INTRODUCTION

Neurogenesis primarily operates in two specific areas of the adult brain: the subgranular zone of the hippocampal dentate gyrus (DG) and the subventricular zone of the lateral ventricles. Hippocampal neurogenesis is well known to be especially important for memory and learning. Aging and neurogenesis in the brain are negatively correlated; it has been suggested that neurogenesis in older adults is reduced by approximately 80% compared to young adults. Deficits in adult neurogenesis due to aging may compromise the structure and function of the entorhinal-hippocampal circuit. This area is particularly vulnerable and impacted in Alzheimer's disease (AD). In addition, many studies have reported that AD pathogenesis may be associated with dysfunctions in the neural stem cells (NSCs) in neurogenic niches, such as the DG of the hippocampus. Neurogenesis also declines from environmental factors affecting the brain.
factors including amyloid β (Aβ), and it has been reported that several cell signaling pathways involved in neurogenesis are decreased by Aβ, which has a primary role in the pathogenesis of AD.9

The phosphatidylinositol 3-kinase (PI3K) pathway is known to be a key enzyme in neuronal cell survival.9-12 It is the major intracellular signal pathway responsible for the transmission of anti-apoptotic components and the control of cell survival, and it is activated by several kinds of neuroprotective stimuli.13-15 This pathway also possesses an important role in the control of tau phosphorylation in AD and is highly involved in adult neurogenesis.16

Candesartan is an angiotensin II receptor antagonist used for the treatment of hypertension. Recent studies reported that it has potent antioxidant, anti-inflammatory and anti-apoptotic properties, possibly by inhibiting the production of the pro-inflammatory cytokines and suppression of the apoptotic signals.17,18 Taken together, these actions may provide candesartan with the ability to have potential neuroprotective effects against Aβ toxicity. Thus, we hypothesize that candesartan might contribute to the restoration of proliferative activity of NSCs injured by Aβ25–35 (Aβ25–35).

In this study, we investigated whether candesartan restores the Aβ25–35-inhibited proliferation of NSCs and the mechanisms by which it did so, with a special focus on the PI3K pathway.

**METHODS**

**Materials**

The Dulbecco's Modified Eagle's Medium (DMEM, high glucose) was purchased from GIBCO (Invitrogen Corporation, Grand Island, NY, USA). The Aβ25–35 proteinase inhibitor cocktail, trypan blue solution, insulin, and DNase I were obtained from Sigma-Aldrich (St. Louis, MO, USA). The candesartan was a generous gift from the Chong Kun Dang Pharmaceutical Corporation (Seoul, Korea). Before use, the candesartan was dissolved in methanol to 500 mM, diluted with DMSO to 100 mM, and then diluted with a culture medium to the desired concentrations.

**Cultures of neural stem cells and treatment**

All procedures using animals were consistent with the Han-yang University’s guidelines for the care and use of laboratory animals. We made every effort to minimize both the number of animals used and animal suffering. Each animal was utilized only once.

Embryonic brain tissue was dissected from the cortex, lateral ganglionic eminence (anlage of striatum), and ventral midbrain of embryonic day 12–14 (E12–E14) rats (Sprague-Dawley, KOATECK, Seoul, Korea).19 After mechanical trituration, 20,000 cells per cm² were plated in culture dishes that were pre-coated with poly-L-ornithine (PO)/fibronectin (FN) in N2B medium [DMEM/F12, 4.4 μM insulin, 100 mg/L transferrin, 30 nM selenite, 0.6 μM putrescine, 20 nM progesterone, 0.2 mM ascorbic acid, 2 mM L-glutamine, 8.6 mM D(+)-glucose, 20 mM NaHCO3, B27 (Invitrogen, Carlsbad, CA, USA)] that were then supplemented with a basic fibroblast growth factor (bFGF; 20 ng/mL, R&D Systems, Minneapolis, MN, USA) and an epidermal growth factor (EGF, 20 ng/mL, R&D Systems). They were cultured for 4–6 days as a monolayer on the adherent surface. To obtain a uniform population of NSCs, clusters of cells formed by the proliferation of NSCs with mitogens (bFGF and EGF) were passaged by dissociating them into single cells and plating them onto freshly PO/FN-coated coverslips (12-mm diameter; Marienfeld GmbH & Co. KG, Lauda-Knigshofen, Germany).20 All data in this study were obtained from passaged cultures that were grown on the adherent surface. The cultures were maintained at 37°C in a 5% CO2 incubator. The media were changed every other day, and mitogens were added daily.

To measure the effects of the Aβ25–35 oligomers on the proliferation of NSCs, the NSCs were simultaneously treated with several concentrations of Aβ25–35 oligomers (0, 2.5, 5, 10, 20, or 40 μM) for 48 hours. The soluble oligomeric forms of Aβ25–35 were prepared as reported by Dahlgren et al.21 Briefly, the Aβ25–35 was dissolved at a concentration of 1 mM in hexafluoroisopropanol (Sigma) and separated into aliquots in sterile microcentrifuge tubes. The hexafluoroisopropanol was removed with a vacuum via a Speed-Vac, and the peptide film was stored in a desiccated state at -20°C. To prepare the oligomers, the peptide was first suspended in dry dimethyl sulfoxide (Me2SO, Sigma) at a concentration of 5 mM, and Ham's F-12 (phenol red-free, BioSource, Camarillo, CA, USA) was added to bring the final concentration to 1 mM. This procedure was followed by 24 hours of incubation at 4°C. The plates were washed carefully more than three times with a phosphate-buffered saline (PBS), and cell viability was measured with MTT and trypan blue assays.

**MTT assay and trypan blue staining to measure cell viability**

MTT is absorbed into cells and transformed into formazan by mitochondrial succinate dehydrogenase. The accumulation of formazan directly reflects mitochondrial activity, which is an indirect measurement of cell viability. The cells were plated in 96-well plates at a density of 1×10⁴ cells/well in 200 μL of culture medium, and 50 μL of 2 mg/mL MTT
analysed by Western blotting. Briefly, 5(Ser9), and heat shock transcription factor-1 (HSTF-1) were for 1 hour, and the cell proliferation assay was performed using a plate reader. All results were normalized to OD values measured from an identically treated well without cultured cells. For trypan blue staining (TBS), 10 μL of trypan blue solution (BMS, Seoul, Korea) was incubated for 2 minutes with 10 μL of dissociated cells from each sample. Unstained live cells were counted on a hemocytometer.12

**BrdU cell proliferation assay**

After 48 hours of treatment, the cells were incubated in a bromodeoxyuridine (BrdU)-labeling medium (10 μM BrdU) for 1 hour, and the cell proliferation assay was performed using the BrdU Labeling and Detection Kit (Roche Boehringer–Mannheim, IN, USA), according to the manufacturer’s instructions. The results were calculated as the proportion of stained cells out of 1000 cells counted under a light microscope at ×400 magnification.

**Western blot analyses**

The levels of p85α PI3K, phosphorylated Akt (pAkt) (Ser473), phosphorylated glycogen synthase kinase-3β (pGSK-3β) (Ser9), and heat shock transcription factor-1 (HSTF-1) were analyzed by Western blotting. Briefly, 5×10^6 cells were washed twice in cold PBS and incubated in a lysis buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.2% sodium dodecyl sulfate (SDS), 100 μg/mL phenylmethylsulfonylfluoride (PMSF), 50 μg/mL aprotinin, 1% Igepal 630, 100 mM NaF, 0.5% sodium deoxycholate, 0.5 mM EDTA, and 0.1 mM EGTA] for 10 minutes on ice. The cell lysates were centrifuged at 10000 μg and evaluated for the levels of p85α PI3K, pAkt, pGSK-3β, and HSTF-1. The protein concentrations of the cell lysates and postmitochondrial fractions were determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Samples containing equal amounts (20 μg) of protein were resolved by a 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membranes were blocked using 5% skim milk before incubation with specific primary antibodies against p85α PI3K (1:1000, Sigma), pAkt (1:500, Cell Signaling, Beverly, MA, USA), pGSK-3β (Ser9) (1:1000, Santa Cruz Biotech, Santa Cruz, CA, USA), and HSTF-1 (1:1000, Santa Cruz Biotech). The membranes were washed with a Tris-buffered saline containing 0.05% Tween-20 (TBST) and then processed using an HRP-conjugated anti-rabbit or anti-mouse antibody (Amersham Pharmacia Biotech, Piscataway, NJ, USA) followed by ECL detection (Amersham Pharmacia Biotech).13 The results from the Western blots were quantified using an image analyzer (Quantity One-4,2,0, Bio-Rad).

**RESULTS**

**The viability and proliferation of NSCs damaged by Aβ25-35 oligomers**

To evaluate the effect of Aβ25-35 oligomers on NSC viability, the NSCs were treated with different concentrations of Aβ25-35 oligomers (0, 2.5, 5, 10, 20, or 40 μM) for 48 hours. Cell viability was measured with a MTT assay, and surviving cells were counted with TBS. The viability of the NSCs significantly decreased after treatment with 2.5 or 5 μM Aβ25-35 oligomer; however, the viability of the cells treated with concentrations greater than 10 μM significantly decreased in a concentration-dependent manner (Fig. 1).

The BrdU labeling demonstrated that the proliferation of NSCs treated with Aβ25-35 oligomers at concentrations greater than 5 μM significantly decreased in a concentration-dependent manner (Fig. 2). Based on these results, a 10 μM Aβ25-35 oligomer was selected as the optimal concentration for subsequent experiments because, at this concentration, cell viability only slightly decreased; yet, the proliferation of the NSCs significantly decreased (p<0.01 when compared with the non-treated group).

**The effects of candesartan on Aβ25-35 oligomer-inhibited proliferation of NSCs**

To investigate the effects of candesartan on Aβ25-35 oligomer-inhibited proliferation, the NSCs were treated with several concentrations of candesartan (0, 0.01, 0.1, 0.5, 1, 5, or 10 μM) and 10 μM Aβ25-35 Oligomer for 48 hours. Cell proliferation was assessed with BrdU labeling. Compared with only 10 μM Aβ25-35 oligomer-treated NSCs, the cells co-treated with candesartan had significantly higher cell proliferation rates (p<0.05). These results were concentration-dependent up to 10 μM candesartan (maximum 99±8% in the BrdU labeling cell proliferation assay, p<0.01) (Fig. 3). When a PI3K inhibitor (10 μM LY294002) was co-administered
with candesartan and the oligomer for 48 hours, the effect of candesartan on the proliferation of the NSCs was blocked (Fig. 4).

The mechanisms of candesartan restoration of Aβ25-35 oligomer-inhibited proliferation of NSCs

We ascertained the effects of candesartan on intracellular signaling proteins, which are critical for the proliferation of NSCs that are treated with Aβ25-35 oligomers. The levels of p85α PI3K, pAkt (Ser473), pGSK-3β (Ser9), and HSTF-1, which are included in the PI3K pathway that has pivotal roles in the proliferation of NSCs, were examined. Compared with untreated cells (100±10, 100±11, 100±9, and 100±8%, respectively), the immunoreactivities (IRs) of p85α PI3K, pAkt (Ser473), pGSK-3β (Ser9), and HSTF-1 significantly decreased in the 10 µM Aβ25-35 oligomer-treated cells (73±8, 45±9, 67±11, and 75±9%, respectively; p<0.01). However, this decrease was significantly attenuated by co-treatment with 1 µM candesartan (236±13, 172±10, 143±12, and 143±

Fig. 1. Viability of neural stem cells (NSCs) injured by the Aβ25-35 oligomer. The data are means (% of control)±standard deviations from five independent experiments. Treatment groups were compared with the control group using a Tukey’s test after a one-way analysis of variance. The MTT assay and trypan blue staining (TBS) exhibit that the viability of NSCs decreased in a concentration-dependent manner when the cells were treated with more than 10 µM Aβ25-35 oligomer. *p<0.05 and †p<0.01 (vs. control group).

Fig. 2. Proliferation of neural stem cells (NSCs) injured by the Aβ25-35 oligomer. The data are means (% of control)±standard deviations from five independent experiments. The treatment groups were compared with the control group using a Tukey’s test after a one-way analysis of variance. The BrdU assay displayed that the proliferation of NSCs decreased in a concentration-dependent manner when the cells were treated with more than 5 µM Aβ25-35 oligomer. *p<0.05 and †p<0.01 (vs. control group).

Fig. 3. Effect of candesartan on the proliferation of neural stem cells (NSCs) injured by Aβ25-35 oligomer. The data are means (% of control)±standard deviations from five independent experiments. The treatment groups were compared with the control group using a Tukey’s test after a one-way analysis of variance. The BrdU assay displayed that treatment with candesartan restored the proliferation of NSCs inhibited by 10 µM Aβ25-35 oligomers. *p<0.05 and †p<0.01 (vs. control group), ‡p<0.01 and §p<0.01 (vs. the group treated with only 10 µM Aβ25-35 oligomer).

Fig. 4. Role of PI3K activation in the effect of candesartan on the proliferation of neural stem cells (NSCs) injured by Aβ25-35 oligomer. The data are means (% of control)±standard deviations from five independent experiments. The treatment groups were compared with the control group using a Tukey’s test after a one-way analysis of variance. The BrdU assay displayed that co-treatment with LY294002 almost completely blocked the effect of candesartan on the proliferation of NSCs inhibited by 10 µM Aβ25-35 oligomer. *p<0.05 (vs. control group), ‡p<0.05 (vs. the group without the treatment of 10 µM LY294002).
Fig. 5. Effect of candesartan on the proliferation-related intracellular signaling proteins of neural stem cells (NSCs) injured by Aβ25-35 oligomer. The data are means (% of control) ± standard deviations from five independent experiments. The treatment groups were compared with the control group using a Tukey’s test after a one-way analysis of variance. Western blotting demonstrated that treatment with candesartan significantly enhanced the expression of the proliferation-related intracellular signaling proteins of NSCs inhibited by 10 µM Aβ25-35 oligomer. *p<0.05 and †p<0.01 (vs. control group), ‡p<0.01 and §p<0.01 (vs. the group treated with only 10 µM Aβ25-35 oligomer).
10% at 1 µM candesartan, respectively, p<0.01) (Fig. 5). However, these effects were slightly lessened if the cells were co-treated with 10 µM of candesartan (160±7, 139±11, 128±8, and 127±8%, respectively, p<0.01 or 0.05).

**DISCUSSION**

Aβ is considered to be one of the most important pathological mechanisms to induce the development of AD and has been reported to affect mitochondrial function, NMDAR endocytosis, excessive calcium influx, tau hyperphosphorylation, synaptic dysfunction, neuronal stress, and apoptosis.22-25 In addition, several studies suggested that Aβ had a strong correlation with oxidative stress.26,27 Recent studies have reported that candesartan has potent antioxidant properties that may ameliorate oxidative stress.28,29 Candesartan has been proven to decrease ROS production and enhance the activity of the antioxidant enzymes and lipid peroxidation. Moreover, Nishida et al.30 reported that candesartan may improve renal functions, possibly through its effect on lipid peroxidation. Also, Luo et al.31 suggested that treatment with antioxidant agents that affect the rennin-angiotensin system (RAS) may resolve the imbalance of renal RAS created by oxidative stress. This suggestion is in agreement with the present study where candesartan induced a significant increase in tissue CAT and GR compared to the carboplatin group. In the present study, the NSC viability significantly decreased with treatment of more than 10 µM oligomeric Aβ25-35 compared to the untreated cells (Fig. 1). NSC proliferation also decreased in a concentration-dependent manner, especially when the NSCs were treated with more than 5 µM oligomeric Aβ25-35 (Fig. 2). The combined treatment with candesartan at concentrations ranging from 1 µM to 10 µM for 48 hours increased the proliferation of NSCs injured by 10 µM Aβ25-35 oligomer in a concentration-dependent manner (Fig. 3), which suggests that candesartan treatment restores the Aβ oligomer-inhibited proliferation of NSCs. A PI3K inhibitor (10 µM LY294002) blocked the effect of candesartan on the proliferation of NSCs (Fig. 4), indicating that the effect of candesartan on the proliferation of NSCs is mediated by the activation of the PI3K pathway. This finding and the fact that the PI3K pathway possesses an important role in the proliferation of NSCs led us to hypothesize that candesartan activation of the PI3K pathway restores the Aβ oligomer-inhibited proliferation of NSCs, which we were able to confirm.

In the first step of the PI3K pathway, the activated PI3K phosphorylates its downstream target, Akt and GSK-3β. The pAkt also inhibits GSK-3β by phosphorylating it at Ser932. While the active form of GSK-3β inhibits HSTF-1 and activates the mitochondrial death pathway,32,33 inactivation of the pGSK-3β by pAkt is important for functioning in a variety of stem cell types.24 It was reported that the PI3K pathway is essential for the self-renewal of embryonic stem cells (ESCs) in a mouse,25 and the activation of this pathway is important for maintaining pluripotency in mice and primate ESCs.26 Several studies demonstrated that the phosphatase and tensin homolog deleted on chromosome 10 (PTEN), an antagonist of PI3K, negatively regulates the NSCs proliferation, survival, and self-renewal both in vivo and in vitro.37-41 Contrary to PTEN, the components of the PI3K pathway were reported to be involved in the self-renewal of NSCs.42 We demonstrated that candesartan affected the PI3K pathway in NSCs and treatment with 10 µM Aβ25-35 oligomer decreased the IRs of survival proteins such as p85α PI3K, pAkt (Ser473), pGSK-3β (Ser9), and HSTF-1 (Fig. 5). However, candesartan co-treatment significantly increased the expression of these survival proteins (Fig. 5). These findings also provide support for our hypothesis that the effects of candesartan on the proliferative activity of NSCs are mediated by the PI3K pathway.

There are some limitations to this study. First, this study was performed under in vitro conditions; therefore, the results could be different under more complex in vivo conditions. Second, the micromolar dose of candesartan that we used in our experiments is not physiologically relevant. To overcome this limitation, chronic treatment with lower concentrations of candesartan might be more similar to in vivo conditions; although, it is not feasible in an in vitro study. Third, we did not carry out a mechanistic study to confirm the precise target of candesartan. Its exact mechanism of action should be delineated in further studies. Last, AD pathogenesis is associated with adult hippocampal NSCs; however, embryonic cortical NSCs were used in this study due to its availability. Conflicts of Interest

The authors have no financial conflicts of interest.

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