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EXPERIMENTAL STUDY

Phosphoinositid signal pathway mediate neurite outgrowth in PC12 cells by staurosporine

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Abstract: *Objectives:* In previous studies, we showed that staurosporine uses intracellular calcium ions to affect cell death in PC12 cells. The bulk release of intracellular excessive Ca^{2+} from intracellular sources into cytosol contributes to neuronal apoptotic events, which in turn results in neuronal cell death. However, the mechanisms of Ca^{2+}-induced neuronal cell death or neurite elongation is still unclear. Therefore, we investigated the relation between phosphoinositid signal pathway, intracellular calcium, and reactive oxygen species on one hand, with staurosporine-induced neurite outgrowth in PC12 cells on the other.
Results: The inhibition of phospholipase C or IP3 receptor antagonist or phosphoinositid signal transduction antagonist produced cell death and suppressed neurite outgrowth by staurosporine in PC12 cells. The inhibition of these enzymes and pathway results in an increase in intracellular Ca^{2+} although subsequent hydroxyl radical (•OH) production began after inhibitors exposure. •OH production was significantly attenuated in inhibitor supplemented medium treatment, and it was dependent on the intracellular Ca^{2+} concentration. These data indicate that staurosporine activates phosphoinositid signal pathway while endoplasmic $Ca^{2+,}$ and subsequent •OH production are critical events in staurosporine-induced neurite outgrowth in PC12 cells.
Conclusion: We conclude that the fact that staurosporine mobilizes $^{Ca2+}$, probably via activating the subcellular compartment, is responsible for staurosporine-induced (Ca2+]i increase during neurite outgrowth in PC12 cells *(Fig. 7, Ref. 30)*. Text in PDF *www.elis.sk.*
Key words: endoplasmic reticulum, neurite outgrowth, staurosporine, PC12 cells.

Staurosporine, a broad spectrum protein kinase inhibitor (1, 2), has been used to induce cell death in a wide range of cell types (3, 4), or neuronal differentiation at 50–350 nM concentration (5). While the exact mechanism responsible for staurosporine-induced neurite outgrowth is unknown, the exact mechanism responsible for staurosporine-induced cell death is known. Several authors have shown that the exact mechanism responsible for staurosporine-induced cell death in treated cells depends on calcium ions, but several controversial reports exist about the effects of staurosporine on extra or intracellular calcium sources on treated cells of various types. For example, it has been shown that staurosporine induces cell death by increasing cytoplasmic Ca^{2+} concentration, which in turn is responsible for releasing mitochondrial cytochrome C, caspase-3 activation (6), intracellular ROS accumulation (7–9), increase in $(Ca^{2+})_i$ (9) and cell death in various cell types (e.g. PC12 cells) (9, 10).

On the other hand, it has been shown that staurosporine dependent on concentration does not directly release intracellular-Ca^{2+} stores (11). We reported previously that staurosporine could induce neurite outgrowth in PC12 cells and that this apoptosis can be blocked by reducing extracellular calcium stores (12). In contrast, present data suggest that staurosporine can induce apoptosis in cultured cortical neurons and that this apoptosis can be blocked by raising intracellular Ca^{2+} (9). Previous studies showed that local and global elevations in the cytosolic Ca^{2+} level are achieved by ion release from intracellular stores and by influx from the extracellular milieu (13). The major intracellular Ca^{2+} store is the endoplasmic reticulum (ER). Ca^{2+} release is regulated by transmembrane channels on the Ca^{2+} store membrane that are formed by tetramers of inositol (1,4,5)-triphosphate receptor (IP3R) proteins (14–16). The bulk of IP3R channels mediate the release of Ca^{2+} from ER, the emptying of which signals Ca^{2+} influx (14, 16–18).

Further, the authors show that ER and mitochondria Ca2+ stores were shown to be generating inositol 1,4,5-trisphosphate (IP3) and causing the release of Ca^{2+} from ER which would also promote mitochondrial Ca^{2+} increase (19–21). In this study, we try to explain the possible role of Ca^{2+} and ROS in staurosporine-induced neurite outgrowth in PC12 cells.

Materials and methods

Cell culture

Cultures were grown in a 40-cm^2 tissue culture flask in RPMI culture medium supplemented with 10 % heat-inactivated horse serum and 5 % FBS, 1 % NEAA, 2mM L-glutamine, and 100 IU/ml penicillin and 100 µg/ml streptomycin. To obtain neuronally dif-

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ferentiated PC12 cultures, cells were grown on collagen-coated plates (10 µg/ml) supplemented with 2 % heat-inactivated horse serum, 10 % FBS, and 214 nM staurosporine for 24 h. Cultures were maintained at 37 °C in a humidified, 5 % CO_2 incubator. In differentiated PC12 cells, all experiments were performed in the presence of staurosporine to prevent the possibility of extracellular and intracellular Ca^{2+}-deprived inhibition of neurite outgrowth and induction of cell death signaling pathways.

Cell treatment

One day after plating the PC12 cells, the cells were washed with phosphate buffer saline (PBS) with pH 7.4. In order to inhibit phospholipase C, IP3 receptors, and phosphoinositid signal pathway, the cells were preincubated with added 10 µM U73122 for 30 min (treatment 1), 50 µM heparin for 30 min (treatment 2) and 10 mM neomycin sulfate for 30min (treatment 3). In our experiment, we combined all inhibitors for complete compartments of signal pathway inhibition (treatment4). Then, the cells were cultured in differentiation medium containing complete culture medium supplemented with 214 nM staurosporine for 24 h. PC12 cells cultured in differentiation medium without inhibitor preincubation serve as a control group. The cells were placed in the incubator at 37 °C with 5 % CO_2.

Measurement of cytotoxicity

Cell cytotoxicity was measured with LDH-cytoxicity Detection Kit (Roche., Germany). In each treatment, the percentage of cytotoxicity was measured according to the protocol of the company, and the colorimetery of LDH activity was measured by calculating the absorbance of samples at 490 or 492 nm. The reference wavelength should be more than 600 nm.

Quantification of cell death incidence

Hoechst / PI nuclear staining was carried out as previously described (12). Briefly, cells were plated in 24-well culture plates with 10^4 cells/mL density for 12 h. Then cells were pretreated with different treatment mediums for certain time. These were grown for a range of times in differentiation medium (6, 12 and 24 h). Then the cells were incubated for 15 min at 37 °C with Hoechst 33342 dye (10 mg/ml in PBS), and washed twice in PBS. PI (50 mg/ml in PBS) was added just before microscopy. Cells were visualized using an inverted-florescence microscope (Olympus IX-71, Japan). The apoptotic index was calculated by the fraction of numbers of apoptotic cells on the total cell count in 100 (~300 cells), respectively. All experiments were replicated independently at least 3 times. Within each experiment, we replicated each condition 4 times.

Measurement of neurite length, fraction of cell differentiation

Measurement of neurite length and fraction of cell differentiation were carried out as reported by recent studies (5, 23). Cells were plated at a density of 2×10^4 cells/well on 24-well tissue culture plates overnight. The culture media were replaced with different treatment media. After 5h, the cultured differentiated PC12 cells were fixed with 4%w/v Para formaldehyde. Extracellular Ca^{2+} were measured with a calcium ion atomic absorption method. Total neurite length (TNL) and the fraction of neurite-bearing cells (*f%*) was evaluated under an inverted microscope. TNL: Length of the largest neurite on individual PC12 cell was measured. More than 100 cells were analyzed in each well. $f\%=n_+/n_T$; n_+ and n_T are the numbers of neurite-bearing cells with at last one neurite longer than the cell body diameter, and total count (~300 cells). Each experiment was repeated five times.

Measurement of $(Ca^{2+})c$ and $(Ca^{2+})m$

Measurement of cytosolic $(Ca^{2+})c$ and mitochondrial $(Ca^{2+})m$ calcium concentration were carried out as reported by recent studies (6). For $(Ca^{2+})c$ measurements, PC12 cells were loaded with 4 µM fura-2 AM at 37 °C in a 5 % CO_2 incubator for 20 min in a HCO_3^- - buffered solution containing (110 mM NaCl, 4.5 mM KCl, 1 mM NaH_2PO_4, 1 mM $MgSO_4$, 1.5 mM $CaCl_2$, 5 mM HEPES-Na, 5 mM HEPES free acid, 25 mM $NaHCO_3$ and 10 mM D-glucose (pH 7.4)). Cells were then rinsed twice and incubated in the HCO_3^- - buffered solution for at least 20 min before use, while cells were superfused at a constant perfusion rate of 2 ml/min with the HCO_3^--buffered solution equilibrated with 95 % O_2 and 5 % CO_2 to maintain pH of 7.4. All experiments were performed at 37 °C. The excitation wavelength was alternated between 340 and 380 nm, and the emission fluorescence was recorded at 510 nm. $(Ca^{2+})c$ values were calculated using the equation described by Grynkiewicz (24). Relative $(Ca^{2+})m$ was measured with the fluorescent probe rhod 2-AM following methods described previously (25).

Measurement of antioxidant enzyme activities

Antioxidant enzyme activities and protein damage assay were carried out as previously described (26). Briefly, in order to visualize intracellular ROS and examine the effect of extracellular Ca^{2+} on ROS generation, cells were incubated with 214 nM staurosporine for 6 h, then washed three times with Krebs–Ringer–Hepes (KRH) buffer (136 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.25 mM $MgSO_4$, 20 mM Hepes, pH 7.4); 1 µM APF in KRH buffer was added, and cells were incubated for 1 h at 37 °C. Fluorescence (Ex. 490 nm and Em. 525 nm) was visualized using a fluorescence microscope.

Statistical analysis

Results are presented as the means ± SD of three or four independent experiments. When comparing two groups, unpaired Student's t-test was used to address the differences.

p-values less than 0.05 were considered significant.

Results

After 6h, cells in control group exhibited neuron morphology with long neurite outgrowth (Fig. 1). TNL control group was determined as 182±0.85µm, respectively, but in treatments 1-4 the corresponding values decreased (Fig. 2) ($p<0.05$). In addition to an increase in the length of neurite in control group, the neuronal network was seen to be producing in limited environment (Fig. 1).

After 6 h, the fraction of cell differentiation (f%) for control group was 100 %, but in treatments 1–4 were it decreased (96 %, 71 %, 67 % and 54 %, respectively) ($p<0.05$) (Fig. 3). After 24 h, the percentage of cell differentiation in treatments 1–4 compared with control group (98 %) were decreased ($p<0.05$). The documented percentages of cell differentiation after 24 h for treatments 1–4 were 81 %, 53 %, 52 % and 41 %, respectively ($p<0.05$) (Fig. 3). After 6 h, the percentage of cell death for control group was 16 %, but in treatments 1–4 it increased (26 %, 33 %, 38 % and 42 %, respectively) ($p<0.05$) (Fig. 4). After 24 h, the percentages of cell death in treatments 1–4 compared with control group (28 %) were increased ($p<0.05$). while the documented percentages of cell death after 24 h for treatments 1–4 were 46 %, 52 %, 57 % and 62 %, respectively ($p< 0.05$) (Fig. 4).

At the starting point of experiments, the percentage of cell cytotoxicity for PC12 cells were constant, namely 98 % and 99 %, respectively (Fig. 5). After 24 h, The percentage of cytotoxicity

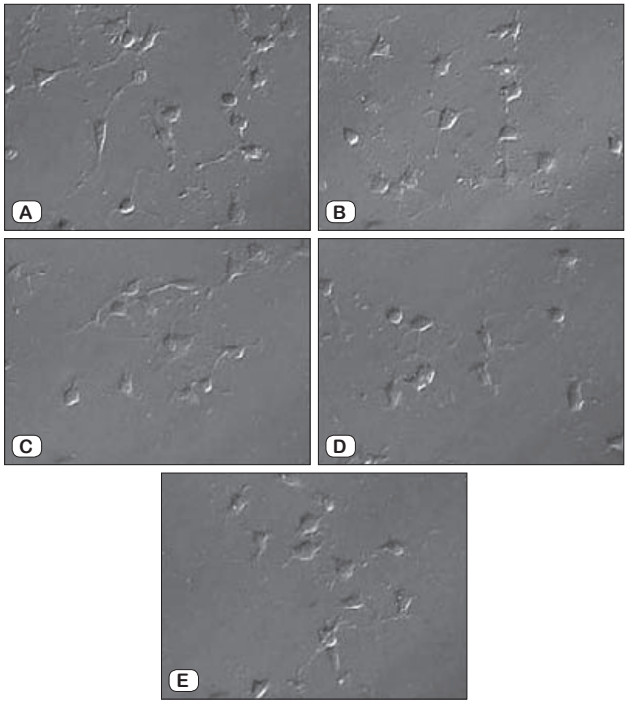

Fig. 1. PC12 cell differentiation induced by different treatment media. PC12 cells were grown on tissue culture plate in 10 % FBS – RPMI 1640 containing 214 nM staurosporine (Control group) at presence of different inhibitors (treatments 1–4) for 24 h. After 6 h, in control group, PC12 cells exhibited a neuron-like morphology with long neurite outgrowth with formed neuronal network (A). After 24 h, PC12 cells in treatments 1–4 did not exhibit an extent neuron-like morphology or neurite-like structure (B–E). After 24 h, treatments 1–4, compared with control group significantly suppressed the neurite in PC12 cells (B-E).
Control group: incubation with 214 nM staurosporine, (A): treatment 1: 10 µM U73122, 30 min, (B): treatment 2: 50 µM heparine, 30 min, (C): treatment 3: 10 mM neomycine sulfate, (D): treatment 4: combination of all inhibitors.

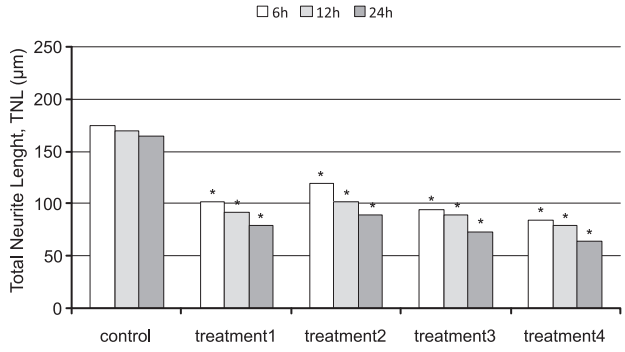

Fig. 2. Neurite length of PC12 cells treated in culture media containing 214 nM staurosporine at different treatment media (µm). PC12 cells were grown on tissue culture plate in 10 % FBS – RPMI 1640 containing 214 nM staurosporine (Control group) at presence of different inhibitors (treatments 1–4) for 6 h. After 24 h, treatments 1–4, compared with control group significantly suppressed the neurite in PC12 cells ($p<0.05$). Control group: incubation with 214 nM staurosporine, treatment 1: 10 µM U73122, 30 min, treatment 2: 50 µM heparine, 30 min, treatment 3: 10 mM neomycine sulfate, treatment 4: combination of all inhibitors. All data represented by mean ± standard.* $p<0.05$ as evaluated by paired ANOVA.

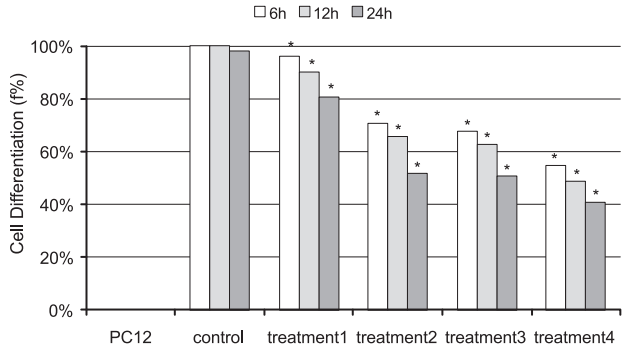

Fig. 3. The percentages of fraction of cell differentiation during PC12 cell differentiation induced by different treatment media. The fraction of cell differentiation for treatments 1–4 compared with control group was decreased ($p<0.05$). Control group: incubation with 214 nM staurosporine, treatment 1: 10 µM U73122, 30 min, treatment 2: 50 µM heparine, 30 min, treatment 3: 10 mM neomycine sulfate, treatment 4: combination of all inhibitors.
All data represented by mean ± standard.* $p<0.05$ as evaluated by paired ANOVA.

in control group compared with PC12 cells (6 %) increased up to 21 %, but in treatments 1–4 compared with PC12 cells and control group it was significantly increased ($p<0.05$) (Fig. 5).

Previous studies indicate that uncontrolled cytosolic or mitochondrial Ca^{2+} overload mediates staurosporine-induced cell death in neuronal cells (8, 9). In order to determine whether the inhibition of phosphoinositid signal pathway is essential for initiating staurosporine-activated cell death signaling, we analyzed $(Ca^{2+})c$ and $(Ca^{2+})m$ in treated cells.

Exposure of PC12 cells to 214 nM staurosporine in the absence of inhibitors had little effect on $(Ca^{2+})c$. In treatments 1–4,

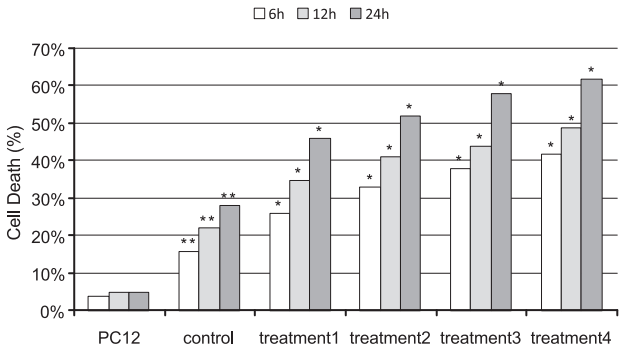

Fig. 4. LDH activities during PC12 cell differentiation induced by different treatment media for 24 h. The percentage of cell cytotoxicity in treatments 1–4 compared with was decreased ($p<0.05$). Control group: incubation with 214 nM staurosporine, treatment 1: 10 µM U73122, 30 min, treatment 2: 50 µM heparine, 30 min, treatment 3: 10 mM neomycine sulfate, treatment 4: combination of all inhibitors. All data represented by mean ± standard.* $p<0.05$ as evaluated by paired ANOVA.

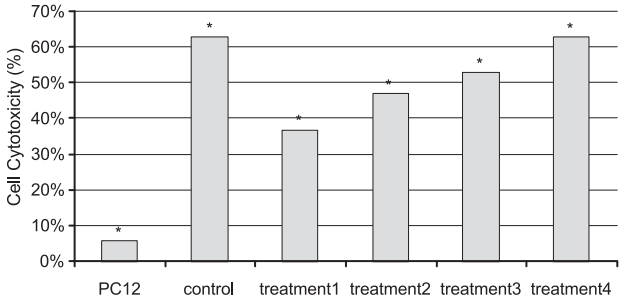

Fig. 5. The percentage of cell death during PC12 cell differentiation induced by different treatment media for 24 h. The percentage of cell death in treatments 1–4 compared with was increased ($p<0.05$). Control group: incubation with 214 nM staurosporine, treatment 1: 10 µM U73122, 30 min, treatment 2: 50 µM heparine, 30 min, treatment 3: 10 mM neomycine sulfate, treatment 4: combination of all inhibitors. All data represented by mean ± standard.* $p<0.05$ as evaluated by paired ANOVA.

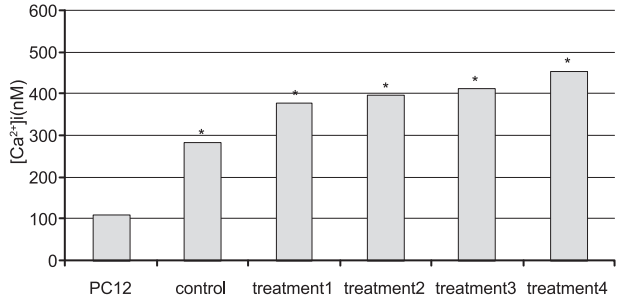

Fig. 6. Determination of $(Ca^{2+})i$ in staurosporine (STS)-treated PC12 cells for 6 h (nM). PC12 cells were incubated for 6 h in the absence (PC12 cells) or in the presence (control cells) of 214 nM STS at the presence of different inhibitors (treatments groups). Control group: incubation with 214 nM staurosporine, treatment 1: 10 µM U73122, 30 min, treatment 2: 50 µM heparine, 30 min, treatment 3: 10 mM neomycine sulfate, treatment 4: combination of all inhibitors. The results are expressed as the mean ± SEM of 4–6 experiments, run in duplicate. Statistical significance: * $p<0.005$ as compared to control cells.

the $(Ca^{2+})c$ were increased compared to control group and PC12 cells ($p<0.05$) (Fig. 6). Since staurosporine caused an early and sustained increase in $(Ca^{2+})c$ in treatment 1–4, we speculated that Ca^{2+} might have accumulated in mitochondria. In this section, we assessed changes in $(Ca^{2+})m$ microscopically in living cells loaded with the mitochondrial Ca^{2+} indicator Rhod 2-AM. Following the treatment inhibitors comparison with control cells, we observed a significant increase in $(Ca^{2+})m$ (Fig. 6).

Exposure of PC12 cells to 214 nM staurosporine in the absence of inhibitors had little effect on ROS (·OH) generation. In treatments 1–4, the (·OH) generation was increased compared with control group and PC12 cells ($p<0.05$) (Fig. 7). It is well shown that overload of cytosolic and mitochondrial Ca^{2+} causes enhanced accumulation and cytochrome c release in ROS pathway in treated cells.

Discussion

In this current study, we investigate the involvement of subcellular compartment which is responsible for the staurosporine-induced neurite outgrowth in PC12 cells. We also used PC12 cells as the best cell model for studying the effect of materials on neurite outgrowth (23). Staurosporine was employed as a strong inducer of neurite outgrowth with inhibition of protein kinases *in vitro* model. we also try to show that inhibition of PLC, IP3R or phosphoinositid signal pathway could effectively inhibit neurite outgrowth induced by staurosporine and increase cell death and cytotoxicity by increased ROS generation and $(Ca^{2+})i$ in PC12 cells.

We observed that when cells were preincubated with U73122, which is a specific phospholipase C antagonist, heparin, which is a specific IP3R antagonist, and neomycin, which is a specific phosphoinositid signal pathway antagonist, they dramatically

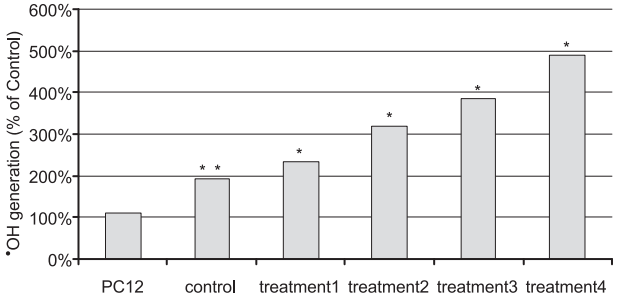

Fig. 7. Effect of antioxidants and reduce agents of endogenous reactive oxygen species (ROS) production on staurosporine (STS)-induced neurite outgrowth for 6 h (% control). PC12 cells were incubated for 6 h in the absence (PC12 cells) or in the presence (control cells) of 214 nM STS at the presence of different inhibitors (treatments groups). Control group: incubation with 214 nM staurosporine, treatment 1: 10 µM U73122, 30 min, treatment 2: 50 µM heparine, 30 min, treatment 3: 10 mM neomycine sulfate, treatment 4: combination of all inhibitors. The results are expressed as the mean ± SEM of 4–6 independent experiments, run in duplicate. Statistical significance: *$p<0.005$ as compared to control cells.

suppressed the neurite outgrowth and increased cell death and cytotoxicity in PC12 cells. It could be suggested that there is a possible involvement of IP3R and PLCγ in staurosporine-neurite outgrowth.

The results showed that staurosporine can regulate and decrease the intracellular calcium concentration by activating the phosphoinositid signal pathway. It was demonstrated that neurite outgrowth in PC12 by staurosporine is induced via the Ca^{2+}-signal transduction pathway by the Ca^{2+} influxes through endoplasmic reticulum channels. On the other hand, recent study showed that staurosporine leads to intracellular calcium overload, which induces apoptosis in PC12 cells (6). In the percent study, we showed that inhibition of IP3R and PLCγ caused a large increase in (Ca^{2+})c, IP3-sensitive Ca^{2+} store, and ROS generation in PC12 cells. This result indicates that IP3-sensitive ER compartments are responsible for staurosporine-induced (Ca^{2+})c increase in PC12 cells.

We reported previously that staurosporine can induce neurite outgrowth in PC12 cells and that this apoptosis can be blocked by reducing extracellular calcium stores (12). In addition, it is known that cytosolic Ca^{2+} increase caused by staurosporine, which in turn mobilizes Ca^{2+} from different sources, might cause apoptosis in astrocytes (27).

In accordance with these results, we detected inhibition of IP3R and PLCγ, as well as increase in intracellular Ca^{2+} (mitochondrial and cytosolic), which then activates ROS (·OH generation) signal pathway (Figs 6 and 7).

In this work, the inhibition of IP3R and PLCγ significantly increased the production of intracellular peroxides, while increasing the intracellular Ca^{2+} (mitochondrial and cytosolic) triggered by staurosporine in PC12 cells. Moreover, the results strongly suggest that ROS generation contributes to this apoptotic and neurite outgrowth suppression mechanism.

On the other side, previous studies demonstrated that intracellular ROS production induced by staurosporine can result from interrupted mitochondrial electron flow as a consequence of cytochrome *c* release (28, 29). However, endoplasmic reticulum has not been investigated as a source of Ca^{2+}.

In this study we showed that inhibition of IP3R and PLCγ caused endogenous ROS production in staurosporine-induced neurite outgrowth resulting in an increase in cell death in PC12 cells. However, authors showed that ROS can interfere with the mechanisms responsible for intracellular Ca^{2+} regulation (30) and staurosporine was previously demonstrated to induce a rise in intracellular Ca^{2+} (9).

In the present study, we show that inhibition of IP3R and PLCγ were shown to attenuate the rise in intracellular calcium triggered by staurosporine thus strongly suggesting that ROS generation can potentiate the observed intracellular calcium overload.

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