INTRODUCTION

Alzheimer’s disease (AD) is a progressive neurodegenerative disease characterized by deficits in cognitive function (Sevush and Leve, 1993; Backman et al., 2001), and accumulations of amyloid β (Aβ) in the extracellular regions and tau aggregates in the intracellular regions of the brain (Ittner and Gotz, 2011; Jin et al., 2011; Lasagna-Reeves et al., 2012).

Plasmin is an important protease in various physiologies, including clearance of blood clots and immune function (Alkjaersig et al., 1959; Draxler et al., 2017). In the brain, plasmin activates brain-derived neurotropic factor (BDNF) (Gray and Ellis, 2008; Rodier et al., 2014). Aβ is a substrate of plasmin, suggesting that plasmin could clear Aβ deposits (Van Nostrand and Porter, 1999; Jacobsen et al., 2008). BDNF protects neurons against Aβ (Arancibia et al., 2008; Criscuolo et al., 2015), and BDNF signaling activation improves memory deficits in AD mouse models (Gao et al., 2016; de Pins et al., 2019). Moreover, small molecule binding of tropomyosin-related kinase B (TrkB), a BDNF receptor, can improve AD-like symptoms in AD models (Castello et al., 2014; Gao et al., 2016). Taken together, this evidence suggests that an agent regulating plasmin activity may be a good candidate for AD therapy.

Spinosin is a flavonoid isolated from Zizyphus jujuba var. spinosa seeds (Shergis et al., 2017). Previously, we reported that spinosin ameliorated oligomeric Aβ-induced memory impairments (Ko et al., 2015), reduced oligomeric Aβ-induced inflammation, and ameliorated choline-acetyl transferase in the hippocampus. Recently, we found that the ethanol extract of Zizyphus jujuba var. spinosa seeds activated plasmin activity (Park et al., 2019). Because spinosin is an active compound of Zizyphus jujuba var. spinosa seeds and has a protective effect in Aβ-induced AD models, we hypothesized that spinosin may regulate plasmin activity. In the present study, we tested whether spinosin affected plasmin activity.

Abstract

Hippocampal synaptic dysfunction is a hallmark of Alzheimer’s disease (AD). Many agents regulating hippocampal synaptic plasticity show an ameliorative effect on AD pathology, making them potential candidates for AD therapy. In the present study, we investigated spinosin as a regulating agent of synaptic plasticity in AD. Spinosin attenuated amyloid β (Aβ)-induced long-term potentiation (LTP) impairment, and improved plasmin activity and protein level in the hippocampi of 5XFAD mice, a transgenic AD mouse model. Moreover, the effect of spinosin on hippocampal LTP in 5XFAD mice was prevented by 6-aminocaproic acid, a plasmin inhibitor. These results suggest that spinosin improves synaptic function in the AD hippocampus by regulating plasmin activity.

Key Words: Spinosin, Alzheimer’s disease, Plasmin, LTP, 5XFAD

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MATERIALS AND METHODS

Materials
Donepezil was donated DAEHWA pharmaceutical CO., LTD (Seoul, Korea). Aβ1-42 was purchased from Anaspec (CA, USA). Spinosin was purchased from Sigma-Aldrich (MO, USA). The antiplasmin, anti-plasminogen, and anti-glycer-aldyhyde 3-phosphate dehydrogenase (GAPDH) antibodies were purchased from Snata Cruz Biotechnology (CA, USA). The 6-aminocaproic acid was purchased from Sigma-Aldrich. Plasmn assay kit was purchased from Abcam (Cambridge, UK).

Animals
Seven ICR mice (6 weeks old) were purchased from SAM-TAKO Biokore (Osan, Korea). Male 5XFAD mice were obtained from the Jackson Laboratory (CA, USA) and crossbred with female hybrid B6SJLF1 mice (Taconic, Seoul, Korea). The male heterozygous transgenic and littermate wild-type (WT) offspring were used for the experiments. Mice were housed in individual ventilated cages with access to water and food ad libitum, under a 12-h light/dark cycle (lights on from 07:30 to 19:30). For examine the effect of spinosin on Aβ-induced synaptic deficit, hippocampal slice isolated from one ICR mouse was treated with vehicle, Aβ+vehicle, Aβ+spinosin (3), Aβ+spinosin (30) or Aβ+donepezil for 2 h. Then, the hippocampal slice was subjected to electrophysiology. This experiment was conducted repeatedly seven times with seven different mice. For figure 2, 4 of 6-month-old 5XFAD and 4 of WT mice were used. Hippocampal slices from a 5XFAD mouse were treated with spinosin for 2 h, and then subjected to measuring plasmin activity or western blot. For blocking experiments, 4 of 6-month-old 5XFAD and 4 of WT mice were used. Hippocampal slices from a 5XFAD mouse were treated with spinosin and/or 6-aminocaproic acid for 2 h, and then subjected to electrophysiology. The treatment and maintenance of the animals were performed out in accordance with the Animal Care and Use Guidelines of Kyung Hee University (Seoul, Korea). All of the experimental protocols using animals were approved by the Institutional Animal Care and Use Committee of Kyung Hee University (KHUASP(SE)-18-046). Behavioral experiments and data analysis were conducted by different persons who did not know group difference.

Acute hippocampal slice preparation
Artificial cerebrospinal fluid (ACSF) was comprised of 124 mM NaCl, 3 mM KCl, 26 mM NaHCO3, 1.25 mM NaH2PO4, 2 mM CaCl2, 1 mM MgSO4, and 10 mM D-glucose. We rapidly removed the brain and isolated the mouse hippocampus. Mouse hippocampal tissues were sliced using a McIlwain tissue chopper. 400-μm-thick hippocampal slices were made and incubated in ACSF (20-25°C) for 2 h before the experiment.

Electrophysiology
Field potential responses were recorded in the Schaffer collateral-commissural pathway in area CA1. Stimuli (constant voltage) were delivered at 30 s intervals. The slope of
the evoked field potential responses (fEPSP) was averaged from four consecutive recordings evoked at 30 s intervals. To induce LTP, two trains of high frequency stimulation (HFS: 100 Hz, 100 pulses in 1 s, 30 s interval) were introduced at 20 min after the initiation of a stable baseline. LTP was quantified by comparing the mean fEPSP slope at 80 min after the HFS period with the mean fEPSP slope during the baseline period and calculating the percentage change from the baseline. For the experiments with Aβ, Aβ dissolved in DPBS at 1 mg/mL and agitated at 37°C for 24 h for aggregation. Hippocampal slices were incubated in ACSF containing vehicle or drugs for 30 min, and then further incubated in ACSF containing Aβ oligomer (1 μM) and/or drugs for 2 h before recording. For the blocking experiments with 5XFAD mice, slices were incubated in ACSF containing inhibitor (100 μM), and then further incubated in ACSF containing inhibitor+spinosin 2 h before recording.

Plasmin activity assay
Plasmin activity was measured using commercial plasmin activity assay kit (Abcam, ab204728). All procedures were followed to protocol presented from Abcam. Hippocampal slices were incubated with spinosin containing ACSF for 2 h. After then hippocampal slices were homogenized in ice-chilled Tris–HCl buffer (20 mM, pH 7.4), sucrose (0.32 M), ethylene-diaminetetraacetic acid (EDTA) (1 mM), ethylene glycol bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) (1 mM)). Debris was removed by microcentrifugation (4200×g, 20 min). A mixture of 50 μl of sample (10 μg of protein) and 50 μl of reaction mix (48 μl plasmin assay buffer+2 μl of plasmin substrate) was made. Measure output on a fluorescent microplate reader at Ex/Em=360/450 nm in a kinetic mode, every 2-3 min, for 10-20 min at 37°C protected from light.

Western blot analysis
Hippocampal slices were incubated with spinosin containing ACSF for 2 h. Afterwards hippocampal slices were homogenized in ice-chilled M-PER buffer (Thermo, Rockford, IL, USA), a containing protease inhibitor, and phosphatase inhibitor cocktail (Thermo). Debris was removed by microcentrifugation (4200×g, 20 min). Proteins from whole-cell lysates were quantified using a BCA protein assay kit following the manufacturer’s instructions. Samples (30 μg of protein) were then subjected to SDS-PAGE (12% gel) under reducing conditions. Proteins were transferred to PVDF membranes using transfer buffer (25 mM Tris-HCl, pH 7.4 containing 192 mM glycine and 20% v/v methanol) at 400 mA for 2 h (4°C). Next, blots were incubated for 2 h with blocking solution (5% skimmed milk for total proteins, 5% BSA for phosphorylated proteins) and then placed at 4°C overnight with 1:1000 dilutions of anti-goat plasmin antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-rabbit plasminogen antibody (Santa Cruz Biotechnology Inc.) or anti-rabbit GAPDH antibody (Santa Cruz Biotechnology Inc.). After serial washing, blots were incubated with a 1:5000 dilution of horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology Inc.) for 1 h at room temperature.

Statistics
Values are expressed as the mean ± SEM. Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test for multiple comparisons. Statistical significance was set at p<0.05.

RESULTS
Spinosin attenuated Aβ-induced long-term potentiation (LTP) impairment in the hippocampus
To investigate the effect of spinosin, LTP was measured in hippocampal slices treated with Aβ and/or spinosin. Aβ-treated slices showed significantly lower LTP levels than did control slices (Fig. 1A, 1F). Spinosin (30 μM) and donepezil (DNP), a positive control, attenuated this Aβ-induced LTP reduction in a concentration dependent manner (F5, 36 =11.42, p<0.05, n=7/group, Fig. 1).

Spinosin regulated plasmin activity in the 5XFAD hippocampus
Previously, we found that Zizyphus jujuba var. spinosa seeds increased plasmin activity in the hippocampus. Since spinosin is an active compound isolated from Zizyphus jujuba...
ba var. spinosa seeds, we tested whether spinosin regulates hippocampal plasmin activity. Plasmin activity was significantly lower in the hippocampus of 5XFAD than in that of WT ($F_{3,20}=4.296$, $p<0.05$, n=3-4/group, Fig. 2A). Spinosin-treated hippocampal slices of 5XFAD showed significantly higher plasmin activity than did vehicle-treated hippocampal slices of 5XFAD ($p<0.05$, Fig. 2A). Plasmin protein levels were significantly lower in the hippocampus of 5XFAD mice than in that of WT mice ($F_{2,9}=4.483$, $p<0.05$, n=4/group, Fig. 2B, 2C) while plasminogen levels were unaffected ($F_{2,9}=0.005$, $p>0.05$, n=4/group, Fig. 2B, 2C). Spinosin treatment rescued this plasmin level reduction (Fig. 2B, 2C).

DISCUSSION

In the present study, we found that spinosin improves LTP in the Aβ-treated hippocampus of normal mice or the hippocampus of 5XFAD mice. Spinosin improves plasmin activity, which is down-regulated in the hippocampus of 5XFAD mice. 6-aminocaproic acid blocked this spinosin-improved LTP in the hippocampus of 5XFAD mice.

The tissue plasminogen activator (tPA)/plasmin system has been suggested as a therapeutic target for AD (Angelucci et al., 2019). Plasmin can cleave Aβ and Aβ deposits, suggesting that it may be involved in Aβ clearance (Ledesma et al., 2000; Jacobsen et al., 2008; Baranello et al., 2015). It was found that there is less plasmin activity in the AD brain than in the normal brain (Dotti et al., 2004; Barker et al., 2010). tPA administration, which is believed to activate plasmin, protected against memory loss in AD mouse models (Tucker et al., 2000; ElAli et al., 2016). Taken together, this information suggests that agents, who increase plasmin activity directly or indirectly, may be good candidates for AD therapy. In the present study, we found that spinosin ameliorated deficits in plasmin activity in the 5XFAD mouse hippocampus. Interestingly, we found that spinosin increase plasmin level and its activity without affecting the level of plasminogen. These suggest that spinosin might activate plasminogen cleavage system. Spinosin could up-regulate activity of tPA. Otherwise, spinosin may suppress activity of neuroserpin or PAI-I, inhibitors of plasminogen activators. Although we still do not know the precise mechanism of the effect of spinosin on plasmin and its side effects, spinosin could be considered a candidate for AD therapy.

Synaptic plasticity is a cellular mechanism of learning and
memory (Maren and Baudry, 1995; Sutton and Schuman, 2006). Aβγ induces internalization of the AMPA receptor, a major target of synaptic plasticity, and this is believed to be a mechanism of Aβ-induced synaptic deficits and memory impairment (Lee et al., 2003; Parameshwaran et al., 2008; Guntupalli et al., 2016). Because monomeric Aβ does not show synaptotoxicity, oligomeric Aβ is believed to be the toxic species (Walsh et al., 2002; Ono et al., 2009; Sengupta et al., 2016). Therefore, if an agent could dissociate preformed oligomeric Aβ, it could negate the synaptotoxicity of oligomeric Aβ. In the present study, spinosin blocked Aβ-induced synaptic deficits. Because spinosin increased plasmin activity, which is believed to be involved in Aβ clearance, this is hypothesized to be the mechanism of action of spinosin on synaptic deficit.

We still do not know how spinosin regulates plasmin activity, as plasmin could be regulated by various mechanisms. Plasmin is produced by proteolytic cleavage of plasminogen (Vassalli et al., 1991), and various plasminogen activators, including tPA, are involved in this process (Sappino et al., 1993; Li et al., 2003). These plasminogen activators are regulated by neuroserpin and plasminogen activator inhibitor 1 (Vassalli et al., 1991; Krueger et al., 1997; Lebeurrier et al., 2005). However, plasmin may be inactivated by α2-antiplasmin, a serine protease inhibitor (Schaller and Gerber, 2011). Regulation of these molecules could control plasmin activity. Therefore, these molecules may be targets of spinosin. Further research is needed to answer these questions.

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