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ORIGINAL ARTICLE

Candesartan Restores the Amyloid Beta-Inhibited Proliferation of Neural Stem Cells by Activating the Phosphatidylinositol 3-Kinase Pathway

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Background and Purpose Neurogenesis in the adult brain is important for memory and learning, and the alterations in neural stem cells (NSCs) may be an important aspect of Alzheimer's disease (AD) pathogenesis. The phosphatidylinositol 3-kinase (PI3K) pathway has been suggested to have an important role in neuronal cell survival and is highly involved in adult neurogenesis. Candesartan is an angiotensin II receptor antagonist used for the treatment of hypertension and several studies have reported that it also has some neuroprotective effects. We investigated whether candesartan could restore the amyloid-β(25−35) ($A\beta_{25-35}$) oligomer-inhibited proliferation of NSCs by focusing on the PI3K pathway.

Methods To evaluate the effects of candesartan on the $A\beta_{25-35}$ oligomer-inhibited proliferation of NSCs, the NSCs were treated with several concentrations of candesartan and/or $A\beta_{25-35}$ oligomers, and MTT assay and trypan blue staining were performed. To evaluate the effect of candesartan on the Aβ-inhibited proliferation of NSCs, we performed a bromodeoxyuridine (BrdU) labeling assay. The levels of p85α PI3K, phosphorylated Akt (pAkt) (Ser473), phosphorylated glycogen sinthase kinase-3β (pGSK-3β) (Ser9), and heat shock transcription factor-1 (HSTF-1) were analyzed by Western blotting.

Results The BrdU assays demonstrated that NSC proliferation decreased with $A\beta_{25-35}$ oligomer treatment; however, a combined treatment with candesartan restored it. Western blotting displayed that candesartan treatment increased the expression levels of p85α PI3K, pAkt (Ser473), pGSK-3β (Ser9), and HSTF. The NSCs were pretreated with a PI3K inhibitor, LY294002; the effects of candesartan on the proliferation of NSCs inhibited by $A\beta_{25-35}$ oligomers were almost completely blocked.

Conclusions Together, these results suggest that candesartan restores the $A\beta_{25-35}$ oligomer-inhibited proliferation of NSCs by activating the PI3K pathway.

Key Words candesartan, amyloid beta, neural stem cells.

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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.[1,2] Aging and neurogenesis in the brain are negatively correlated[3,4]; it has been suggested that neurogenesis in older adults is reduced by approximately 80% compared to young adults.[5] 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.[6,7] Neurogenesis also declines from environmental

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.[8]

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 Aβ(25−35) ($A\beta_{25-35}$).

In this study, we investigated whether candesartan restores the $A\beta_{25-35}$-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 $A\beta_{25-35}$, protein protease 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 methyl chloride 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 Hanyang 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 cm^2 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-Knlgshofen, 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% CO_2 incubator. The media were changed every other day, and mitogens were added daily.

To measure the effects of the $A\beta_{25-35}$ oligomers on the proliferation of NSCs, the NSCs were simultaneously treated with several concentrations of $A\beta_{25-35}$ oligomers (0, 2.5, 5, 10, 20, or 40 μM) for 48 hours. The soluble oligomeric forms of $A\beta_{25-35}$ were prepared as reported by Dahlgren et al.[21] Briefly, the $A\beta_{25-35}$ 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 (Me_2SO, 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 1×10^4 cells/well in 200 μL of culture medium, and 50 μL of 2 mg/mL MTT

(Sigma) was added to each well. An aliquot (220 μL) of the resulting solution was removed from each well, followed by the addition of 150 μL dimethyl sulfoxide. The precipitate from each well was re-suspended on a microplate mixer for 10 minutes, and the optical densities (OD) at 540 nm were measured 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 μL/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).[11] The results from the Western blots were quantified using an image analyzer (Quantity One-4,2,0, Bio-Rad).

Statistical analysis

All data are presented as means±standard deviations from five or more independent experiments. The statistical comparisons of viability among the different treatment groups were performed with a Tukey's test after a one-way analysis of variance. *p*-values of less than 0.05 were considered statistically significant.

RESULTS

The viability and proliferation of NSCs damaged by $A\beta_{25\text{-}35}$ oligomers

To evaluate the effect of $A\beta_{25\text{-}35}$ oligomers on NSC viability, the NSCs were treated with different concentrations of $A\beta_{25\text{-}35}$ 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 $A\beta_{25\text{-}35}$ 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 $A\beta_{25\text{-}35}$ oligomers at concentrations greater than 5 μM significantly decreased in a concentration-dependent manner (Fig. 2). Based on these results, a 10 μM $A\beta_{25\text{-}35}$ 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 $A\beta_{25\text{-}35}$ oligomer-inhibited proliferation of NSCs

To investigate the effects of candesartan on $A\beta_{25\text{-}35}$ 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 $A\beta_{25\text{-}35}$ oligomer for 48 hours. Cell proliferation was assessed with BrdU labeling. Compared with only 10 μM $A\beta_{25\text{-}35}$ 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

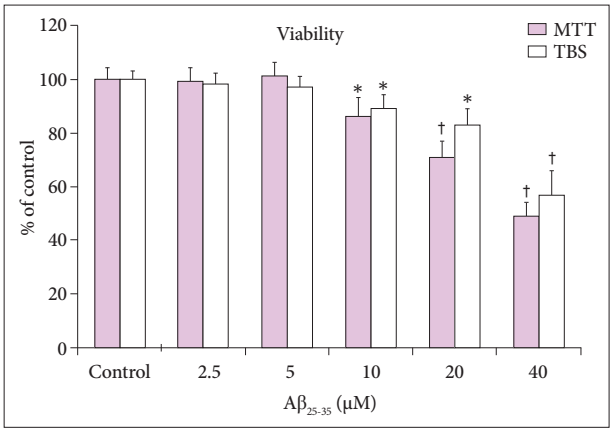

Fig. 1. Viability of neural stem cells (NSCs) injured by the $A\beta_{25\text{-}35}$ oligomer. The data are means (% of control)±standard deviations from five independent experiments. Treatment groups were compared with the control group using a Tukey's test after a one-way analysis of variance. The 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 $A\beta_{25\text{-}35}$ oligomer. *p<0.05 and †p<0.01 (vs. control group).

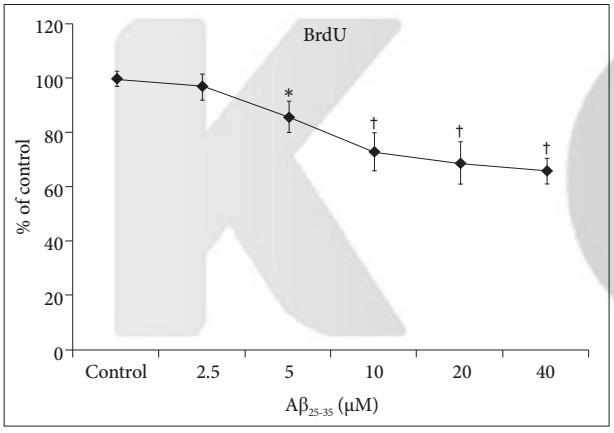

Fig. 2. Proliferation of neural stem cells (NSCs) injured by the $A\beta_{25\text{-}35}$ oligomer. The data are means (% of control)±standard deviations from five independent experiments. The treatment groups were compared with the control group using a Tukey's test after a one-way analysis of variance. 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 $A\beta_{25\text{-}35}$ oligomer. *p<0.05 and †p<0.01 (vs. control group).

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 $A\beta_{25\text{-}35}$ 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 $A\beta_{25\text{-}35}$ oligomers. The levels of p85α PI3K, pAkt (Ser473), pGSK-3β (Ser9), and HSTF-1, which are included in the PI3K pathway that has pivotal

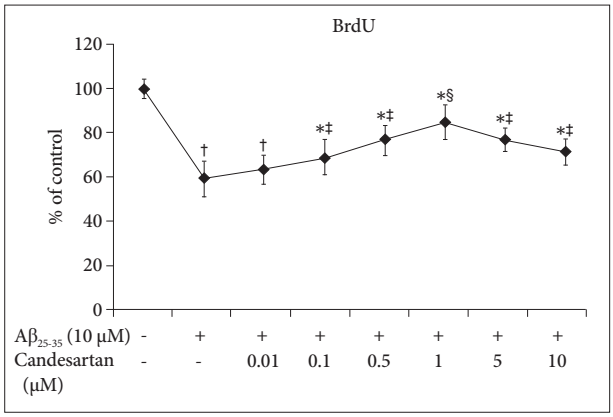

Fig. 3. Effect of candesartan on the proliferation of neural stem cells (NSCs) injured by $A\beta_{25\text{-}35}$ oligomer. The data are means (% of control)±standard deviations from five independent experiments. The treatment groups were compared with the control group using a Tukey's test after a one-way analysis of variance. The BrdU assay displayed that treatment with candesartan restored the proliferation of NSCs inhibited by 10 μM $A\beta_{25\text{-}35}$ 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 $A\beta_{25\text{-}35}$ oligomer).

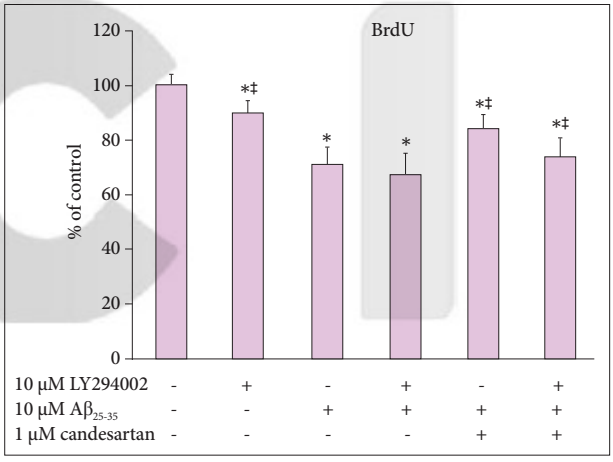

Fig. 4. Role of PI3K activation in the effect of candesartan on the proliferation of neural stem cells (NSCs) injured by $A\beta_{25\text{-}35}$ oligomer. The data are means (% of control)±standard deviations from five independent experiments. The treatment groups were compared with the control group 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 $A\beta_{25\text{-}35}$ oligomer. *p<0.05 (vs. control group), ‡p<0.05 (vs. the group without the treatment of 10 μM LY294002).

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 $A\beta_{25\text{-}35}$ 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±

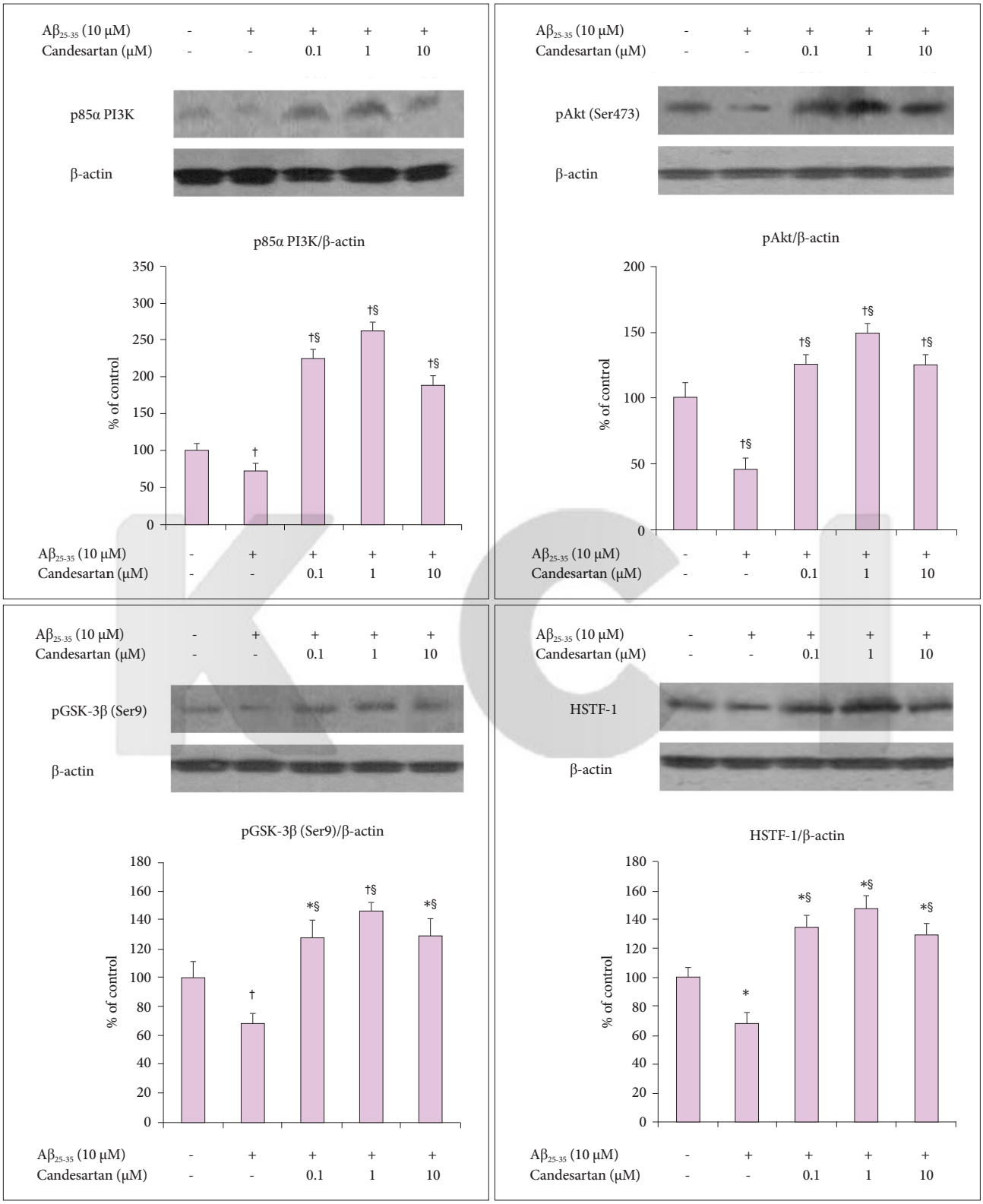

Fig. 5. Effect of candesartan on the proliferation-related intracellular signaling proteins of neural stem cells (NSCs) injured by $A\beta_{25-35}$ oligomer. The data are means (% of control)±standard deviations from five independent experiments. The treatment groups were compared with the control group 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 $A\beta_{25-35}$ 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 $A\beta_{25-35}$ 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, neuronalstress, 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 $A\beta_{25-35}$ 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 $A\beta_{25-35}$ (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 $A\beta_{25-35}$ oligomer in a concentration-dependent manner (Fig. 3), which suggests that candesartan treatment restores the $A\beta_{25-35}$ 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 $A\beta_{25-35}$ 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.[34] It was reported that the PI3K pathway is essential for the self-renewal of embryonic stem cells (ESCs) in a mouse,[35] and the activation of this pathway is important for maintaining pluripotency in mice and primate ESCs.[36] 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 $A\beta_{25-35}$ 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 for the *in vitro* study. To overcome this problem, an *in vitro* study using AD model mice needs to be performed in future studies.

Taken together, the results of this study indicate that candesartan restores the $A\beta_{25-35}$ oligomer-inhibited proliferation of NSCs by activating the PI3K pathway and can be applied as potential NSC-based therapies for AD.

Conflicts of Interest

The authors have no financial conflicts of interest.

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