Redox-dependent regulation of neurite outgrowth induced by staurosporine and Y-27632 in PC12 cells

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This certifies that the Master's Thesis
of Kim Du Sik is approved.

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‘감사의 글’을 읽는 모든 지인들이 한 모금의 미소를 지을 수 있기를 바라며...

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Redox-dependent regulation of neurite outgrowth induced by staurosporine and Y-27632 in PC12 cells

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The cellular redox state is thought to play an important role in a wide range of cellular signaling pathways. In this study, I investigated the regulatory roles of ROS in staurosporine (STS)- and Y-27632-induced neurite outgrowth, which is a major hallmark of the differentiation phenotype, in PC12 cells. In comparison to NGF-induced neurite outgrowth, STS, a broad spectrum inhibitor of protein kinases, or Y-27632, a Rho-associated kinase inhibitor rapidly induced neurite outgrowth within 1 h. Recent studies indicate that Rho and Rac

have opposite functions in neuronal differentiation. In agreement with this, it was shown that inactivation of Rho downstream by treatment STS and Y-27632 had an effect on Rac1 activity in PC12 cells within 30 min. In addition, the expression of dominant negative Rac1N17 blocked morphological differentiation caused by STS and Y-27632. To investigate the role of Rac1-induced ROS production in STS- and Y-27632-induced neurite outgrowth, N-acetylcysteine (NAC), an antioxidant, was used. Pretreatment with NAC effectively suppressed 100 nM STS- and 100 μM Y-27632- induced neurite outgrowth. Also, Diphenylen-iodonium (DPI), which inhibits a flavo protein such as NADPH oxidase, showed the similar inhibitory effect on the neurite outgrowth to NAC. Next, H_2O_2 (100 μM) in the presence of 10nM STS or 10 μM Y-27632 (lower concentrations than those inducing neurites) was treated, to examine whether it facilitated the neurite outgrowth. The co-treatment notably stimulated the rapid neurite outgrowth. Using DCF-DA, the increase of ROS production by Y-27632 and STS was also detected. In addition, constitutively active form of Rac1, Rac1V12, increased the intracellular ROS level. These results implicate that STS and Y-27632 stumulate the generation of an oxygen radical, perhapse H_2O_2, via Rac1 activation, which is required for STS- and Y-27632-induced morphological changes. However, treatment of cells only with H_2O_2 did not cause the neurite outgrowth. In

addition, only overexpression of wild type Rac1 and CA-Rac1V12 in PC12 cells could not induce neurites from the cells, indicating that the activation of Rac1 and ROS production are not sufficient for inducing neurite outgrowth in PC12 cells. Therefore, the Rac1 localization upon STS and Y-27632 treatment was examined in PC12 cells. Using immunocytochemistry, the appropriate relocalization of Rac1 at the end tips of neurite was observed after the treatment of STS and Y-27632. Newly formed actin filaments which are required for the morphological changes were also located in the end tips of neurites with Rac1. In conclusion, these results suggest that both the activation of Rac1 at the tips of neurites and Rac1-mediated ROS production may play an important role in the neuritogenesis induced by STS and Y-27632 in PC12 cells.

Key words : PC12 cells, Staurosporine, Y-27632, Reactive oxigen species (ROS), Rac1, Neurite outgrowth

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I. Introduction

A neural crest-derived tumor cell line isolated from a rat pheochromocytoma, PC12, is widely used as a study model for neuronal functions. Upon stimulation with growth factor such as nerve growth factor (NGF), PC12 cells differentiate into sympathetic neuron-like cells with neurites; the cells stop dividing, attach more strongly to the substratum, and extend long neuritic processes[1,2]. NGF binds to its receptor, tyrosine receptor kinase (TrkA), and activates its tyrosin kinase, which leads to activation of a guanine nucleotide-binding protein, Ras, in PC12

cells[3,4]. Expression of the proto-oncogenic Ras activates the Raf-MAPK/ERK kinase (MEK)- mitogen-activated protein (MAP) kinase signaling pathway, which has been shown to play an important role in the induction of neurite outgrowth[3]. Activation of MAP kinase also known as extracellular signal-regulated kinase (ERK) by growth factors can trigger either cell growth or differentiation. The transient activation of MAP kinase by epidermal growth factor (EGF) stimulates proliferation of PC12 cells, whereas activation by NGF causes differentiation, which acts partly by inducing a sustained activation of MAP kinase[5]. PC12 cells can also be induced to differentiate by other agents such as cyclic-AMP[6,7,8], depolarization [9,10] and the protein kinase inhibitor, staurosporine[11]. Especially, protein kinase inhibitors such as staurosporine and Y-27632 induce the rapid neurite outgrowth in neuronal cells and are involved in the processing of neurite remodeling independent of the cellular signaling stimulated by growth factors (NGF, EGF) [12,13].

It is known that staurosporine is able to inhibit various kinases, such as protein kinase C (PKC), protein kinase A (PKA), protein kinase G (PKG), and myosin light chain (MLC) kinase. Recent studies have defined the effect of staurosporine on apoptosis-arresting cell cycle at G1 check point in normal cells through the inhibition of cyclin dependent kinase (CDK) and inducing programmed cell death by release of cytochrome c[14]. Under the

lower concentrations than that inducing cell death, staurosporine did not show the typical morphology of cell death such as membrane blebbing and cytoskeletal fragmentation, and induced the rapid neurite outgrowth in most neuronal cells and PC12 cells[15]. Although staurosporine potently inhibits a number of protein kinases, it is not elucidated about the exact target related to rapid morphological change in neurons and the related cell types.

On the other hand, Y-27632 has been reported as a specific inhibitor of Rho-dependent serine/threonine protein kinase, p160ROCK[16], and was widely used to investigate the signal pathway involving Rho/ROCK such as morphological change and cell motility. Y27632 also inhibits chondrocyte proliferation and accelerates hypertrophic differentiation [17].

The Rho family of small GTPases has been implicated in diverse cellular events such as membrane trafficking, transcriptional regulation, growth control, and cytoskeletal organization[18]. Presently, at least 15 mammalian Rho family proteins have been identified: Rho A, -B, -C, -D, -E, and -G, Rac1, -2, and -3, Cdc42, Rnd 1, -2, and -3, TC10, Rho H/TTF. Among them, the functions of Rho, Rac, and Cdc42 have been extensively characterized. In fibroblasts, the activation of Rho leads to formation of actin stress fibers and assembly of focal adhesions[19], whereas the activation of Rac and Cdc42 induces

formation of filopodia and lamellipodia, respectively[20]. Studies on neuronal cells have shown that Rac and Cdc42 are involved in the formation of lamellipodia and filopodia of the growth cone, respectively, and that they are required for the outgrowth of neurites. On the other hand, Rho is required for the collapse of the growth cone and the retraction of neurites[21,22]. Many studies have reported that the reciprocal balance between these two opposing activities is a critical determinant of cellular morphology and migratory behavior in various cell types[23].

Reactive Oxygen Species (ROS), such as H_2O_2, super oxide ($O2^-$), and hydroxyl radicals (OH^-), are generated in cells by several pathways, including electron transfer reactions, xanthine oxygenase, NADPH oxidase, and x-ray and UV light irradiation. Many studies about ROS have generally been viewed as cytotoxic effect in cells, referring to the cell death by apoptosis or necrosis. Also, ROS are well-known for the host defense mechanism in neutrophils[24] and possess carcinogenic potential associated with tumor promotion[25]. Recent studies, however, indicate that small nontoxic amounts of ROS may play a normal role as a second messenger in the various signaling pathways[26]. Generation of ROS was observed in a number of cells stimulated with cytokines such as interleukin-1 and TNF-1 or peptide growth factors such as NGF, EGF, and platelet-derived growth factor (PDGF)[27].

Rac1 is a component of the NADPH oxidase complex and has been shown to contribute to the ROS production required for integrin-mediated cell adhesion and spreading in HeLa cells[23]. It was also reported that Rac1 is involved in NADPH oxidase-mediated ROS signaling during the phagocytosis of immunocyte. Although the role of ROS has been extensively studied in mitogenesis, inflammation, and apoptosis, little is known about its functional role in the differentiation process of neuronal cells. Some studies have suggested that a strong antioxidant, N-acetylcysteine (NAC), has an inhibitory effect on the neuronal differentiation induced by NGF or by the expression of oncogenic *ras* in PC12 cells[28].

In this study, I focused on the role of a small GTP-binding protein, Rac1, in those kinase inhibitors, staurosporine and Y-27632 ,-induced neurite outgrowth. Also, I investigated if the regulation of redox state has an effect on the neurite outgrowth induced by staurosporine and Y-27632.

II. Materials and Methods

1. Materials

The rat pheochromocytoma (PC12) cells were obtained from ATCC (Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM), Fetal Bovine Serum (FBS), Horse serum (HS), Antibiotics were from GIBCO (Grand Island, NY, USA). Y-27632 dihydrochloride was from TOCRIS (Ellisville, MO, USA). staurosporine (STS), N-acetylcysteine (NAC), Poly-l-lysine (PLL), Diphenylene iodonium (DPI) were obtained from Sigma-aldrich (St. Louis, MO, USA). Anti-Rac1 antibody was from BD Transduction Laboratories (San Jose, CA USA). FITC-conjugated anti-mouse antibody was obtained from Jackson Immuno Research (West Grove, PA, USA). Anti-Phospho-myosine light chain 2 antibody was from Cell signaling (Beverly, MA, USA). Lipofectamine 2000 was obtained from Invitrogen (Carlsbad, CA, USA). 2`,7`-dichlorofluorescein diacetate (DCFHDA) was from Molecular Probes (Eugene, OR, USA). Mounting medium was obtained from Biomeda (Foster City, CA, USA). Rhodamine-phalloidin was from Molecular Probes (Eugene, OR, USA).

2. Methods

2. 1 Cell culture

PC12 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and 10% horse serum, and 1% antibiotics under humidified conditions in 95% air and 5% CO_2 at 37℃. The medium was changed every 2 days and the subculture was performed every 7 days. Cells were plated in 12-well plates for analyses of neurite outgrowth, 35-mm dishes for ROS measurement and transfection, and 100-mm dishes for GST-pull down assay.

Astrocyte cultures were prepared from the cerebral hemispheres of 1 day old rats by mechanical dissociation. Tissue was suspended in Hanks' balanced salt solution (HBSS) and cells were dissociated by trituration and washed by centrifugation. Cells were cultured in minimum essential medium supplemented with 26 mM glucose, 25 mM sodium bicarbonate, 2 mM L-glutamine, 5% fetal bovine serum and 5% horse serum. After 1-2 weeks of culture, when full confluence had been obtained, the cultured cells were trypsinized, dispersed in MEM containing 20% fetal bovine serum and 10% DMSO, and stored in liquid nitrogen until use.

2. 2 Evaluation of neurite outgrowth

Cells were plated on 12-well plates at a density of 5 $x10^4$ cells

/well. A neurite was identified as a process that is greater than one cell body in length. The percentage of cells with neurites was calculated by counting 100 cells per well in triplicate wells.

2. 3 Western blot analysis

For immunoblot analysis, cells were collected, and then washed out with phosphate-buffered saline (PBS) twice. Collected cells were allowed to incubate for 1 h on ice in 50 μl of lysis buffer (50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1% TritonX-100, 100 g/ml phenylsulfonyl fluoride (PMSF), 1 g/ml aprotinin, 1 mM dithiothreitol (DTT), and 1 mM Na_3VO_4). The resulting cell lysates were resolved on 8-12% SDS-PAGE and transferred onto nitrocellulose membrane (Protran, Bioscience). The membranes were blocked with Tris-buffered saline with 5% skim milk in tween 20 [10 mM Tris-Cl (pH 7.5), 150 mM NaCl, 0.1% Tween 20] and then hybridized with different antibodies. Proteins were detected by using luminol chemiluminescent substrate (LumiGLO, Cell Signaling Technology).

2. 4 Transfection

PC12 cells were transfected with pEXV-wt Rac1, pEXV-Rac1N17, pEXV-Rac1V12, and pEGFP vector. For transfection, cells were seeded in poly-L-lysine-coated glass coverslips (22 ×22 mm) in 6-well plates at a density of 1×10^5 cells/well and

cultured for 24-36 h. Then cells were transfected with 0.8 mg of total DNA using Lipofectamine 2000 according to the manufacturer's instructions.

2. 5 Measurement of Intracellular ROS

PC12 cells were plated in a poly-L-lysine-coated 12-well plate and serum-starved in 0.5% horse serum and 0.25% fetal bovine serum for 12-18h. After treating the indicated drugs, cells were loaded with 2`,7`-dichlorofluorescein diacetate (DCFHDA; 5 mg/ml, Molecular Probes) for 5 min at 37℃. The DCF fluorescence intensity was measured by a confocal microscope (Carl zeiss 500) (excitation wavelength, 485 nm; emission wave-length, 530 nm).

2. 6 GST-pull down assay

Cells were then stimulated with 100 nM STS and 100 μM Y-27632 for the indicated times and lysed for 5 min with the ice-cold cell lysis buffer (Tris (pH 7.4) 50 mM, NaCl 100 mM, $MgCl_2$ 2mM, Glycerol 10%, NP-40 1%, DTT 1 mM, BM cocktail 20x) containing 10 mg of GST-CRIB. Cell lysates were then centrifuged for 5 min at 13,000 rpm at 4 °C, and the supernatant was incubated with glutathione-Sepharose beads for 2 hr at 4 °C. After the beads were washed with the cell washing buffer (Tris (pH 7.4) 50 mM, NaCl 100 mM, $MgCl_2$ 2mM, Glycerol 10%,

NP-40 1%, DTT 1 mM), the bound proteins were eluted in Laemmli sample buffer and separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). To detect GTP-bound Rac1, anti-Rac1 antibody was used.

2. 7 Immunocytochemistry

PC12 cells were fixed with 3.7% paraformaldehyde (PFA) for 20 min, washed with PBS, and permeabilized with 0.1% (v/v) Triton X-100. Normal goat serum was used to block unspecific reactions. Then, cells were incubated with a monoclonal mouse anti-Rac1 antibody. The resulting immune complexes were visualized with a FITC-conjugated anti-mouse IgG. For confocal image analysis, a Zeiss LSM510 confocal system (Zeiss, Oberkochem, Germany) was used.

III. Results

1. Rapid neurite outgrowth induced by STS and Y-27632 is different from neuritogenesis elicited by NGF

As shown in the fig. 1 A, in comparison to NGF induced-neurite outgrowth, STS and Y-27632 induced-neurite outgrowth was proceeded rapidly within 1 hr. And also, the difference on neurite outgrowth induced by STS, Y-27632, and NGF was examined by observing the ERK activation depending on the time. PC12 cells were treated with 100 nM STS, 100 μM Y-27632, and NGF (50 ng/ml) for 5, 10, and 30 min and then each cell lysate was subjected to Western blot analysis using anti-phospho ERK. The same blots were subsequently stripped and reprobed with antibody that recognizes total ERK to verify equal amounts of the protein in the various samples. As shown in the fig. 1 B, treatment with 50 ng/ml NGF led to an activation of ERK from 5min to 30min but treatments of STS and Y-27632 did not show any effect on ERK activation.

2. The effect of STS and Y-27632 on the activity of ROCK and Rac1.

It is known that Lysophosphatidic acid (LPA) activates the GTPase Rho and its target Rho-kinase to induce myosin light-chain (MLC) phosphorylation[30]. To assess whether STS and Y-27632 could induce down regulation of Rho signaling activated

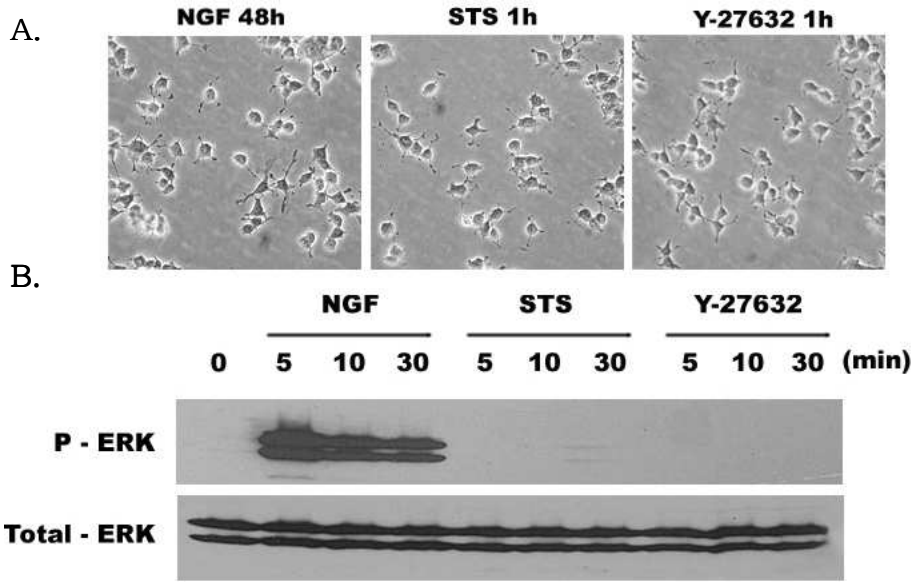


Figure 1. The mechanism of the neuritogenesis by STS and Y-27632 was different from that induced by NGF. Cells were incubated with 50ng/ml NGF, 100 nM STS, and 100 μM Y-27632 for the indicated time. A, STS and Y-27632 induce the rapid neurite outgrowth in PC12 cells. B, after cells were harvested and lysated, western blotting was performed (n=3). Unlikely NGF, treatment of STS and Y-27632 did not show any effect on ERK activation.

by LPA stimulation, the amounts of phosphorylated MLC were measured by Western blotting. STS, a non-specific potent kinase inhibitor, and Y-27632, a specific ROCK (Rho associated kinase) inhibitor, reduced the amounts of phosphorylated MLC stimulated by LPA.

To assess direct modulation of Rac1 activity by Rho down-stream inhibition effect of STS (100 nM) and Y-27632 (100 μM), the amounts of cellular GTP-bound Rac1 were measured by using the GST-fused CRIB domain of aPAK (GST-CRIB). STS and Y-27632 induced a rapid increase in the amount of cellular GTP-bound Rac1 at 30 min (Fig 2).

3. The effect of Rac1 on morphological changes

To investigate the effect of Rac1 on morphological changes , PC12 cells were transfected with pEXV-Rac1N17, pEXV-RacV12 , and pEGFP by using Lipofectamin 2000 reagent. In the cells co-transfected with a dominant negative Rac1N17 and pEGFP, STS- and Y-27632-induced neurite outgrowth was abrogated by transient expression of the dominant negative Rac1N17. On the other hands, pEGFP had no suppressive effect on the morphological changes in PC12 cells. These results suggested that Rac1 activity was required for the neuritogenesis induced by STS and Y-27632.

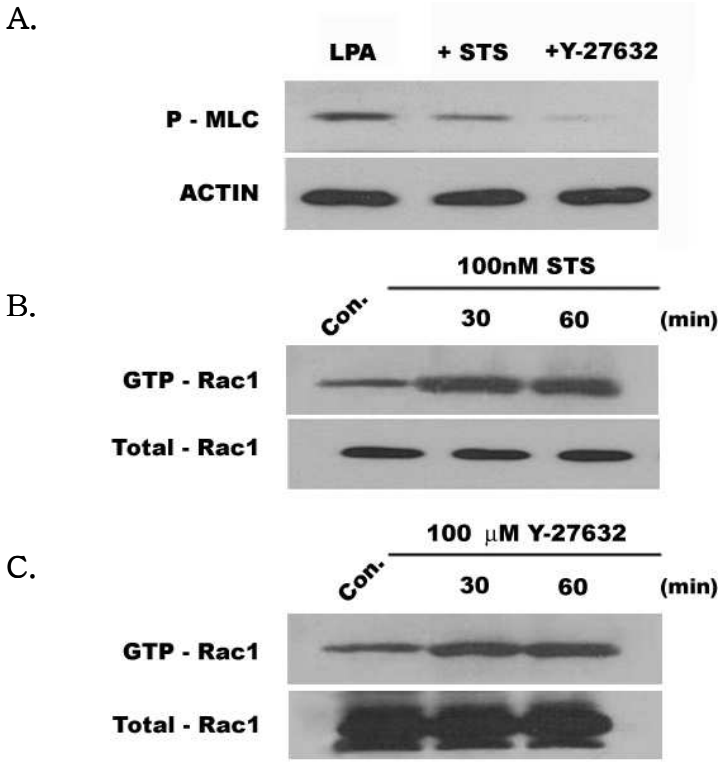


Figure 2. The effects of staurosporine and Y-27632 on between Rho and Rac signaling in PC 12 cells. A, Serum-starved PC12 cells were stimulated by STS, Y-27632, and LPA for 1 h. Cell lysates were probed with anti-phospho MLC antibody after 15% SDS-PAGE and Western blot. To verify the same amounts of cell lysates, anti-actin antibody was used. LPA induced-MLC phosphorylation was inhibited by STS and Y-27632. B, the cell lysates were incubated with GST-CRIB after 100 nM STS stimulation, and the amounts of GTP-bound Rac1 were determined by immunoblotting using a monoclonal antibody against Rac1. Total amounts of Rac1 in cell lysates are also

shown. Treatment of 100 nM STS increased the amounts of GTP binding-Rac1. C, the cell lysates were treated with 100 μM Y-27632 for the indicated time and then determined by using GST-pull down assay. Treatment of Y-27632 had an effect on Rac1 activity in PC12 cells.

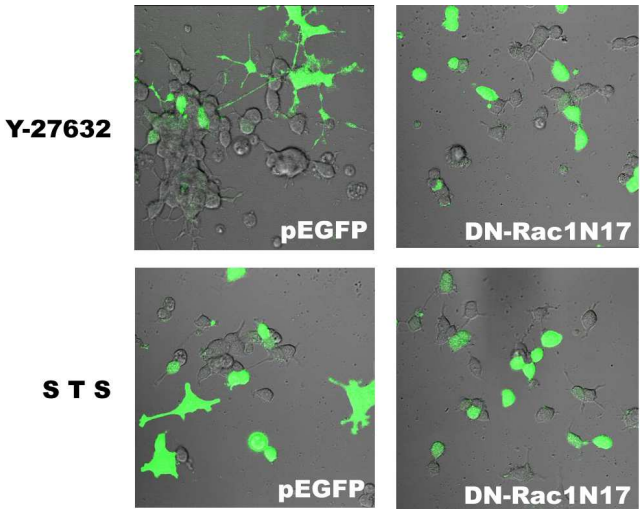


Figure 3. The effect of Rac1 on the morphological changes. A, PC12 cells were co-transfected with pEGFP and DN-Rac1N17, using Lipofectamin 2000 reagent. The cells co-expressing GFP and DN-Rac1N17 are visualized in green. Neuritogenesis induced by STS and Y-27632 was blocked by co-transfection with a dominant negative Rac1N17 and pEGFP.

4. Rapid neurite outgrowth by STS and Y-27632 was suppressed by pretreatment of NAC and DPI.

To assess whether Rac1-mediated ROS production is involved in the neurite outgrowth induced by STS and Y-27632, PC 12 cells were treated with NAC, a strong antioxidant, and DPI, a flavo protein inhibitor. STS or Y-27632 caused rapid neurite outgrowth even 1hr after treatment, which was inhibited by 20 mM NAC and 10 μM DPI (Fig 4, 5).

5. H_2O_2 facilitated the neurite outgrowth stimulated by STS and Y-27632.

To examine whether the ROS production was involved in the processing of neurite outgrowth, the effect of H_2O_2 (100 μM) on the neurite processing was investigated in the presence of 10 μM Y-27632 and 10nM STS to minimize the effect on neurite outgrowth. The co-treatment with H_2O_2 (100 μM) notably stimulated the rapid neurite outgrowth in serum-starved PC12 cells.

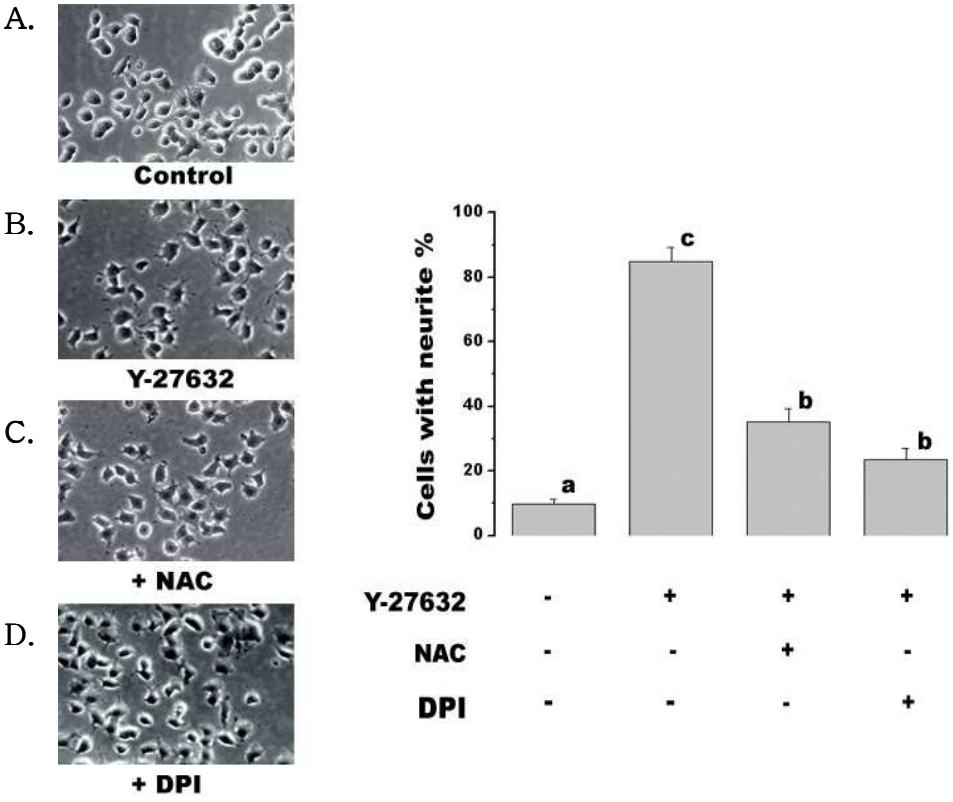


Figure 4. Neurite outgrowth was induced by 100 μM Y-27632(B), a Rho-associated kinase inhibitor. PC12 cells were seeded on poly-L-lysine coated 12-well plates. Pretreatment with 20 mM N-acetylcysteine (C), a strong antioxidant, effectively suppressed Y-27632-induced neurite outgrowth. Also, DPI 10 uM (D), which inhibits a flavo protein such as NADPH oxidase, showed the similar inhibitory effect on the neurite outgrowth (n=6).

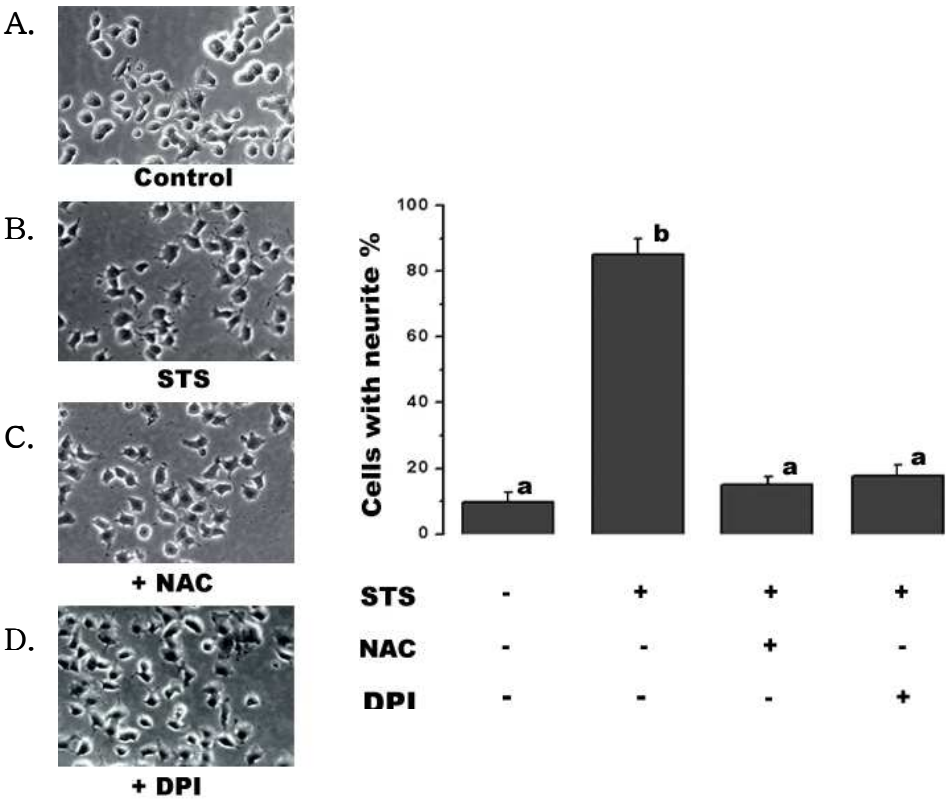
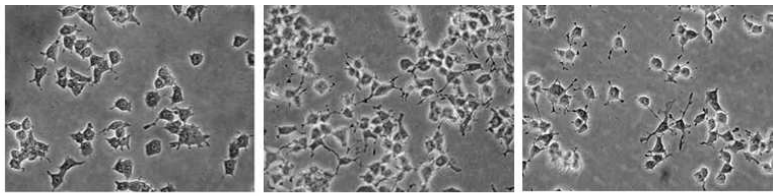


Figure 5. Staurosporine (B), at concentrations of 100 nM, caused a rapid neurite outgrowth in PC12 cells.

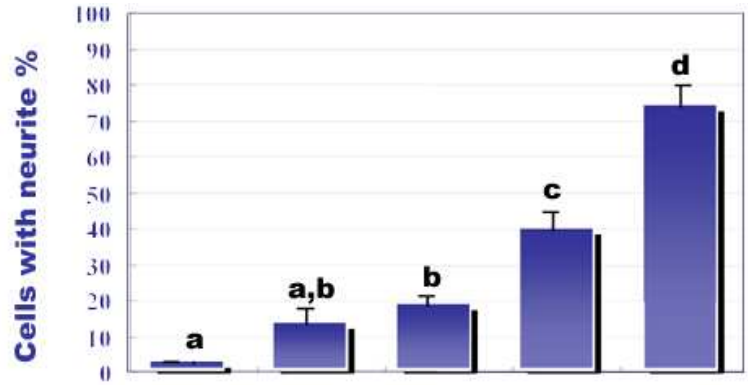
After the pretreatment with 20 mM NAC (C) and 10 μM DPI (D) for 20min, respectively, followed by Staurosporine for 1 h, cells were also allowed to inhibit rapid neurite outgrowth (n=6).

A.



Y-27632 (μM)	10	10	100
H_2O_2 (μM)	-	100	-

B.



Y-27632 (μM)	-	10	10	10	100
H_2O_2 (μM)	-	-	10	100	-

Figure 6. Co-treatment with 100 μM H_2O_2 facilitated rapid neurite outgrowth induced by Y-27632. PC12 cells were seeded on poly-L-lysine coated 12-well plates and then starved with DMEM containing 0.25% FBS, 0.5% HS for 12 h. The effect of Y-27632 for neurite outgrowth was enhanced with co-treatment 100 μM H_2O_2. Values represent mean ±S.E (n=3).

6. STS and Y-27632 stimulate intracellular ROS production, and its inhibition by NAC and DPI blocks both kinase inhibitors-induced production of ROS in PC12 cells.

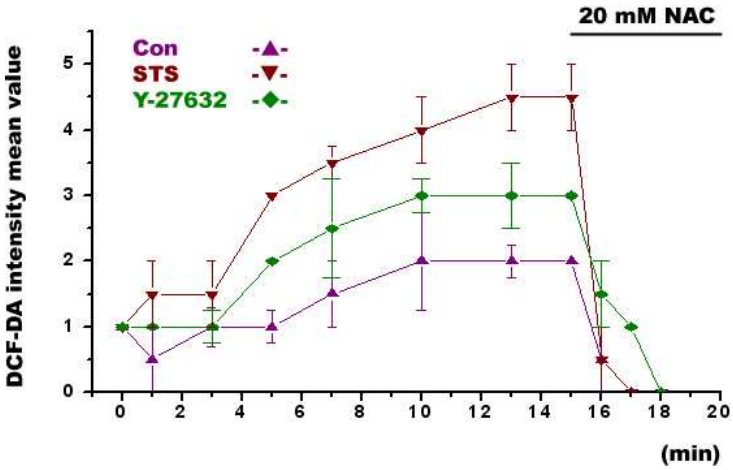
When using a membrane-permeable fluorescent probe, 2',7'-H_2DCFDA, loaded into PC12 cells to monitor the level of ROS, treatment with STS and Y-27632 quickly increased the intracellular level of ROS (Fig. 7). STS (100 nM) significantly increased the level of intracellular ROS $\geq$ 5min after treatment. Y-27632 also stimulated the ROS production although its amounts were smaller than that stimulated by STS. When cells were treated with NAC, STS-and Y-27632-stimulated DCF fluorescence was significantly reduced.

To determine whether the NADPH oxidase is involved in STS- and Y-27632-induced ROS generation in PC12 cells, I treated PC12 cells with DPI, a specific inhibitor for flavoprotein that is a constituent of the NADPH oxidase complex. DPI significantly inhibited the ROS production, after 15 min-treatment with STS and Y-27632 stimulation.

7. Rac1 activation is required for the ROS production in PC12 cells.

Because the NADPH oxidase-induced ROS production is regulated by Rac1 in phagocytic cells, the role of Rac1 in ROS generation in PC12 cells was examined by using transfection

A.



B.

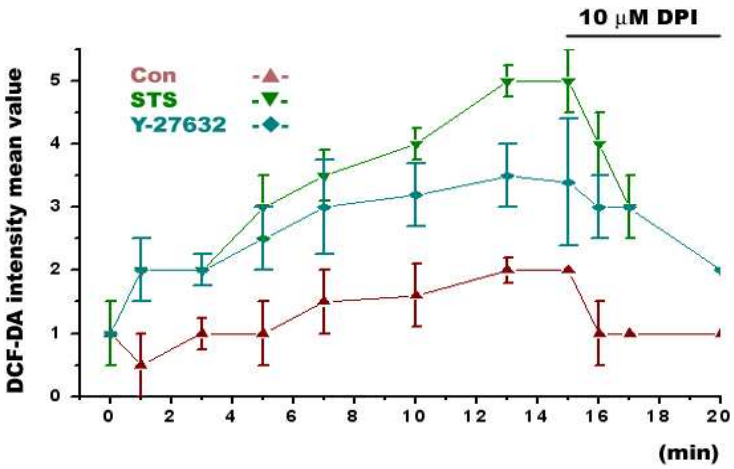


Figure 7. ROS production by STS and Y-27632 was decreased by the treatment of NAC, an ROS scavenger, and DPI, a flavoprotein inhibitor. Treatment with 20 mM NAC suddenly decreased ROS production mediated by STS and Y-27632 (A) during monitoring by DCF fluoroscence. DPI also inhibited this increase after stimulation (B). Each symbol represents the mean SEM (bar) of 4 observations.

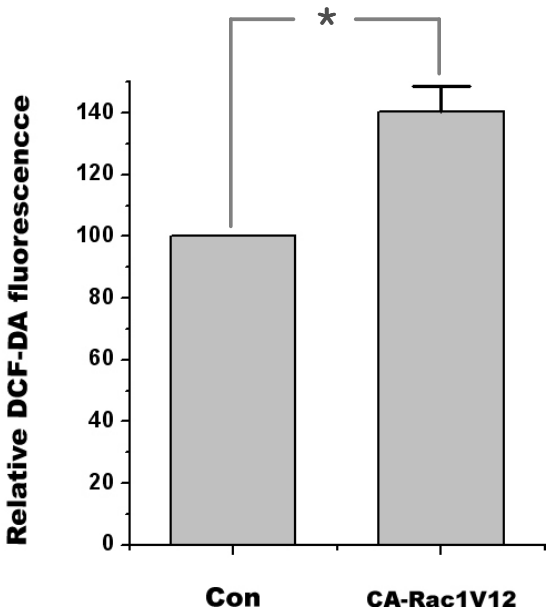


Figure 8. Constitutively active form of Rac1, Rac1V12, increase the intracellular ROS level. PC12 cells were transfected with pEXV-Rac1V12, using Lipofectamine 2000 reagent. After 36-48 h, intracellular ROS production in PC12 cells expressing CA-Rac1V12 was determined by DCF fluorescence. The level of ROS in CA-Rac1V12-transfected cells was increased to nearly 40%, compared to non-transfected cells.

technique with DN-Rac1N17 and CA-Rac1V12. The level of ROS in DN-Rac1N17-transfected PC12 cells was slightly lower than that observed in control cells (data not shown), whereas the level of ROS in CA-Rac1V12-transfected cells was higher than that.

8. The neurite outgrowth induced by inhibition of ROCK requires not only an increase in Rac1 activity but also their appropriate localization to the sites where neurites are formed and extend.

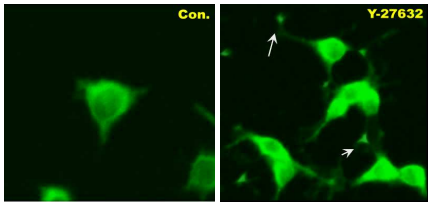
Using immunofluorescence and a monoclonal anti-Rac1 antibody, the distribution of Rac1 in PC12 cells during neurite processing was analyzed. When cells were treated with STS (data not shown) and Y-27632 for 1 h (Fig. 9, A), Rac1 was localized at the tip of neurites in PC12 cells.

To confirm whether Rac1 redistribution is required for the STS- and Y-27632-induced morphological changes, astrocytes ,which are relatively larger than PC12 cells, were examined. The treatment of STS and Y-27632 stimulated the morphological changes, inducing Rac1 localization in a dose-dependent manner at the sites to be stellations. Furthermore, pretreatment with 20 mM NAC and 10 μM DPI has an inhibitory effect on Rac1 distribution during STS- (data not shown) and Y-27632-induced morphological changes (Fig. 9, B).

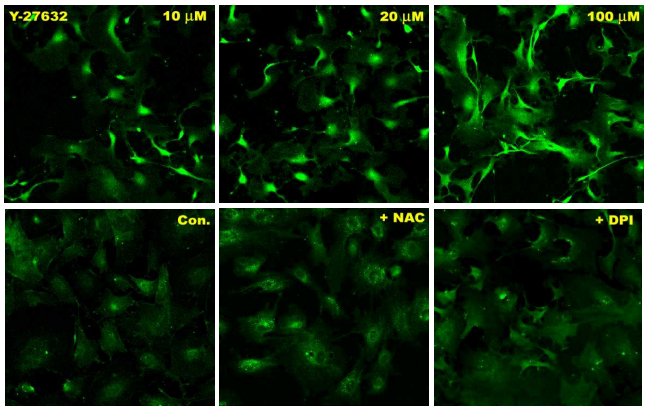
To test some possibilities that the localized Rac1 could induce

actin rearrangement through Rac1 induced-ROS production, the co-localization of Rac1 and actin was investigated, using immuno-double staining. Enrichment of F-actin at the tips of the Y-27632 induced-extension was observed with Rac1 (Fig. 9, C).

A.


Con.
Y-27632

B.


Y-27632
10 μM
20 μM
100 μM
Con.
+ NAC
+ DPI

C.

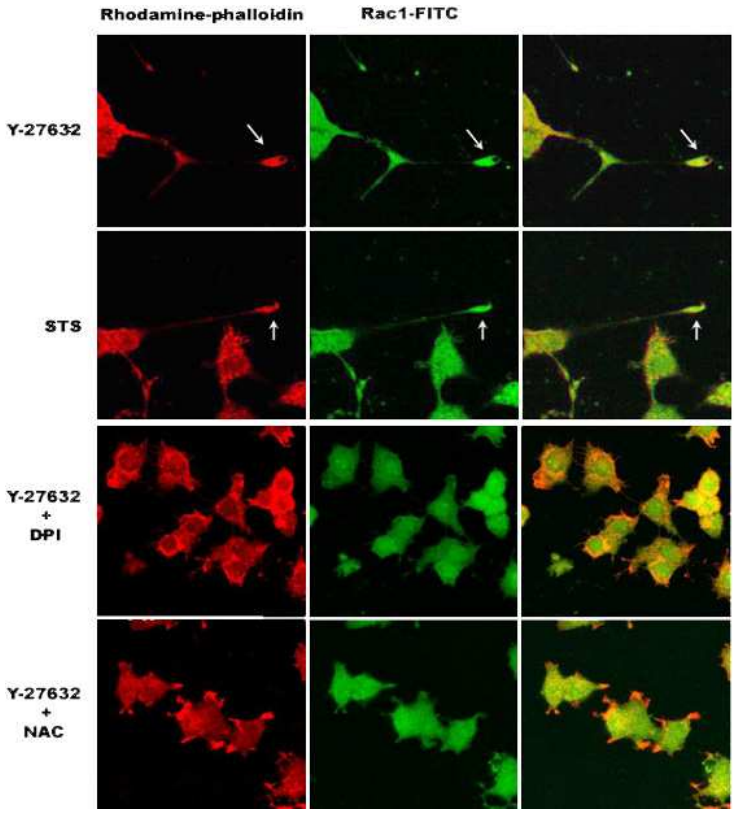

Rhodamine-phalloidin
Rac1-FITC
Y-27632
STS
Y-27632 + DPI
Y-27632 + NAC

Figure 9. Distribution of Rac1 during STS and Y-27632 treatment. A, PC12 cells were treated with 100 μM Y-27632 for 1 h and then were fixed by using 3.7% PFA (paraform-aldehyde). PC12 cells were then labeled for Rac1, using FITC-conjugated secondary antibody. Rac1 was localized at the tip of neurites (*arrows* in A). B, after treatment of Y-27632 for 1 h, dose-dependent Rac1 re-distribution were induced in astrocytes. Also, treatment with 20 mM NAC and 10 μM DPI inhibited Rac1 re-distribution in astrocytes. C, Organization of the actin cytoskeleton and Rac1 in PC12 cells. rhodamine-phalloidin staining and Rac1-FITC staining of the corresponding cells are shown. After treatment of STS and Y-27632 in PC12 cells, both Rac1 and actin were observed at the end tip of neurite (*arrows* in C). Also, treatment with 20 mM NAC and 10 μM DPI suppressed not only Rac1 distribution at the tips of neurites but also morphological changes in PC12 cells (Fig. 9, C)

IV. Discussion

In PC 12cells, NGF induces cell differentiation into the neuronal phenotype through the activation of Ras. It was already reported that neurotropic effect of STS involved neither the activation of established signalling pathways by NGF, such as tyrosine phosphorylation of ERK[29], nor gene expression through transcription activation[11]. Also, ROCK inhibitor, Y-27632 induces the rapid neurite outgrowth in neuronal cells and is involved in the processing of neurite remodeling independent of the cellular signaling stimulated by growth factors (NGF, EGF) [12,13]. However, the molecular mechanisms regulating the cytoskeletal changes necessary for NGF-, STS-, and Y-27632-induced neurite outgrowth are still largely obscure. In this study, I investigated how kinase inhibitors, such as STS and Y-27632, might induce the rapid neurite outgrowth and whether the redox state could regulate the STS- and Y-27632-induced morpho- logical changes.

As shown in fig. 1, STS and Y-27632 rapidly induced the morphological changes within 1 hr. However, neither the MEK inhibitor of PD98059 nor PI3K inhibitor, wortmanin was able to inhibit the induction of neurite outgrowth by STS or Y-27632 (data not shown). In addition, STS and Y-27632 did not affect the phosphorylation of ERK. These results indicated that neurite

outgrowth by STS or Y-27632 was independent of NGF signaling.

Generally, the morphological changes seem to be determined by the crosstalk of Rac and Rho proteins in various cell types such as fibroblasts, epithelial cells, and neuronal cells. To address whether Rac and Rho regulate their activities in a reciprocal manner or whether Rac activation is mediated by the inhibition of Rho downstream, the effect of STS and Y-27632 on ROCK and Rac1 activity in PC12 cells was examined, using Western blotting and GST pull down assay. Lysophosphatidic acid (LPA) was shown to activate the GTPase Rho and its target Rho-kinase to induce myosin light-chain (MLC) phosphorylation, leading to platelet shape change. Rho kinase directly phosphorylates MLC[30]. As shown in fig. 2 A, LPA-induced Rho activation did not recover the inhibitory effect of STS and Y-27632 on the ROCK activity. These findings support the notion that STS and Y-27632 could have the inhibitory effects on the downstream of Rho pathway, decreasing the phosphorylation of myosin light chain.

In Swiss 3T3 fibroblasts, it was shown that activation of Rho did not affect Rac activity by using dominant negative N19Rho or the Rho-kinase inhibitor, Y-27632[31]. However, the data obtained in this study showed that inactivation of Rho downstream by treatment of STS and Y-27632 had an effect on Rac1 activity in PC12 cells within 30 min. These findings are in agreement with a

recent study in hippocampal neurons, where the authors concluded that the inactivation of Rho leads to increase Rac1 activity within 2 hr[32]. Also, according to the other report, inhibition of the p160 Rho kinase induced a shift from focal contacts to focal complexes, which was accompanied by enhanced membrane ruffling[33]. The authors suggested that inhibiting downstream of Rho leads to activation of Rac1.

Next, the effect of Rac1 on morphological changes induced by STS and Y-27632 in PC12 cells was assessed. Using dominant negative Rac1 mutant, Rac1 activity was shown to serve as an essential regulator in STS- and Y-27632-induced neurite outgrowth. The involvement of Rac1 in neurite outgrowth was also reported previously in various cell types [20,21,22,23].

It was reported that Rac1 is a component of the NADPH oxidase complex and has been shown to contribute to the ROS production required for cell adhesion and spreading in HeLa cells[23]. To determine whether the NADPH oxidase-like enzyme is involved in STS and Y-27632-induced neurite outgrowth, PC 12 cells were treated with DPI, a specific inhibitor for flavoprotein that is a constituent of the NADPH oxidase complex. DPI pretreatment could suppress the STS- and Y-27632-induced morphological changes in PC 12 cells. These data suggest that activation of an enzyme functionally similar to the NADPH oxidase could be involved in STS and Y-27632 induced-neurite

outgrowth.

It was also reported that ROS generation appears to be modulated by Rac1 in fibroblast cells[23]. To investigate whether ROS regulated by Rac1 activation was involved in STS and Y-27632 induced-neurite outgrowth, the cells were pretreated with 20mM NAC, a chemical antioxidant in this study and demonstrated that found that ROS production, presumably via an NADPH oxidase-like enzyme, could serve as an important factor during process of neurite outgrowth.

Furthermore, the co-treatment of STS or Y-27632 with H_2O_2 (100 μM) stimulated the rapid neurite outgrowth in serum-starved PC12 cells. This result implicated that ROS, such as H_2O_2, helped Y-27632 and STS induced-neurite outgrowth. In addition, according to the Leni Moldova et al.[42], the localized production of oxidants could selectively affect the function of actin monomer- sequestering proteins, -capping proteins, or -severing proteins. Therefore, in this study, H_2O_2 might act as a facilitator to process the neurite outgrowth.

In the previous reports, STS increased the production of ROS at 200 nM, which caused apoptosis, and STS-induced apoptosis could be prevented by antioxidants[34]. Although there are no proven results about the ROS production stimulated with Y-27632, some possibility has been reported that ROCK inhibition by Y-27632 could affect the ROS-induced

atherosclerotic cellular processes (e.g., proliferation, apoptosis) at downstream of signal transduction[35]. Corresponding with these results, STS and Y-27632 quickly induced an increase in DCF fluorescence in PC12 cells. In addition, the inhibitory effect of NAC and DPI on the ROS increasement was observed. Furthermore, it was reported that phosphatidylinositol 3-kinase, beta Pix, Rac1, and Nox1 are involved in growth factor-induced production of H_2O_2[36]. Therefore, it was examined whether Rac1 was directly related to ROS production induced by STS and Y-27632 in PC12 cells, using constitutively active Rac1 mutants. Through confocal microscopy, the level of ROS in constitutively active RacV12-transfected cells was found to be slightly higher than that observed in control cells. These results suggest that STS and Y-27632 stimulated ROS production in short period and Rac1 activity was required for the ROS production in PC12 cells as well as an NADPH oxidase-like enzyme-mediated ROS signaling could be inhibited by the treatment of NAC and DPI.

These data provide evidence that STS and Y-27632 stimulate the generation of an oxygen radical, perhaps H_2O_2, via Rac1 activation, which is required for STS and Y-27632-induced morphological changes. However, treatment of cells only with H_2O_2 did not cause the neurite outgrowth. After inhibition by NAC and DPI, treatment of H_2O_2 did not also recover the morphological changes induced by STS and Y-27632 (data not

shown). In experiments using transfection, only overexpression of wild type Rac1 and CA-Rac1V12 in PC12 cells could not induce neurites from the cells, indicating that both the activation of Rac1 and ROS production are not sufficient for inducing neurite outgrowth in PC12 cells.

A possible explanation for this inconsistency requires the presence of the other factors to form and extend neurites, besides Rac1 activation. According to previous reports, both Rac1 activation and its localization may be required for the cell motility and cell-cell adhesion in endothelial cells[37]. In PC12 cells, Hironori katoh et al.[38], suggested that neurite outgrowth might require not only an increase in Rac1 and Cdc42 activities but also their appropriate localization to the sites where neurites are formed and extend[38]. In the present study, the Rac1 localization upon STS and Y-27632 treatment was examined in PC12 cells. Using immuno-cytochemistry, the appropriate re-localization of Rac1 at the end tips of neurite was observed after the treatment of STS or Y-27632.

Because PC12 cells are too small to detect Rac1 localization precisely, I used astrocytes which are relatively larger than PC12 cells to investigate Rac1 localization by STS and Y-27632. Both STS and Y-27632 dose-dependently induced Rac1 distribution at the sites where stellations were formed.

A number of key cellular functions, such as morphological

differentiation and cell motility, are closely associated with changes in cytoskeletal dynamics. Especially, actin filaments have been known as a key molecule for the morphological changes. Because several studies have reported the role of ROS in the organization of the actin cytoskeleton in different cell types[39, 40, 41], it was examined whether the localized oxidants produced by Rac1 at the end tips could be required for actin polymerization during neurite processing.

Confocal microscopy revealed that newly formed actin filaments were located in the end tips of neurites with Rac1. Based on the results, the appropriate Rac1 re-localization with actin filaments to the sites where neurites formed and extended might have a meaning that GTP binding-Rac1 has a function to proceed actin polymerization, via ROS production. This results is in agreement with previous report that the localized production of oxidants could selectively affect the function of actin monomer[42].

In summary, the shifted balance between Rho and Rac signaling stimulated by STS and Y-27632 appear to induce ROS production in PC 12cells. Therefore, the activation and localization of Rac1 at the tip of neurite have an effect to coordinate actin cytoskeletal rearrangements required for STS- and Y-27632-induced neurite outgrowth, via Rac1- mediated ROS production.

V. References

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Abstract (in Korean)

PC12 세포에서 staurosporine 과 Y-27632 에 의해 유도되는 신경 돌기 성장의 산화 환원 조절 기전

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김 두 식

세포 내 산화 환원 상태는 다양한 세포의 신호 전달 기전에 매우 중요한 역할을 하고 있다고 알려져 있다. 본 실험에서는 PC12 세포에서 staurosporine (STS)과 Y-27632에 의해 유도되는 신경 돌기 성장 기전에 활성 산조종의 관여 여부와 그 역할에 대한 연구를 수행하였다. 다양한 단백질 kinase 억제제인 STS와 Rho-associated kinase 억제제인 Y-27632는 신경 성장 인자 (NGF)가 유도하는 신경 돌기 성장과는 달리 1시간 안에 빠르게 신경 돌기 성장을 유도하였다. 최근의 연구 결과에 따르면 신경성 세포의 분화에 있어 Rho와 Rac은 상반되는 기능을 가진다고 보고 되었다. 이러한 결과와 일치하게 PC12 세포에서 STS와 Y-27632로 Rho의 하위 신호 전달을 억제 하였을 때 30분 안에 Rac1 단백의 활성이 확인되었다. 게다가, dominant negative Rac1N17 돌연변이 유전자를 과발현 시킨 PC12 세포에 STS와 Y-27632를 처리하였을 때 형태 변화가 유도되지 않았다. Rac1 활성에 의해 유도되는 활성 산소종이 STS와 Y-27632가 유도하는 신경 돌기 성장에 관여 하는지를 확인하기 위해 항산화제인 N-acetyl- cysteine

(NAC)와 flavo-단백 억제제인 diphenyleneiodonium (DPI)를 전 처리하였을 때 STS와 Y-27632에 의해 유도되는 신경 돌기 성장이 억제 되었다. 또한 신경 돌기 성장을 유도하는 최소 농도의 STS와 Y-27632에, 100 μM H_2O_2를 함께 처리하는 경우 신경 돌기 성장이 촉진 됨을 확인하였다. DCF-DA를 이용하여 세포내 활성 산소종을 탐지하는 실험에서도 STS와 Y-27632에 의한 활성 산소종의 증가가 확인되었고 constitutively active (CA) Rac1V12 돌연변이 유전자를 PC12 세포에 과발현 시켰을 때 역시 세포내 증가된 활성 산소 종을 확인 할 수 있었다. 이러한 결과들을 통해 STS와 Y-27632이 유도하는 PC12 세포의 신경 돌기 성장에 있어 Rac1 단백의 활성이 유도하는 세포내 활성 산소종이 중요한 조절자라는 것을 확인하였다. 그러나 wild type Rac1 유전자와 CA Rac1V12 돌연변이 유전자를 세포내 주입하여 발현 시켰을 때나, H_2O_2 를 단독으로 세포 외부에서 처리하였을 때 PC12 세포의 신경 돌기성장이 유도되지 않았다. 면역 염색법을 통해 Rac1 단백의 분포를 확인한 결과, STS와 Y-27632에 의해 유도된 신경 돌기 말단에 Rac1이 재분포 되는 것을 확인되었다. 또한 세포 형태 변화를 위해 새롭게 형성되는 actin 단백과 Rac1 단백이 신경 돌기 말단에 함께 분배되어 있었다. 결론적으로 신경 돌기 말단에 위치한 Rac1 단백의 활성과 그에 따른 활성 산소종의 생성이 STS와 Y-27632가 유도하는 PC12 세포의 신경 돌기 성장 기전에 있어서 중요한 역할을 하는 것으로 보인다.

핵심되는 말 : PC12 세포, Staurosporine, Y-27632, 활성 산소종, Rac1, 신경 돌기 성장