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Full-length article

Neuroprotective effect of sodium ferulate and signal transduction mechanisms in the aged rat hippocampus[1]

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Key words

ferulic acid; interleukin-1β; c-Jun N-terminal kinases; extracellular signal-regulated kinase; proto-oncogene proteins c-akt

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Abstract

Aim: To investigate whether the age-related increase in interleukin-1β (IL-1β) and c-Jun N-terminal kinases (JNK) pathway was coupled with a decrease in cell survival signaling pathways and whether sodium ferulate (SF) treatment was effective in preventing these age-associated changes. **Methods:** Groups of young and aged rats were fed for 4 weeks on a diet enriched in SF (100 mg/kg and 200 mg/kg per day). At the end of the period of dietary manipulation, Western blotting analysis was used to determine the expressions of IL-1β, phosphorylated mitogen-activated protein kinase kinase (MKK)4, phospho-JNK, phospho-c-Jun, phosphorylated extracellular signal-regulated kinase (ERK1/2), phospho-MEK, phospho-Akt, phosphorylated ribosomal protein S6 protein kinase (p70S6K), and activated caspase-3 and caspase-7. Nissl staining was used to observe the morphological change in hippocampal CA1 regions. Immunohistochemical techniques for glial fibrillary acidic protein (GFAP) and integrin αM (OX-42) were used to determine the astrocyte and microglia activation. **Results:** IL-1β protein levels, and phospho-MKK4, phospho-JNK1/2, and phospho-c-Jun were significantly enhanced in hippocampus prepared from age-matched control rats. Increased IL-1β production and JNK1/2 activation was accompanied by down-regulation of MEK/ERK1/2 pathway and Akt/p70S6K pathway, leading to cell apoptosis assessed by activation of caspase-3. Significantly, treatment of aged rats with SF (100 mg/kg and 200 mg/kg per day) for 4 weeks prevented the age-related increase in IL-1β and IL-1β-induced JNK signaling pathway and also the age-related changes in ERK and Akt kinase. **Conclusion:** SF plays neuroprotective roles through suppression of IL-1β and IL-1β-induced JNK signaling and upregulation of MEK/ERK1/2 and Akt/p70S6K survival pathways.

Introduction

The hippocampus is a target of age-related physiological and structural changes. Alterations in the hippocampus during aging are paralleled by behavioral and functional deficits in hippocampus-dependent learning and memory tasks[1]. This impairment is associated with inflammatory and oxidative changes. It has recently been reported that there is an increase in hippocampal concentration of the proinflammatory cytokine, interleukin-1β (IL-1β) and an increase in reactive oxygen species production[2,3] in aged rats. A number of cellular responses are shared by IL-1β and reactive oxygen species, which have been shown to activate certain mitogen-activated protein kinases (MAPK)[4,5]. Three subfamilies of these kinases have been clearly identified in mammals: extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 MAPK. Although ERK is stimulated by growth factors and activation results in neurite outgrowth, cell proliferation or differentiation[6], JNK and p38 MAPK are activated by environmental stress including oxidative stress and heat shock[7]. The age-related decrease in ERK1/2 and increase in p38 MAPK and JNK1/2 were observed in hippocampus of aged rats, which in turn impacts on the

ability of the aged rats to sustain long-term potentiation (LTP)[8,9].

An increase in stress-induced cell signaling in hippocampus may lead to cell death in the aged brain but it seems reasonable to propose that this may be coupled with a decrease in cell survival signaling pathways. The serine/threonine kinase Akt/protein kinase B (PKB) is activated via a phosphoinositide-3 kinase (PI3K)-dependent signaling pathway when cells or tissues are exposed to growth factors, insulin, and certain cytokines[10]. PI3K is an intracellular signaling enzyme that has been implicated in cell survival[11]. Akt/PKB has received widespread attention as an important anti-apoptotic protein[12].

Sodium ferulate (SF), extracted from a traditional Chinese herbal medicine, has potent antioxidant[13] and anti-inflammatory activities[14]. It has recently been reported that long-term administration of ferulic acid protected mice against learning and memory deficits induced by centrally administered β-amyloid[15]. The primary site of action of ferulic acid could be microglia[16] and astrocytes[17]. It has been recently reported that ferulic acid inhibited formation of Aβ fibrils and destabilized preformed fibrillary Aβ[18]. Sultana *et al* reported that ferulic acid ethyl ester significantly inhibited $A\beta_{1-42}$-induced cytoxicity, intracellular reactive oxygen species accumulation, lipid peroxidation, and induction of inducible nitric oxide synthase in primary hippocampal cultures[19]. Significantly, we have recently reported that SF had inhibitory effects on Aβ-induced increases of IL-1β expression and p38 MAPK pathway and apoptosis in rat hippocampus[20,21]. In addition, treatment with SF protected against glutamate-induced apoptosis of cultured cortical neurons[22]. In the present study, we set out to investigate whether SF treatment was effective in reversing age-associated changes in IL-1β and the JNK signaling pathway in the hippocampus of aged rats.

Materials and methods

Sodium ferulate, a colorless powder with purity >99%, was obtained from Suzhou Changtong Chemical Co (Suzhou, China). The enhanced chemiluminescence kit was from Pierce Biotechnology Inc (Rockford, IL, USA). Phospho-mitogen-activated protein kinases kinase (MKK) 4 (Thr261, No 9912), phospho-JNK1/2 (Thr183/Tyr185, No 9912), phospho-c-Jun (Ser63, No 9261), phospho-MEK1/2 (Ser217/221, No 9121), phospho-ERK1/2 (Thr202/Tyr204, No9101), MKK4, JNK1/2, c-Jun, MEK1/2, ERK1/2, caspase-3 (No 9662), caspase-7 (No 9492), and rabbit IgG, horse radish peroxidase (HRP)-linked antibodies and biotinylated protein ladder detection pack (No 7727) were purchased from Cell Signaling (Beverly, MA, USA). IL-1β, phospho-Akt/PKB (Thr-308), phosphorylated ribosomal protein S6 protein kinase (p70S6K; Thr389), p70S6K, GFAP, integrin αM (OX-42) antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). β-actin antibody was from Sigma Chemical Co. SeeBlue Plus2 Pre-stained Standard (Catalog no. LC5925) was from Invitrogen Life Technologies (USA).

Animal maintenance and drug exposure protocol Male Sprague-Dawley rats (Experimental Animal Center of Liaoning Medical University, Liaoning, China) at mean age of 3 months (210−270 g, young) or 21 months (500−600 g, aged) were used in these experiments. Animals were housed in pairs (aged rats) or groups of 4 (young rats) at an ambient temperature of 22−24 °C under a 12:12 h light:dark cycle and rats were maintained under veterinary supervision throughout the study. Body weights were monitored and animals that demonstrated external evidence of disease or significant loss of body weight were eliminated from the experiment. Drug doses and durations of treatments used for the experiments were based on our published study[21]. The aged animals were subdivided into three groups. Two groups were fed on a diet enriched in SF (100 mg/kg and 200 mg/kg, daily for 4 weeks). The non-drug-treated control group received standard laboratory chow. The groups of young rats (3 months) received SF (100 mg/kg) or standard laboratory diet. At the end of this period rats were 4 (230−300 g) and 22 (550−650 g) months old, respectively. In addition, 21-month-old rats were used for control animals in the Western blot experiments. Feed was prepared freshly each day and food and water intake did not vary between groups. Hippocampal slices (500 μm thick) were prepared and immediately frozen on dry ice. The CA1 region was microdissected as previously described[23] for Western blot (5 rats in each group). Animals (5 in each group) used for Nissl staining and GFAP immunohistochemical staining, and fluorescent double immunostaining were anesthetized and perfused transcardially with 4% paraformaldehyde.

Western blot analysis Western blot analysis was carried out to analyze the expression of IL-1β, phospho-MKK4, phospho-JNK1/2, phospho-c-Jun, phospho-MEK1/2, phospho-ERK1/2, phospho-Akt, phospho-p70S6K caspase-7, and caspase-3. The fresh hippocampal CA1 region was homogenized in RIPA buffer [1% Triton, 0.1 % SDS, 0.5 % deoxycholate, 1 mmol/L

EDTA, 20 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 10 mmol/L NaF, 1 mmol/L Na_3VO_4, 0.1 mmol/L PMSF]. The nuclear fractions were first isolated by centrifuging the homogenates at 7500×*g* for 30 min at 4 °C. The supernatant was further centrifuged at 12 000×*g* for 20 min at 4 °C to remove insoluble materials. Protein concentrations were quantified by the method of Lowry. Tissue samples were equalized for protein concentration. Proteins were resolved by 10%–12% SDS-PAGE and transferred to nitrocellulose membranes. Gels were also loaded with colored molecular weight markers to assess electrophoretic transfer and biotinylated protein ladder marker to estimate the molecular weights of bands of interest. The membranes were blocked with 3% BSA in TBS (pH 7.6) for 1 h and incubated overnight at 4 °C with suitably diluted primary antibodies. After extensive washing with TTBS , the membranes were incubated with anti-rabbit IgG, HRP-linked antibody and anti-biotin antibody for 1 h at room temperature. The blots were detected using the enhanced chemiluminescence (ECL) reaction. Following Western immunoblotting for phospho-MKK4, phospho-JNK1/2, phospho-c-Jun, phospho-MEK1/2, phospho-ERK1/2, phospho-Akt, and phospho-p70S6K, blots were stripped and reprobed for total MKK4, JNK1/2, c-Jun, MEK1/2, ERK1/2, Akt, and p70S6K. Following Western immunoblotting for IL-1β, blots were stripped and reprobed for β-actin antibody to ensure equal loading of protein. Quantification of protein bands was achieved by densitometric analysis using Chem Image 5500 software (UVP, USA). The ratio of phosphorylated MKK4, JNK1/2, c-Jun, MEK1/2, ERK1/2, Akt, and p70S6K to the total abundance of the respective proteins of young control rats was normalized to 1.

Nissl staining The rats (5 rats of each group) were perfused transcardially with 4% paraformaldehyde in phosphate-buffered saline (PBS). The brains were post-fixed for 24 h and embedded in paraffin. Serial coronal sections (5 μm thickness) were taken from various sections of the brain, stained for Nissl body using cresyl violet, and examined for pathological changes.

To assess hippocampal injury, the number of neurons in the pyramidal layer of the hippocampal CA1 region was counted under a light microscope at 400× magnification according to the method described by Zhang *et al*[24]. Briefly, two continuous fields in the hippocampal CA1 subregion were selected for each section and the neurons were counted. The mean of the two fields was taken as the neuron number of this section and the mean of four sections was taken as the neuron number of this specimen.

Immunohistochemical staining and double immunofluorescence study Tissue sections were processed for immunohistochemistry and double immunofluorescence study. Endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 for 10 min. The sections were incubated with 10% normal goat serum. The blocking serum was removed, and sections were incubated with polyclonal antibody against GFAP (1:100 in TBS) overnight at 4 °C, then with biotinylated secondary antibody at 37 °C for 20 min. The GFAP-positive cells were detected by using SABC and DAB kits. The sections for double immunofluorescence labeling were incubated in IL-1β (dilution 1:50) or phospho-JNK (dilution 1:50) antibody with integrin αM (dilution 1:50) or GFAP (dilution 1:50) overnight at 4 °C. After incubation, fluorescein isothiocyanate (FITC) conjugated and Cy3 conjugated secondary antibodies (Santa Cruz, CA, USA) were added after washing in PBS. All images were captured under a Leica TCS-SP2 (Germany) confocal microscope. For quantitative image analysis of hippocampal integrin αM immunostaining, serial sagittal sections of one hemisphere taken from lateral (+0.5 to +2.25) were examined. Integrin αM immunostaining was evaluated on sagittal brain sections of 5 animals from each group. For each animal, antigens were detected in 5 parallel sections having a defined distance of 35 μm showing hippocampus. The number of integrin αM positive cells was counted and is shown as immunopositive cells per mm^2.

Statistical analysis All data are presented as mean± SD. Statistical analysis was carried out with one-way ANOVA, followed by least significant difference (LSD) *post hoc* test, which was provided by SPSS 11.5 statistical software. The level of significance was accepted as $P<0.05$.

Results

Age-related alteration in IL-1β protein expression and glial activation were reversed by sodium ferulate in the hippocampus As shown in Figure 1A, the basal level of IL-1β protein expression in hippocampus of young, control-treated rats was very low. IL-1β protein expression was significantly higher in hippocampus from 22-month-old control-treated rats compared with young control-treated rats. SF treatment inhibited the age-related increase in IL-1β protein expression, but it did not affect IL-1β expression in hippocampus prepared from young rats. Quantitative counting of microglial cells showed that oral SF treatment effectively reduced the number of microglia

in hippocampus of aged rats (Figure 1B). Confocal immunofluorescence for IL-1β and integrin αM (OX-42), a specific marker of microglia, or for IL-1β and GFAP showed that the immunostaining of IL-1β predominantly co-localized with integrin αM (OX-42) in the hippocampus (Figure 1C). The vast majority of IL-1β-positive microglial cells showed a round to oval morphology, indicating an activated state of inflammation in hippocampus of aged rats. However, IL-1β-positive astrocytes were not found in hippocampus of rats (Figure 1D). Astrocytes were visualized by means of immunohistochemistry for GFAP, a specific marker of astrocytes. In aged hippocampus and cortex, a marked infiltration of astrocytes was found as well as transformation of astrocytes from a resting to an activated state, highlighted by phenotypic changes characterized by long, thick branching. Oral SF treatment at doses of 100 mg/kg and 200 mg/kg daily for 4 weeks significantly reduced the age-related astrocytic reaction in hippocampus and cortex (Figure 2). In addition, IL-1β protein expression was significantly enhanced in hippocampus from 21-month-old control rats compared with young 4-month-old control rats (data not shown).

Sodium ferulate inhibited age-related increase in phospho-MKK4, phospho-JNK and phospho-c-Jun expressions A good deal of evidence suggests that one consequence of increased IL-1β in several cell types is activation of JNK and this has been reported in hippocampus[5]. Therefore, hippocampal tissue prepared from young and aged rats was assessed for phosphorylated MKK4 (p-MKK4), phospho-JNK1/2 (p-JNK) and phospho-c-Jun expressions. Figure 3A shows a sample immunoblot indicating that expressions of p-MKK4 (but not total MKK4), which is known to activate JNK, was enhanced in 22-month-old rats compared with young rats. The age-related increase in MKK4 phosphorylation was paralleled by the changes in p-JNK (Figure 3B) and p-c-Jun (Figure 3C); assessment of the data obtained from densitometric analysis indicated that the age-related increase in p-JNK and p-c-Jun was statistically significant, but the expressions of total JNK and c-Jun were not affected by treatment. The expressions of p-MKK4, p-JNK, and p-c-Jun in hippocampus prepared from 21-

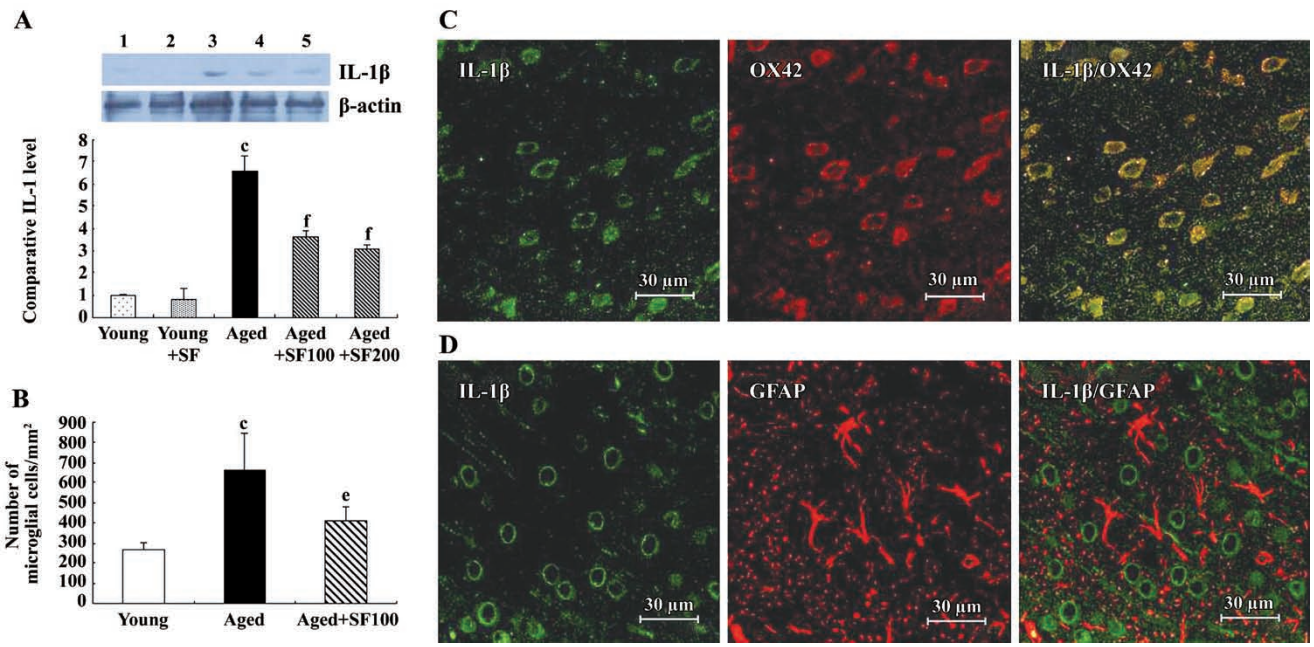

Figure 1. Sodium ferulate (SF) inhibited inflammatory reaction in the hippocampus of aged rats. The immunoreactity of IL-1β in hippocampus CA1 areas of young and aged rats treated with or without SF were determined by Western blotting. β-actin was analyzed as a sample loading control. Lane 1, control young rats; lane 2, young rats treated with SF 100 mg/kg; lane 3, control aged rats; lane 4 and 5, aged rats treated with SF 100 mg/kg and 200 mg/kg, respectively. The bar chart shows the semiquantitative analysis of the expressions of IL-1β (A). The quantitative image analysis of hippocampal integrin αM (OX-42) immunostaining shows SF treatment (100 mg/kg daily for 4 weeks) effectively reduced the number of microglia in hippocampus of aged rats (B). Confocal analysis of co-staining with IL-1β and integrin αM or GFAP revealed that integrin αM positive microglial cells expressed IL-1β (C) and GFAP positive astrocytes almost did not express IL-1β (D). Data are expressed as mean±SD of 5 independent preparations. $^{c}P<0.01$ *vs* young-control rats. $^{e}P<0.05$. $^{f}P<0.01$ *vs* aged-control rats. Bar=30 μm.

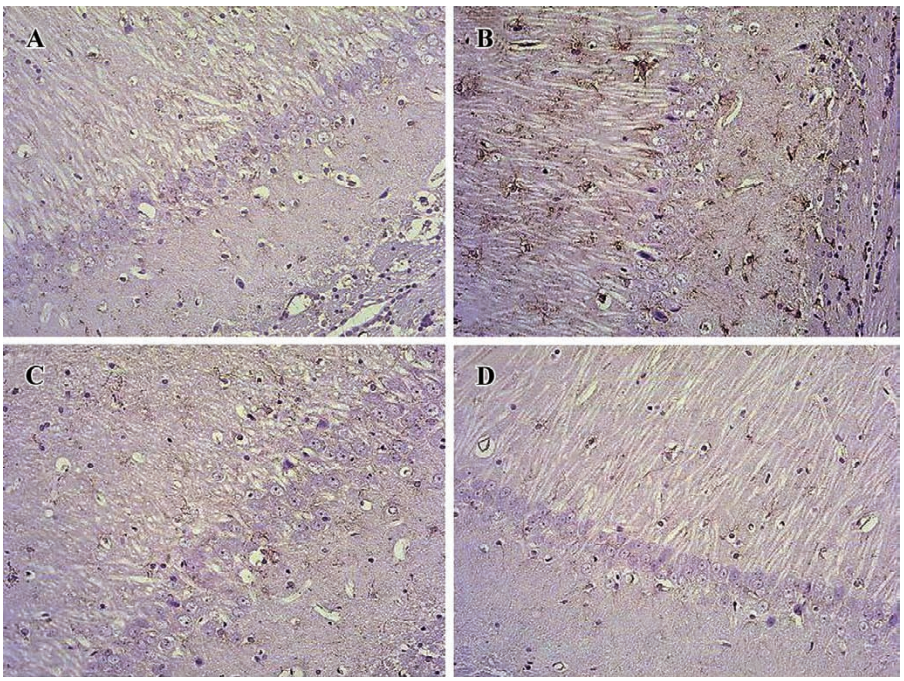

Figure 2. Photographs of GFAP immunohistochemical staining showing inhibitory effects of SF on aged-related change of astrocyte activation in rat hippocampus. In aged hippocampus and cortex, a marked infiltration of astrocytes was found, highlighted by phenotypic changes characterized by long, thick branching (B). SF treatment at doses of 100 mg/kg (C) and 200 mg/kg (D) daily for 4 weeks significantly reduced the age-related astrocytic reaction. (A) Young control-treated rats. Magnification: ×400.

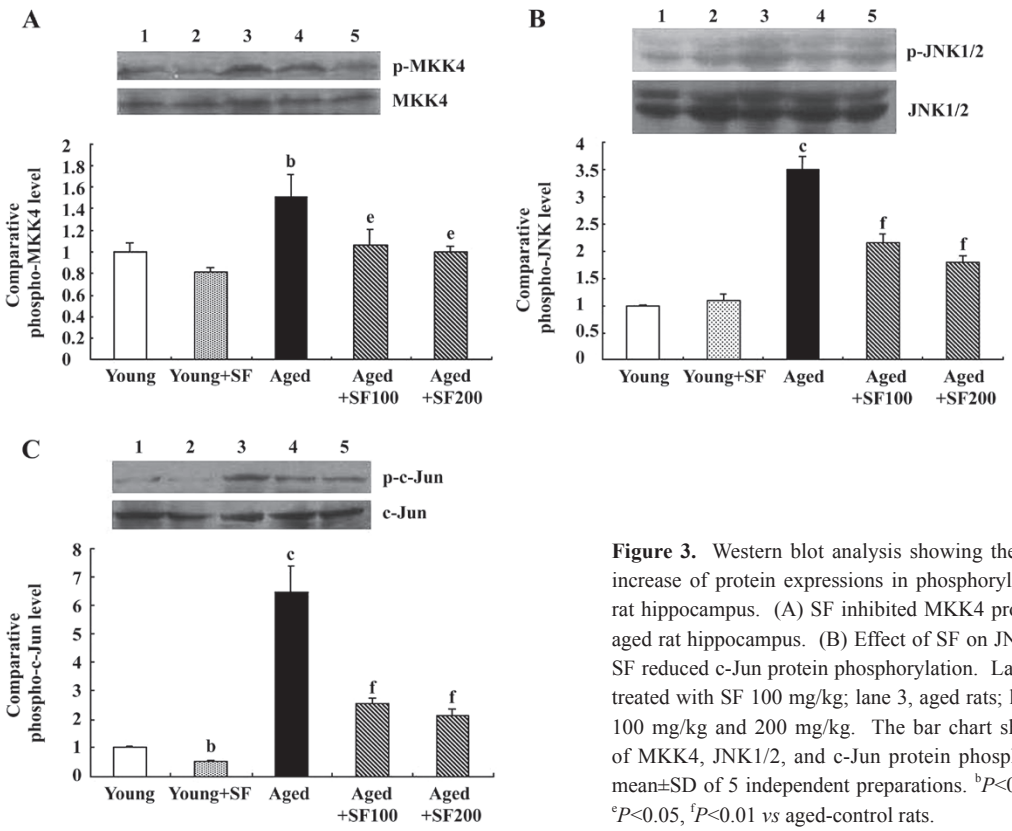

Figure 3. Western blot analysis showing the effects of SF on the aged-related increase of protein expressions in phosphorylated MKK4, JNK1/2, and c-Jun in rat hippocampus. (A) SF inhibited MKK4 protein phosphorylation in young and aged rat hippocampus. (B) Effect of SF on JNK1/2 protein phosphorylation. (C) SF reduced c-Jun protein phosphorylation. Lane 1, young rats; lane 2, young rats treated with SF 100 mg/kg; lane 3, aged rats; lanes 4–5, aged rats treated with SF 100 mg/kg and 200 mg/kg. The bar chart shows the semiquantitative analysis of MKK4, JNK1/2, and c-Jun protein phosphorylation. Data are expressed as mean±SD of 5 independent preparations. $^{b}P<0.05$, $^{c}P<0.01$ *vs* young-control rats. $^{e}P<0.05$, $^{f}P<0.01$ *vs* aged-control rats.

month-old rats were enhanced compared with that in young control rats (data not shown). SF (100 mg/kg and 200 mg/kg, daily for 4 weeks) treatment abrogated the aged-related increase of p-MKK4, p-JNK, and p-c-Jun. Meanwhile,

SF had significant inhibitory effects on p-MKK4, p-JNK, and p-c-Jun expressions in hippocampus prepared from young rats. To determine the cell type that expresses phospho-JNK, a double immunofluorescent study was carried out in the hippocampus of rats. Co-staining of integrin αM and phospho-JNK confirmed that phospho-JNK was predominantly expressed in microglia (Figure 4). In addition, co-staining of GFAP and phospho-JNK showed that phospho-JNK positive astrocytes were small (Figure 4).

Effects of sodium ferulate on MEK1/2 and ERK1/2 phosphorylation in the hippocampus of aged rats One consistent finding was that ERK activation decreased in the hippocampus and cortex of aged rats[25,26]. This age-related decrease in ERK1/2 is likely to contribute to the age-related deficit in long-term potentiation[27]. Therefore, we analyzed phosphorylated MEK1/2 and ERK1/2 protein expressions. The results revealed that ERK1/2 phosphorylation was decreased in hippocampal preparations obtained from control-treated aged rats compared with control-

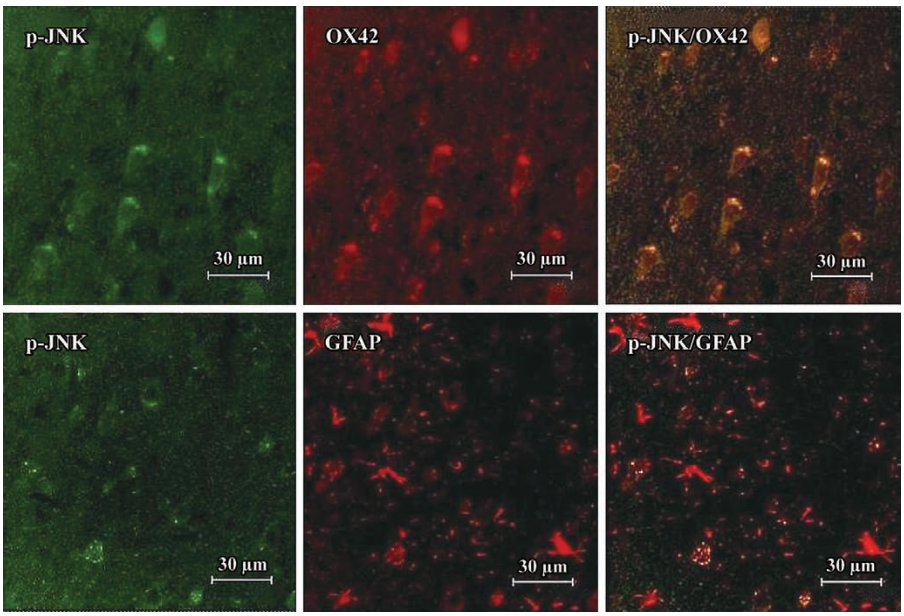

Figure 4. Double-labeled immunofluorescent staining of phospho-JNK and integrin αM (OX-42) or GFAP in the hippocampus of aged rats. Slices (5 μm thickness) were stained with antibodies of phospho-JNK and integrin αM or phospho-JNK and GFAP. Merged images (yellow) showed that most of phospho-JNK immunoreactivity co-localized with microglia-speccific protein integrin αM (OX-42). Bar=30 μm. Representative results of four different experiments are shown.

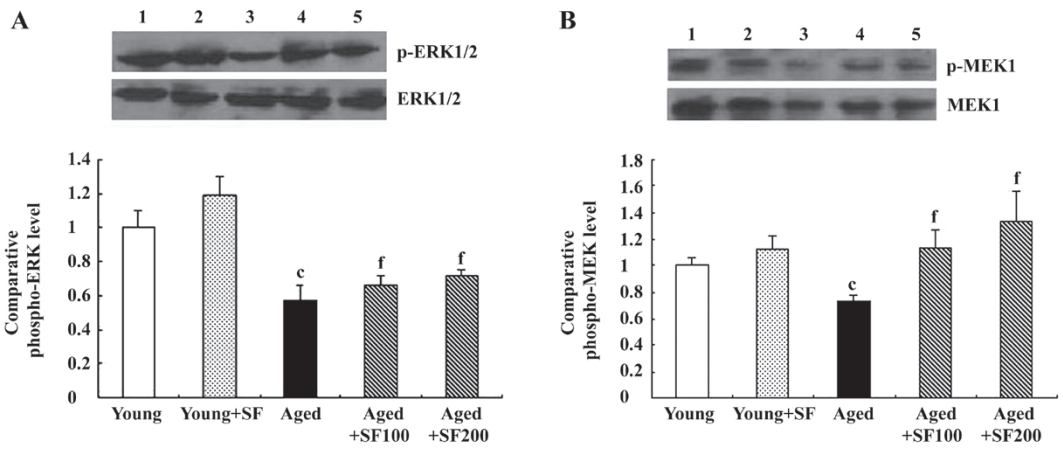

Figure 5. Effects of SF on the aged-related decrease in phosphorylated ERK1/2 and MEK1/2 protein expressions revealed by Western blot. (A) SF prevented the aged-related decrease in phosphorylated ERK1/2 expression. (B) SF prevented the aged-related decrease in phosphorylated MEK1 expressions. Lane 1, young rats; lane 2, young rats treated with SF 100 mg/kg; lane 3, aged rats; lanes 4–5, aged rats treated with SF 100 mg/kg and 200 mg/kg. The bar chart shows the semiquantitative analysis of the expressions of phosphorylated ERK1/2 and phosphorylated MEK1. Data are expressed as mean±SD of 5 independent preparations. $^{c}P<0.01$ *vs* young-control rats. $^{f}P<0.01$ *vs* aged-control rats.

treated young rats. SF partly inhibited the aged related decrease in p-ERK1/2 (Figure 5A). In parallel with the change of ERK1/2 phosphorylation, we found that p-MEK1/2 expression, an upstream kinase of ERK1/2, was significantly decreased in hippocampus obtained from control-treated aged rats and SF suppressed this change (Figure 5B). Equal protein loading was verified by reprobing immunoblots for total MEK1/2 and ERK1/2 and the data indicate that their expressions were similar in all treatment groups examined.

Sodium ferulate prevented the age-related decreases in phosphorylated Akt/PKB and p70S6K protein expression in the hippocampus of aged rats It has been shown that cell death is accompanied by a decrease in survival signals[28]. We considered that the age-related increase in IL-1β concentration in hippocampus, which is associated with upregulation of phospho-JNK1/2, might be accompanied by an age-related decrease in Akt/p70S6K pathway. Therefore, we analyzed protein expressions of phosphorylated Akt/PKB and p70S6K. The sample immunoblot and mean data in Figure 6 indicated that activation of both kinases was significantly reduced in hippocampus prepared from control-treated aged, compared with control-treated young rats. SF treatment did not significantly affect kinase activation in hippocampus prepared from young rats, but it prevented the age-related decreases so that the values were similar to those in hippocampus prepared from young rats, but the expressions of total Akt/PKB and p70S6K were not affected by treatment (Figure 6A, 6B).

Sodium ferulate inhibited the age-related alterations in caspase-3 and caspase-7 expression in the hippocampus of aged rats The activation of caspase-3 is a hallmark of apoptosis**.** In the apoptotic pathway, caspase-9 activity is responsible for procaspase-3 and procaspase-7 activation. The activated caspase-3 and caspase-7 of 20 kDa were observed in Western blot analysis. The results showed that expressions of activated caspase-3 and caspase-7 were markedly increased in hippocampus prepared from aged, compared with young, rats. SF treatment prevented this age-related change. Thus, protein expression of activated caspase-3 and caspase-7 in the hippocampus of aged rats treated with SF was similar to that in the hippocampus of young control-treated or SF-treated rats (Figure 7A, 7B).

Effect of sodium ferulate on age-related morphological change and number of hippocampal CA1 pyramidal neurons The arrangement of hippocampal CA1 pyramidal neurons of the young-control group is clearly discernible (Figure 8A). The Nissl body in the hippocampal pyramidal neurons of the aged-control group was decreasing or dissolving (Figure 8B). The arrangement of hippocampal pyramidal neurons of SF-treated aged rats was better than that of aged-control rats (Figure 8C, 8D). However, no obvious age-related changes in neuron numbers in hippocampal CA1 regions were found (data not shown).

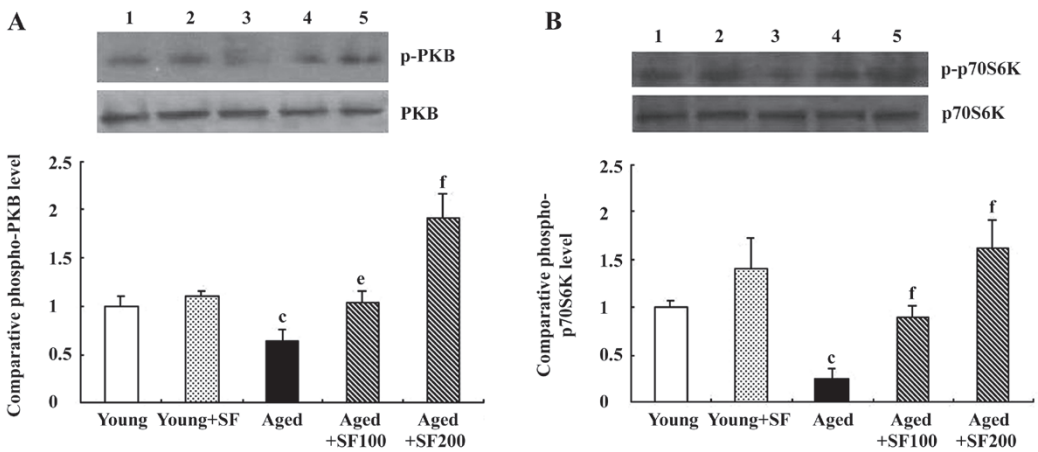

Figure 6. Effects of SF on the aged-related decrease in phosphorylated Akt/PKB and p70S6K protein expressions in rat hippocampus revealed by Western blot. (A) SF inhibited the aged-related decrease in phosphorylated Akt/PKB protein expressions. (B) SF prevented aged-related decrease in phosphorylated p70S6K protein expressions. β-actin was analyzed as a sample loading control. Lane 1, young rats; lane 2, young rats treated with SF 100 mg/kg; lane 3, aged rats; lanes 4–5, aged rats treated with SF 100 mg/kg and 200 mg/kg. The bar chart shows the semiquantitative analysis of the expressions of phosphorylated Akt/PKB and p70S6K. Data are expressed as mean±SD of 5 independent preparations. $^{c}P<0.01$ *vs* young-control rats. $^{f}P<0.01$ *vs* aged-control rats.

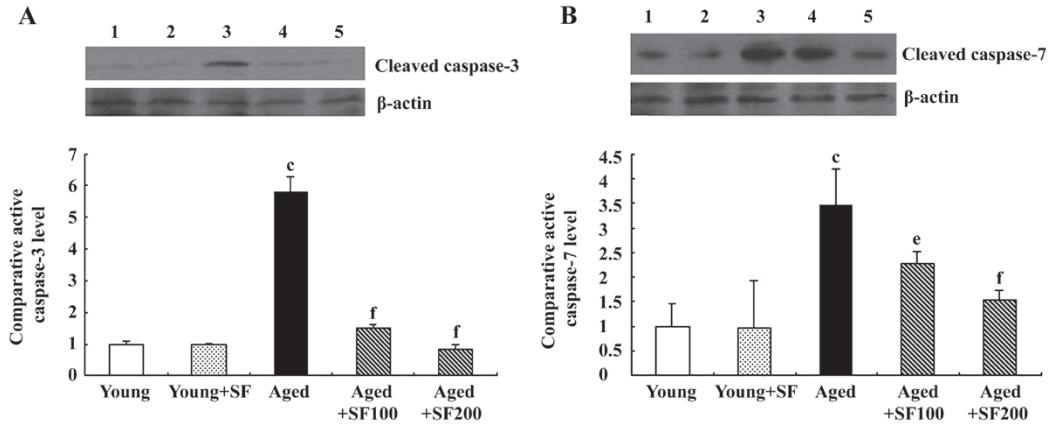

Figure 7. Western blot analysis showing age-related increases in activated caspase-3 and caspase-7 protein expressions were blocked by SF. (A) SF significantly reduced the age-related increase in activated caspase-3 (p19 fragment). (B) SF inhibited age-related activation of procaspase-7. The active fragment of caspase-7 is indicated as cleaved caspase-7 (20 kDa). β-actin was analyzed as a sample loading control. Lane 1, young rats; lane 2, young rats treated with SF 100 mg/kg; lane 3, aged rats; lanes 4–5, aged rats treated with SF 100 mg/kg and 200 mg/kg. The bar chart shows the semiquantitative analysis of the expressions of activated caspase-3 and caspase-7. Data are expressed as mean±SD of 5 independent preparations. $^{c}P<0.01$ *vs* young-control rats. $^{e}P<0.05$, $^{f}P<0.01$ *vs* aged-control rats.

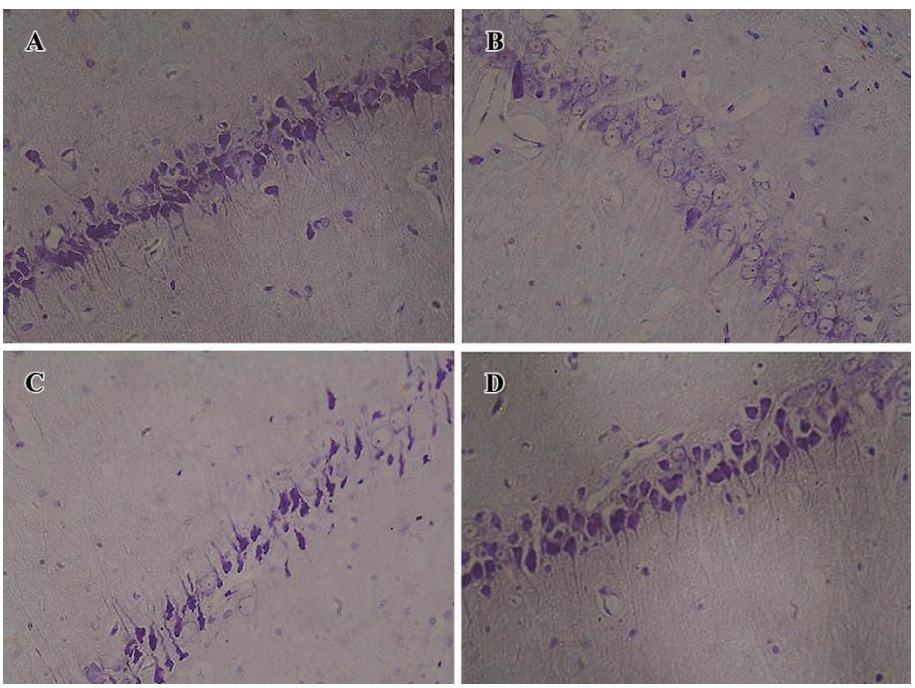

Figure 8. Nissl staining demonstrates the change of arrangement of hippocampus CA1 pyramidal neurons. (A) Young control-treated rats. (B) Aged control-treated rats. (C) Aged SF (100 mg/kg)-treated rats. (D) Aged SF (200 mg/kg)-treated rats. Magnification×400. The Nissl body in hippocampal pyramidal neurons of aged-control group was decreasing or dissolving (B).

Discussion

We present evidence demonstrating that the age-related increases in IL-1β expression and JNK activation were accompanied by downregulation of survival signals in the Akt/p70S6K pathway and MEK/ERK pathway. These findings were coupled with increased expression of activated caspase-3 and caspase-7. Significantly, treatment with SF prevented these age-related changes, including the age-related increase in caspase-3 activation, an indicator of cell death. The increase in IL-1β in hippocampus prepared from aged rats, which confirms previous findings[4,9], was coupled with JNK phosphorylation. The increase in JNK phosphorylation is paralleled by several changes that are hallmarks of cell death. For example, phosphorylation of c-Jun in the hippocampus was enhanced, which is a

downstream consequence of JNK activation and which has been shown to play a significant role in triggering neuronal apoptosis in a variety of cells *in vitro*[29,30]. Similarly, increased caspase-3 and caspase-7 activation was observed in hippocampus prepared from aged rats. A recent study linked activation of JNK with translocation of cytochrome *c* from mitochondria, suggesting that the patency of the mitochondrial membrane was affected by JNK activation[31]. A significant finding of this study is that SF treatment prevented age-related astrocyte activation, increases in MKK4/JNK/c-Jun pathway and caspase-3 activation indicating a potential neuroprotective effect as previously described[20,21]. These findings suggest that the inhibitory effect of SF on the JNK pathway and on caspase-3 activity may be secondary to its ability to suppress the age-related increase in IL-1β.

It has also been reported that cell death is accompanied by a decrease in survival pathways[28]. ERK1/2 plays a role in different types of LTP. Several behavioral studies have also demonstrated the importance of ERK1/2 in learning and memory[32]. Our results showed that, in addition to enhanced JNK and c-Jun activation, the increase in activated caspase-3 expression was associated with attenuated expression of phosphorylated ERK1/2. This observation is in agreement with recent reports showing an age-related decrease in ERK1/2 activity in the hippocampus and cortex of aged rats[9]. ERK1/2 activation and inactivation is regulated by MEK1/2. The age-related decrease in phosphorylated ERK1/2 was paralleled by an age-related decrease in phosphorylated MEK1/2 expression. Significantly, SF treatment prevented the age-related decrease in phosphorylated ERK1/2 and phosphorylated MEK1/2 expressions. Our previous studies showed that SF abolished the Aβ-induced decrease in phosphorylated ERK1/2 in rat hippocampus[20]. It is possible that age-related change in ERK1/2 activation is dependent on MEK1/2 activation. Due to the complexity of the regulation of ERK cascade, it is difficult to delineate mechanisms related to age-associated deficits in this signaling pathway. The activation of the ERK signaling pathway was reported to suppress the proapoptotic activity of JNK, thus protecting rat pheochromocytoma cells (PC-12) from nerve growth factor NGF withdrawal-induced cell death[33]. The MEK/ERK pathway interferes with apoptosis at the level of cytosolic caspase activation, downstream of the release of cytochrome *c* from mitochondria[34].

It has been reported that increased IL-1β concentration was coupled with downregulation of PI3K in cortical tissue prepared from aged rats[9]. Active PI3K catalyses the synthesis of 3′-phosphorylated inositol lipids that control the intracellular localization and activity of a key molecule of neuronal survival, the protein kinase Akt/PKB. Because the phosphorylation of Akt/PKB serves as an indicator for a previous PI3K activation, we set out to investigate age-related changes in Akt/PKB levels by phosphorylation using phospho-specific antibody. The results showed that Akt/PKB activation decreased with age. Therefore, the age-associate increase in activated caspase-3 may arise from the coupled increase in JNK activation and decrease in ERK and Akt/PKB activation. It has been reported that the anti-inflammatory cytokine IL-4 can lead to activation of PI3K[35]. IL-4 concentration in cortical tissue decreased with age and IL-4 had the capability of increasing the activity of PI3K[9]. A recent study found that IL-4 prevented apoptosis through Akt activation and the p70S6K survival signaling pathway[36]. We considered that an age-related downregulation of Akt may contribute to the decrease of p70S6K. The evidence presented is consistent with that hypothesis. SF treatment reversed the age-associated decreases in cell survival signals Akt and p70S6K.

In conclusion, this study demonstrated that in aged hippocampus, in addition to enhanced JNK/c-Jun pathway, the increases of caspase-3 and caspase-7 were associated with the decrease of the MEK/ERK pathway and the Akt/p70S6K survival signaling pathways. SF treatment prevented these age-related changes, including the increase in caspase-3 activity.

Author contribution

Ying JIN designed research; En-zhi YAN, Xiao-ming LI, Ying FAN performed research; Yan-jie ZHAO, Wan-zhu LIU contributed new analytical reagents and tools; En-zhi YAN analyzed data; Ying JIN wrote the paper.

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