, 479–500 (1999).
- Mills J, Laurent Charest D, Lam F, Beyreuther K, Ida N, Pelech SL, Reiner PB. Regulation of amyloid precursor protein catabolism involves the mitogen-activated protein kinase signal transduction pathway. *J. Neurosci.*, **17**, 9415–9422 (1997).
- Desdouits-Magnen J, Desdouits F, Takeda S, Syu LJ, Saltiel AR, Buxbaum JD, Czernik AJ, Nairn AC, Greengard P. Regulation of secretion of Alzheimer amyloid precursor protein by the mitogen-activated protein kinase cascade. *J. Neurochem.*, **70**, 524–530 (1998).
- Cheung EC, Slack RS. Emerging role for ERK as a key regulator of neuronal apoptosis. *Sci. STKE*, **2004**, PE45 (2004).
- Subramaniam S, Zirrgiebel U, von Bohlen Und Halbach O, Strelau J, Laliberté C, Kaplan DR, Unsicker K. ERK activation promotes neuronal degeneration predominantly through plasma membrane damage and independently of caspase-3. *J. Cell Biol.*, **165**, 357–369 (2004).
- Ji RR, Befort K, Brenner GJ, Woolf CJ. ERK MAP kinase activation in superficial spinal cord neurons induces prodynorphin and NK-1 upregulation and contributes to persistent inflammatory pain hypersensitivity. *J. Neurosci.*, **22**, 478–485 (2002).
- Zhuang ZY, Gerner P, Woolf CJ, Ji RR. ERK is sequentially activated in neurons, microglia, and astrocytes by spinal nerve ligation and contributes to mechanical allodynia in this neuropathic pain model. *Pain*, **114**, 149–159 (2005).
- Akiyama H, Barger S, Barnum S, Bradt B, Bauer J, Cole GM, Cooper NR, Eikelenboom P, Emmerling M, Fiebich BL, Finch CE, Frautschy S, Griffin WS, Hampel H, Hull M, Landreth G, Lue L, Mrak R, Mackenzie IR, McGeer PL, O'Banion MK, Pachter J, Pasinetti G, Plata-Salaman C, Rogers J, Rydel R, Shen Y, Streit W, Strohmeyer R, Tooyoma I, Van Muiswinkel FL, Veerhuis R, Walker D, Webster S, Wegrzyniak B, Wenk G, Wyss-Coray T. Inflammation and Alzheimer's disease. *Neurobiol. Aging*, **21**, 383–421 (2000).
- Wyss-Coray T, Mucke L. Inflammation in neurodegenerative disease—a double-edged sword. *Neuron*, **35**, 419–432 (2002).
- Glass CK, Saijo K, Winner B, Marchetto MC, Gage FH. Mechanisms underlying inflammation in neurodegeneration. *Cell*, **140**, 918–934 (2010).
- Weitz TM, Town T. Microglia in Alzheimer's disease: It's all about context. *Int. J. Alzheimer's Dis.*, **2012**, 314185 (2012).
- Schwab C, Klegeris A, McGeer PL. Inflammation in transgenic mouse models of neurodegenerative disorders. *Biochim. Biophys. Acta*, **1802**, 889–902 (2010).
- Dahlgaard K, Jung A, Qvortrup K, Clausen H, Kjaerulff O, Wandall HH. Neurofibromatosis-like phenotype in *Drosophila* caused by lack of glucosylceramide extension. *Proc. Natl. Acad. Sci. U.S.A.*,

- 109, 6987–6992 (2012).
- 34) McGeer PL, Schulzer M, McGeer EG. Arthritis and anti-inflammatory agents as possible protective factors for Alzheimer's disease: a review of 17 epidemiologic studies. *Neurology*, **47**, 425–432 (1996).
- 35) Cole GM, Frautschy SA. Mechanisms of action of non-steroidal anti-inflammatory drugs for the prevention of Alzheimer's disease. *CNS Neurol. Disord. Drug Targets*, **9**, 140–148 (2010).
- 36) Mangialasche F, Solomon A, Winblad B, Mecocci P, Kivipelto M. Alzheimer's disease: clinical trials and drug development. *Lancet Neurol.*, **9**, 702–716 (2010).
- 37) Bensky D, Gamble A. *Chinese Herbal Medicine (Materia Medica)*, 1st ed., Eastland Press, Seattle (1986).
- 38) Koo BS, Lee SI, Ha JH, Lee DU. Inhibitory effects of the essential oil from SuHeXiang Wan on the central nervous system after inhalation. *Biol. Pharm. Bull.*, **27**, 515–519 (2004).
- 39) Kim KD, Jeong DG. Experimental Study on the change of stress-related hormone contents by Kyogamdan and Sohaphyangwon. *J. Orient. Neuropsychiatry*, **3**, 121–134 (1993).
- 40) Jeon S, Bose S, Hur J, Jun K, Kim YK, Cho KS, Koo BS. A modified formulation of Chinese traditional medicine improves memory impairment and reduces A β level in the T $_{\text{g}}$ -APPswe/PS1dE9 mouse model of Alzheimer's disease. *J. Ethnopharmacol.*, **137**, 783–789 (2011).
- 41) Lee MJ, Park SH, Han JH, Hong YK, Hwang S, Lee S, Kim D, Han SY, Kim ES, Cho KS. The effects of hempseed meal intake and linoleic acid on *Drosophila* models of neurodegenerative diseases and hypercholesterolemia. *Mol. Cells*, **31**, 337–342 (2011).
- 42) Yuan J, Yankner BA. Apoptosis in the nervous system. *Nature*, **407**, 802–809 (2000).
- 43) Kuran E. Keeping the CNS clear: glial phagocytic functions in *Drosophila*. *Glia*, **59**, 1304–1311 (2011).
- 44) Selkoe DJ. Translating cell biology into therapeutic advances in Alzheimer's disease. *Nature*, **399** (Suppl.), A23–A31 (1999).
- 45) Keller M, Enot DP, Hodson MP, Igwe EI, Deigner HP, Dean J, Bolouri H, Hagberg H, Mallard C. Inflammatory-induced hibernation in the fetus: priming of fetal sheep metabolism correlates with developmental brain injury. *PLoS ONE*, **6**, e29503 (2011).
- 46) Atri A, Shaughnessy LW, Locascio JJ, Growdon JH. Long-term course and effectiveness of combination therapy in Alzheimer's disease. *Alzheimer Dis. Assoc. Disord.*, **22**, 209–221 (2008).
- 47) Parachikova A, Green KN, Hendrix C, LaFerla FM. Formulation of a medical food cocktail for Alzheimer's disease: beneficial effects on cognition and neuropathology in a mouse model of the disease. *PLoS ONE*, **5**, e14015 (2010).
- 48) Patel L, Grossberg GT. Combination therapy for Alzheimer's disease. *Drugs Aging*, **28**, 539–546 (2011).