

Original Research

Streptozotocin induces neurite outgrowth via PI3K-Akt and glycogen synthase kinase 3 β in Neuro2a cells

T. Nishimoto^{1*}, R. Kimura², A. Matsumoto³, H. Sugimoto⁴

¹ Department of Immunology, Kawasaki Medical School, Okayama, 701-0192, Japan

² Arts and Sciences, Faculty of Engineering, Tokyo University of Science, Yamaguchi, 756-0884, Japan

³ Faculty of Life and Medical Sciences, Doshisha University, Kyoto, 610-0394, Japan

⁴ Nervous Disease Research Center, Graduate School of Brain Science, Doshisha University, Kyoto, 610-0394, Japan

Abstract: Streptozotocin (STZ), a naturally occurring chemical, is toxic to the various kinds of cells such as insulin-producing beta cells. However, the beneficial effect of STZ on neuronal cells such as neurite outgrowth-inducing activity has been unknown. In this study, we examined the effect of STZ on neurite outgrowth in mouse neuronal Neuro2a cells. STZ (0.01 mM~5 mM) exerted remarkable neurite outgrowth-inducing activity in Neuro2a cells in a concentration dependent manner. STZ also had the same neurite outgrowth-inducing activity as that of retinoic acid (RA), which is well known neurite outgrowth inducer. As with the result of RA treatment, STZ administration increased MAP2-positive cells. The MAP2-positive cells reflect neurite outgrowth-induced cells. STZ (0.01 mM~5 mM) did not induce cell death, but significantly decreased cell proliferation. The serine/threonine kinase Akt, a downstream target of phosphatidylinositol-3 kinase (PI3K), was transiently phosphorylated at Ser473 and at Thr303 by STZ (5 mM) administration. Glycogen synthase kinase 3 β (GSK3 β), which has been reported to be inactivated by Akt, was also transiently phosphorylated at Ser9 by STZ (5 mM) administration. In addition, a blocker of PI3K, LY294002 (10 μ M), significantly attenuated STZ-induced neurite outgrowth. These results suggest that STZ induces neurite outgrowth via activation of PI3K-Akt signaling pathway and GSK3 β inhibition.

Key words: Streptozotocin, PI3K, Akt, glycogen synthase kinase 3 β , neurite outgrowth.

Introduction

Neuronal differentiation is an essential for neuronal development of nervous system and for functional networks of neurons during development as well as neuronal plasticity (1, 2). Neurite outgrowth is thought to be a typical marker of neuronal differentiation. As developing neurons that do not make correct synaptic connections lead to neuronal cell death and neuronal dysfunction, regulation of neurite outgrowth is therapeutically important for promotion of neuronal regeneration from nerve injury or neuronal disorders (3).

The serine/threonine kinase Akt, also known as protein kinase B, is a signaling kinase downstream of phosphoinositide-3-kinase (PI3K) (4). The PI3K-Akt signaling pathway is a critical transducer for several major survival signals in central nervous system neurons (5). Indeed, Kihara *et al.* have previously reported that nicotinic acetylcholine receptor stimulation protects neurons against glutamate excitotoxicity through the PI3K-Akt system (6). Also, we have demonstrated that prestimulation of the glutamate receptor protects neurons from excess glutamate-induced excitotoxicity (7) via activation of Akt. The PI3K-Akt signaling pathway also plays a key role in the neuronal outgrowth and differentiation induced by several kinds of neurotrophins such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (NT3) (1). These neurotrophins bind to Trk receptor tyrosine kinases, which then activate PI3K-Akt signal transduction (8). In adult dorsal root ganglion neurons, for example, PI3K-Akt is involved in NGF-induced neurite outgrowth through the NGF-specific receptor TrkA (9).

The drug streptozotocin (STZ) is a chemical that is

particularly toxic to the insulin-producing beta cells of the pancreas in mammals. It is used in medical research to produce an animal model for type I diabetes (10). Also, STZ administration in rat brain by intracerebroventricular (*i.c.v.*) route induces memory deficit (11). However, so far, the beneficial effect of STZ on neuronal cells is not well known.

Glycogen synthase kinase 3 β (GSK3 β), which is highly expressed in brain tissue and is one of the downstream key targets of the Akt, is multifunctional serine/threonine kinase. GSK3 β regulates diverse biological processes including glycogen metabolism, insulin signaling and cell cycle/survival regulation. The activity of GSK3 β is negatively regulated by phosphorylation at N-terminal serine 9 (12).

It has been reported that PI3K-Akt-GSK3 β pathway regulate the early stages of dendrite formation in hippocampal neurons (13). Also, inhibition of Akt-GSK3 β signaling pathway in PC12 cells blocks neurite length and frequency (14). Other report shows that Akt activation and subsequent GSK3 β inactivation induces neurite outgrowth in Neuro2a cells (15). These reports suggest that regulation of GSK3 β plays crucial roles in neuronal differentiation and development.

More recent study shows that after intracerebroventricular administration (*i.c.v.*) of STZ has been shown to

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* **Corresponding author:** Takaaki Nishimoto, Ph.D., Department of Immunology, Kawasaki Medical School, Okayama, 701-0192, Japan. Email: t-nishi12@med.kawasaki-m.ac.jp

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alter in Akt and GSK3 β activity (16).

In this report, we studied the beneficial effect of STZ on neuronal cells and showed STZ causes neurite outgrowth. To our knowledge, this is the first report that STZ have the neurotrophic effect involving neurite outgrowth.

Materials and Methods

Materials

The sources of drugs and materials were as follows: Dulbecco's modified Eagle's medium (DMEM) and Fetal Bovine Serum (Invitrogen, Carlsbad, CA, USA); anti-phospho-GSK3 β (Ser9) antibody, anti-GSK3 β antibody, anti-phospho-Akt (Ser 473) antibody, and anti-Akt antibody (Cell Signaling Technology, Inc, Beverly, MA, USA); Hoechst33342 and STZ (Sigma-Aldrich, St. Louis, MO, USA).

Cell culture

Murine neuroblastoma Neuro2a cells were purchased from American Type Culture Collection (Rockville, MD, USA). Cells were cultured in DMEM supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100 μ g/ml) at 37 °C in a humidified atmosphere containing 5 % CO₂.

Quantification of neurite outgrowth

Twenty-four hours after plating, the cells were treated for 48 h with or without STZ in serum-free DMEM. Neurite outgrowth was estimated under phase-contrast microscopy and was quantified as the percentage of cells bearing neurite processes with lengths equivalent to one or more diameters of a cell body. In each independent experiment, at least 300 cells were scored for each condition.

Preparations of cell extract

After each treatment, cells were lysed in a buffer consisting of 20 mM Tris HCl, pH 7.0, 2 mM EGTA, 25 mM 2-glycerophosphate, 1% Triton X-100, 2 mM dithiothreitol, 1 mM vanadate, 1 mM phenylmethylsulfonyl fluoride, and 1% aprotinin and centrifuged at 12,000 rpm for 30 min at 4 °C. The supernatants were used as the cell extracts for immunoblot analysis.

Immunoblotting

SDS-solubilized samples were loaded onto SDS-polyacrylamide gels. After electrophoresis, proteins were electrotransferred to a polyvinylidene difluoride membrane. Nonspecific binding sites were blocked with Western Blot Blocking Buffer (Takara Bio Inc, Shiga, Japan) for 1 hr at room temperature. The blots were probed with a rabbit polyclonal anti-phospho-Akt (diluted 1:1000), rabbit polyclonal anti-Akt antibody (diluted 1:1000), rabbit polyclonal anti-phospho-GSK3 β (Ser9) antibody (diluted 1:1000), rabbit polyclonal anti-GSK3 β antibody (diluted 1:1000) overnight at 4 °C. Subsequently, membranes were incubated with horseradish peroxidase-conjugated (HRP-conjugated) anti-rabbit or HRP-conjugated anti-mouse antibody. The immunoreactive bands were detected with an enhanced chemiluminescence detection kit (Millipore, Billerica,

MA, U.S.A.).

Measurement of lactate dehydrogenase activity

Lactate dehydrogenase (LDH) activity was spectrophotometrically measured using a MTX-LDH assay kit (Kyokuto Pharmaceutical Industrial, Tokyo, Japan) according to the manufacturer's instructions. Briefly, 50 μ l of culture supernatant was mixed with 50 μ l of the LDH substrate mixture in a 96-well plate. After incubation for 45 min at room temperature, the reaction was stopped by addition of 100 μ l of 1 N HCl and the absorbance at 570 nm was determined using a microplate reader (Model 680; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Total LDH activity was defined as the sum of intracellular and extracellular LDH activities obtained by 0.2 % Tween 20 treatment, which killed all cells in our culture (data not shown), and each released LDH was defined as the percentage of extracellular LDH compared with the total LDH activity.

Quantification of cell death

To determine the number of viable cells the trypan blue exclusion test was performed. After STZ treatment, the cell suspension was mixed with trypan blue and then visually examined to count the number of dead cells. Stained cells were scored as dead cells. Cell viability was represented as % of total cells. In each independent experiment, at least 200 cells were scored for each condition.

Determination of cell growth

The effect of STZ on the rate of Neuro2a cells growth was estimated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, after each treatment, cells were incubated at 37 °C for 30 min with media containing 0.5 mg/ml MTT. After incubation, the MTT solution was replaced with 2-propanol (200 μ l) and the cells were submitted to 1 min of shaking. The solution was transferred to a 96 well plate. The absorbance of samples at 570 nm was measured spectrophotometrically using a microplate reader (Model 680; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis

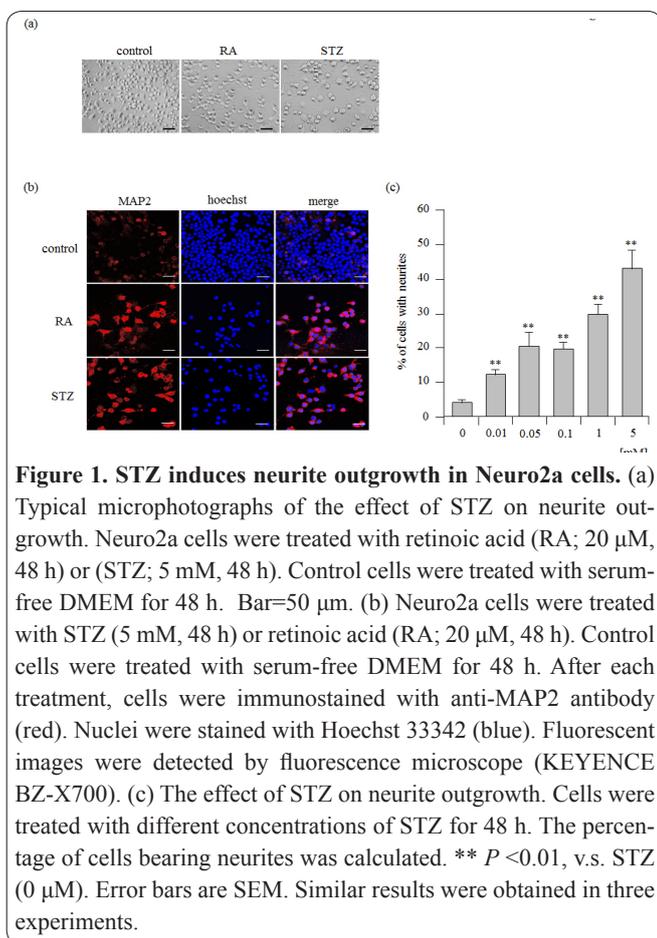
Statistical significance of the differences between groups was determined by Student's *t*-test or one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests.

Results

STZ induces neuronal outgrowth

Neuro2a is a mouse neural crest-derived cell line that has often been used to study neural differentiation, neurite outgrowth (15, 17, 18). Therefore, in the present study, in order to study the effect of STZ on neurite outgrowth and its molecular mechanism, we used Neuro2a cells.

To investigate whether STZ induces neurite outgrowth in Neuro2a cells, the morphological changes after STZ administration were investigated. Control cells showed round shape without neurite extension. On the other hand, STZ (5 mM, 48 h) increased the number of



cells with neurites under the phase contrast microscopy compared to control cells (Figure 1 a). STZ treated cells exerted the same neurite outgrowth activity as that of RA (20 μ M, 48 h) treated cells. To further confirm whether STZ induced neurite outgrowth in Neuro2a cells, we assessed the expression of MAP2 protein, which is the marker of neurite outgrowth. As with the result of RA (20 μ M, 48 h) administration, the cells treated with STZ (5 mM, 48 h) increased the number of MAP2 positive cells compared to control cells (Figure 1 b). Moreover, quantitative examination revealed that STZ significantly increased the outgrowth of neurites in a concentration dependent manner (Figure 1 c). These results indicate that STZ induced neuronal differentiation of Neuro2a cells, resulting in neurite outgrowth.

STZ stimulates cell proliferation rather than inducing cell death

Next, we investigated the effect of STZ on cell toxicity in Neuro2a cells. As shown in Figure 2a, STZ (0.01 mM~5 mM, 48 h) did not induce significant increase of LDH release from the cells compared to control cells. However, treatment with STZ (10 mM) to the cells increased LDH release significantly. Also, trypan exclusion test showed that STZ did not exert cytotoxicity in Neuro2a cells, and as with the result of LDH assay, STZ (10 mM) showed significant cell death (Figure 2b). To study the effect of STZ on cell proliferation in Neuro2a cells, MTT assay was performed. As shown in Figure 2c, STZ significantly reduced cell growth compared to control cells in Neuro2a cells. This STZ-induced reduction of cell growth might reflect that STZ-treated cells increase their proliferative capacity. These results suggest that STZ induces cell proliferation rather than indu-

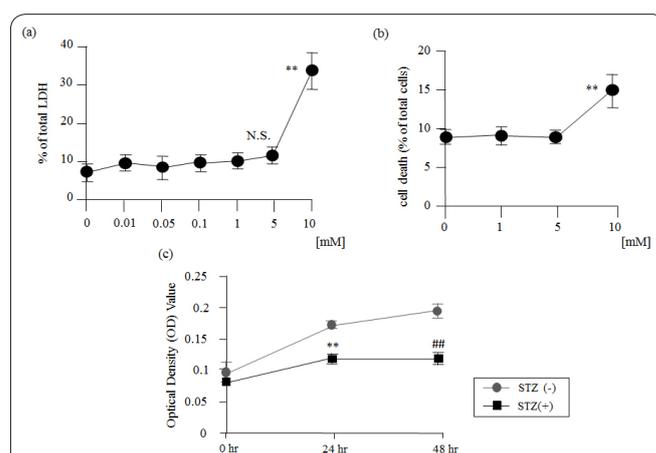
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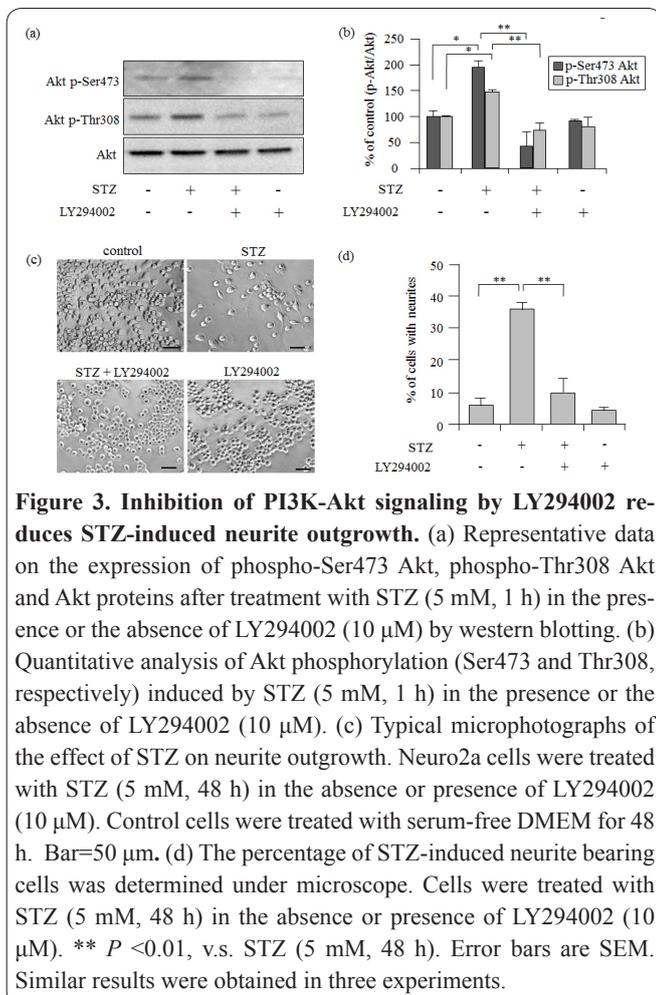
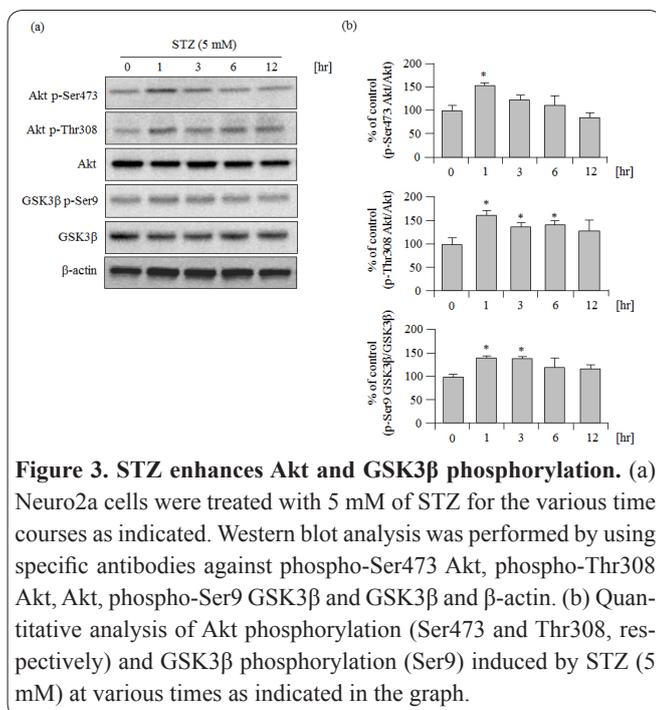
STZ enhances phosphorylation of Akt and GSK3 β

It has been reported that Akt and GSK3 β are important regulator of neurite outgrowth (1, 19). We hypothesized that STZ-induced neurite outgrowth might be mediated by Akt and GSK3 β . We investigated the expression levels of the phosphorylated forms of Akt and GSK3 β by immunoblotting. As shown in Figure 3a, b, STZ (5 mM) administration to Neuro2a cells resulted in an increase in the phosphorylation of Akt at Ser473, which peaked at 1 hr of exposure and returned to the basal level in 3 hrs. STZ also increased the persistent Akt phosphorylation at Thr308 up to 6 hrs. Phosphorylation of Akt at both Ser473 and Thr308 are well known to activate Akt activity. STZ enhanced phosphorylation of GSK3 β at Ser9, which peaked at 1 hr of exposure and returned to the basal level in 6 hrs (Figure 3 a, b). The activity of GSK3 β is negatively regulated by phosphorylation at Ser9. These results indicate that STZ increases Akt activity and decreases GSK3 β activity.

STZ induces neurite outgrowth through PI3K-Akt signaling pathway

Akt is the key downstream effector of PI3K, and PI3K is the critical mediator of neurite outgrowth (20). To identify the effect of PI3K on STZ-induced neurite outgrowth, we further investigated whether LY294002 (10 μ M, 1 h) decrease STZ-induced phosphorylation at Ser473 and Thr308 of Akt. As shown in Figure 4 a, b, LY294002 inhibited STZ-induced phosphorylation of Ser473 and Thr308 of Akt. Furthermore, we showed LY294002 significantly inhibited STZ-induced neurite outgrowth in Neuro2a cells (Figure 4 c, d). These results clearly indicate that STZ induces neurite outgrowth via PI3K-Akt signaling pathway.





Discussion

In the present study, we elucidated the new beneficial aspects of STZ on neuronal cells. To the best of our knowledge, no previous studies have been shown the beneficial effect of STZ on neuronal cells. Thus, the present study is shed light on the new beneficial nature of STZ.

Up to date, many reports show that the various

kinds of toxic effects of STZ on both *in vitro* and *in vivo* models. For example, STZ induces pancreatic beta cell death through transport of STZ into beta cells via GLUT2 transporter (21). Thus, STZ-induced diabetic animal models are useful platforms for the understanding of β cell glucotoxicity in diabetes. STZ also induces impairment of brain energy metabolism and oxidative damage and leads to cognitive dysfunction (22). These reports indicate STZ has just toxic effects on animals. So far, the beneficial effect of STZ on neuronal cells is not well known.

The discrepancy between the previous reports that indicates the toxic effect of STZ and our present report might be result from differences of the number of cell passage times or the timing of STZ administration to the cells. Indeed, we used only Neuro2a cells which are relatively few passage times (less than 10 passages).

Unlike our present results, Sakaue *et al* reported that neurite outgrowth was retarded in STZ induced diabetes rats (23). As they use isolated dorsal root ganglion (DRG) neurons in STZ-induced diabetic rats, the difference between our results and their results might be due to the kind of the cell type.

There are many reports that PI3K-Akt signaling pathways are critical mediator for neurite outgrowth (1). Our present study demonstrated that STZ increased phosphorylation at Ser473 and Thr308 residue of Akt. We also showed that a selective inhibitor of the PI3K pathway, LY294002, completely blocked STZ-induced neurite outgrowth. These results are consistent with a previous report suggesting that PI3K-Akt signaling pathways play a crucial mediator for neurite outgrowth.

The present result also clearly indicated that STZ increased phosphorylation at Ser9 of GSK3 β . This finding is consistent with a previous report that inhibition of GSK3 β activity by retinoic acid and lithium promoted neurite outgrowth in Neuro2a cells (19).

In conclusion, we have demonstrated for the first time that STZ induces neurite outgrowth through the regulation of PI3K-Akt and GSK3 β activities in Neuro2a cells. In the present data, STZ might be useful for developing pharmacological drug to promote neuronal regeneration and/or useful tool for understanding the mechanism of neuronal regeneration in neurodegenerative diseases.

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