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Pinealon Increases Cell Viability by Suppression of Free Radical Levels and Activating Proliferative Processes

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Abstract

The synthetic tripeptide pinealon (Glu-Asp-Arg) demonstrates dose-dependent restriction of reactive oxygen species (ROS) accumulation in cerebellar granule cells, neutrophils, and pheochromocytoma (PC12) cells, induced by oxidative stress stimulated by receptor-dependent or -independent processes. At the same time, pinealon decreases necrotic cell death measured by the propidium iodide test. The protective effect of pinealon is accompanied with a delayed time course of ERK 1/2 activation and modification of the cell cycle. Because restriction of ROS accumulation and cell mortality is saturated at lower concentrations, whereas cell cycle modulation continues at higher concentrations of pinealon, one can conclude that besides its known antioxidant activity, pinealon is able to interact directly with the cell genome.

Introduction

Under the influence of environmental factors, emotional stress, and/or progression of age pathologies, the regulation of body functions is disturbed. The role of regulatory neuropeptides in the formation of the adaptive response of an organism to stress and the disturbance of homeostasis is now discussed broadly.[1] The endogenous components of living cells, peptide bioregulators, demonstrate diverse biological effects; they are effective at low concentrations and show no side effects.[2–4] However, their therapeutic use is limited by their permeability through the blood–brain barrier, relatively rapid metabolization, and effect on the immune system. Short synthetic analogs of neuropeptides that preserve their specific activity do not have these restrictions. Among these short peptides, which are potential modulators of regulatory functions, is the tripeptide pinealon (Glu-Asp-Arg), which has been synthesized after analysis of amino acid composition of bovine brain extracts.[5] The Glu-Asp-Arg sequence is the most common motif in a complex peptide from the cerebral cortex called cortexin that demonstrates neuroprotective properties.[6] This compound was synthesized recently and was found to be capable of stimulating neuronal regeneration[5] and protecting brain neurons from hypoxia.[7] The aim of present study was to characterize the effect of pinealon on cell metabolism under oxidative stress conditions *in vitro*.

Materials and Methods

Three different kinds of cell preparations were used in these experiments: (1) Granule cells isolated from cerebellums of 10- to 12-day-old rats, (2) neutrophils isolated from peripheral blood of intact adult rats, and (3) a commercially available culture of pheochromocytoma (PC12) cells. Cerebellar granule cells represent a standard model for the study of oxidative stress,[8] including their induction of the intracellular accumulation of reactive oxygen species (ROS).[9,10] In contrast, when neutrophils are activated, they generate ROS in the surrounding medium.[11] We have used these two models to estimate the ability of pinealon to diminish the levels of free radicals both inside and outside the cells. To estimate the effect of pinealon on cell cycle division, PC12 cells were used because they are characterized precisely in the literature and the role of ROS in regulation of the PC12 cell cycle is well known.[12]

Experiments using Wistar rats were carried out according to international rules of working with laboratory animals (http://www.nap.edu/books/0309083893/html/R1.html).

Dissociated cerebellar granule cells

Cerebellar granule cells were derived from 7- to 10-day-old pups. A suspension of surviving neurons was obtained after treatment of cerebellar slices by dispase, followed by a wash with standard Tyrode solution (148 mM NaCl, 5 mM

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KCl, mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4) and filtration through a 53-μm nylon filter.[10] Oxidative stress was induced in the presence or absence of pinealon by a 30-min exposure of the cells to 100 nM ouabain or 500 μM homocysteine (HC). Both compounds are known to be able to induce oxidative stress in cerebellum granule cells by interacting with specific receptors on the neuronal membrane. Ouabain affects sodium/potassium–adenosine triphosphatase (Na/K-ATPase) of the outer cell membrane.[13,14] HC is a congener of *N*-methyl-D-aspartate (NMDA), and it acts via the glutamate receptor of the NMDA class.[15] In both cases, an increase in intracellular ROS levels and activation of the key enzyme of the mitogen-activated protein kinase (MAPK) cascade ERK1/2 kinase (extracellular regulated kinase, isoforms 1 and 2) is observed.[14,15] Each ligand was used in a concentration sufficient to induce oxidative stress *via* intracellular accumulation of ROS.[14,15] The cell suspension was incubated with ouabain or HC (in the presence or absence of pinealon), and then flow cytometry analysis was performed.

Primary culture of cerebellar granule cells

Cerebellums from 7- to 10-day-old rats were washed with cold Hanks' solution (PanEko, Russia) and incubated 20 min with 0.05% trypsin solution (PanEko, Russia). The neurons were then washed and cultivated for 11 days in Neurobasal™-A cultivation medium, with 2% Supplement B-27 used as a serum substitute (Invitrogen, USA), GlutaMax (Invitrogen, USA), 50 U/mL penicillin, 50 U/mL streptomycin, and 20 mM KCl (at 5% CO_2 and 37°C). Before the following experiment, cells were removed from the substrate with trypsin-EDTA (PanEko, Russia) and exposed to 500 μM HC for 5–30 min in the presence or absence of pinealon. Then cell suspension was used in western blotting experiments.

Experiments with neutrophils

Before the experiment, adult rats weighing 200–250 grams were treated with chloral hydrate (500 mg/kg intravenously). A blood sample was collected from the jugular vein in a heparin-containing syringe (Spofa, Czech Republic, 50 U/mL blood). Intact neutrophils were obtained by centrifuging the blood samples in MonoPoly medium (ICN Biomedicals, USA) and suspending them in Hanks' solution. Neutrophils were activated by adding of 2 mg/mL zymosan to the incubation medium.

To obtain a suspension of neutrophils activated *in vivo* (peritoneally induced neutrophils), animals were treated with 500 μL of zymosan suspension (4.5 mg/mL of Hanks' solution, intraperitoneally) to induce a local inflammatory area. Activated neutrophils were isolated from peritoneal fluid, and a cell suspension with a purity of 95–98% was obtained. The cells were sedimented by centrifugation, resuspended in 1 mL of Hanks' solution, and stored at 37°C for up to 3 hr.[16]

The functional activity of neutrophils was evaluated by production of ROS, measured as a chemiluminescence response in the presence of 1 mM luminol (Sigma-Aldrich, Germany) on a SmartLum 5773 chemiluminometer (St.-Petersburg, Russia). Neutrophils were activated using HC (Sigma-Aldrich, Germany). In preliminary experiments, we tested the effect of HC on the chemiluminescence of intact cells in the range of 5–30 min and found no effect in this time interval. Thus, for characterization of HC action, we used 30 min of incubation.

PC-12 cell culture

Cells were cultured in 75-mm^3 flasks in RPMI-1640 medium (PanEko, Russia) supplemented with 10% fetal calf serum (FCS; PanEko, Russia), 0.2 mg/mL L-glutamine (PanEko, Russia), and 20 mg/mL gentamicin at 5% CO_2 and 37°C. Cells were removed from the substrate with trypsin-EDTA (PanEko, Russia).

Measuring the levels of free radicals in cerebellar neurons

To determine the intracellular ROS levels, cerebellar granule cells were loaded with the fluorescent dye 2,7-dihlorodihydrofluoresceine-diacetate (DCF-DA; Molecular Probes, USA) at a final concentration 100 μM.[8] Cells were removed from the substrate, washed free of trypsin, and resuspended in Hanks' solution. They were then placed in 1.5-mL Eppendorf tubes, allowed to rest for 30 min, and incubated for 40 min with DCF-DA. After dye loading, the cells were exposed for 20 min to 1 mM hydrogen peroxide (H_2O_2; Sigma, Germany) in the presence or absence of pinealon (Garmonika, Russia) at concentrations noted in the legends to the figures. For 1 min before the measurement, the samples were supplemented with 10 μM propidium iodide (PI; Sigma, Germany). Measurements were performed on BD FACSCalibur flow cytometer (Becton, Dickinson, USA) after gating the cell population corresponding in size to neuronal cells.[8–10]

Western blotting

The activity of ERK 1/2, which is tightly connected with cell viability,[17] was measured as a ratio between the phosphorylated form of ERK 1/2 and an endogenous control (total actin level). Quantification was performed using western blotting. Cells were washed with cold Hanks' solution and were lysed using RIPA buffer (Sigma-Aldrich, USA). The lysates were then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 10% separating and 6% concentrating gels. A prestained protein molecular weight marker (Fermentas, Germany) was run in parallel. After SDS-PAGE, the proteins were electroblotted onto polyvinylydine difluoride (PVDF) membranes (Thermo Scientific, USA). Detection of proteins was performed as described by the manufacturers of the antibodies anti-ERK 1/2 (Thr202/Tyr204) (1:1,000, Cell Signaling Technology, USA), anti-phospho-ERK 1/2 (Thr202/Tyr204) (1:1,000, Cell Signaling Tecnology, USA), and anti-pan-actin (1:2,000 Cell Signaling Tecnology, USA). Secondary antibodies were conjugated with horseradish peroxidase (HRP; 1:1,000, Cell Signaling Tecnology, USA). Membranes were visualized via enhanced chemiluminescence (ECL) with SuperSignal West Femto solution (Thermo Scientific, USA). Data were analyzed using TotalLab Quant software (TotalLab Limited, UK).

Study of the PC12 cell cycle

To measure the parameters of the cell cycle, cells were stained with PI.[18] Cells were removed from the substrate,

washed free of trypsin, and then resuspended in phosphate-buffered saline (PBS; PanEko, Russia); fixation was performed with 70% ethanol (at −20°C overnight). The next day, cells were washed free of ethanol with PBS, and staining was performed in citrate buffer containing 100 μg/mL RNase A from bovine pancreas (Sigma, Germany) (40 mg PI per 1 million cells, 40 min in the dark at 37°C).

Results

As shown earlier, incubation of neurons with a specific inhibitor of Na/K-ATPase ouabain results in accumulation of free radicals and activation of ERK 1/2 kinase,[14] which induces switching on of the so-called early response genes.[18] Incubation of DCF-DA pre-loaded rat cerebellum granule cells with 100 nM ouabain causes an increase in their fluorescence, which is a sign of increasing intracellular levels of free radicals (Fig. 1).

Addition of pinealon to the incubation medium at different concentrations leads to suppression of the level of free radicals in a dose-dependent manner (Fig. 1), which corresponds to the earlier described antioxidant action of pinealon.[19] A 100 nM concentration of pinealon is enough to prevent ouabain-induced ROS accumulation completely. Thus, pinealon in a dose-dependent manner prevents an increase in the ROS accumulation induced by ouabain. When cerebellum cells were incubated with 500 μM HC under the same conditions as above, the stationary ROS level was increased by 92 ± 5%, and this activation was abolished in the simultaneous presence of HC and pinealon (500 nM).

It is known that ROS can act as a secondary messenger in the cells, triggering cascades of cellular signaling, in particular, that of MAPK pathway ERK 1/2.[18,20] Therefore, we evaluated the effect of pinealon on the neuronal level of ERK 1/2 which is activated by HC (Fig. 2). In control samples containing only HC, activation of ERK 1/2 is observed within 2.5 min, whereas in the presence of HC and pinealon, an increase in the level of active forms of ERK 1/2 occurs 20 min later. Thus, pinealon has a suppressing effect on activation of ERK 1/2 in rat cerebellar granule cells exposed to HC.

The ability of pinealon to suppress intracellular levels of free radicals in neurons indicates its possible effects on the other ROS-generating systems, especially those that generate them for extracellular use. To this purpose, we investigated the ability of pinealon to affect the respiratory burst of neutrophils activated by two different modes—*in vitro* by adding zymosan to the reaction medium with freshly isolated neutrophils, and *in vivo* by measuring the chemiluminescence response of neutrophils isolated from rats after 24-hr of intraperitoneal administration of zymosan, as described above. Intraperitoneal administration of zymosan to intact animals promotes inflammation, which accumulates intraperitoneally induced neutrophils. Thus, the cells prepared after such a procedure are characteristic of high stationary levels of ROS production.

Figure 3 shows that under the conditions used both neutrophils activated by zymosan *in vitro* (Fig. 3A) and those prepared from zymosan-treated animals (Fig. 3B) demonstrate a similar ability to generate a chemiluminescent signal. In both models of neutrophil activation, pinealon showed a dose-dependent ability to inhibit accumulation of ROS, and its effect was carried out in the same concentration range (Fig. 3).

In both previous models of oxidative stress, we observed no cell death. In further experiments on pheochromocytoma PC12 cell cultures, we used a less physiological, but a stronger, stress agent, H_2O_2, which induced not only ROS accumulation but also cell death. Exposure of PC12 cells to 1 mM H_2O_2 for 20 min resulted in a five-fold increase in ROS levels (Fig. 4). Preincubation of the cells with pinealon for 60 min decreased the accumulation of ROS induced by H_2O_2 (Fig. 4, gray bars). At the same time, pinealon had no significant impact on the level of ROS in intact cells (Fig. 4, white bars).

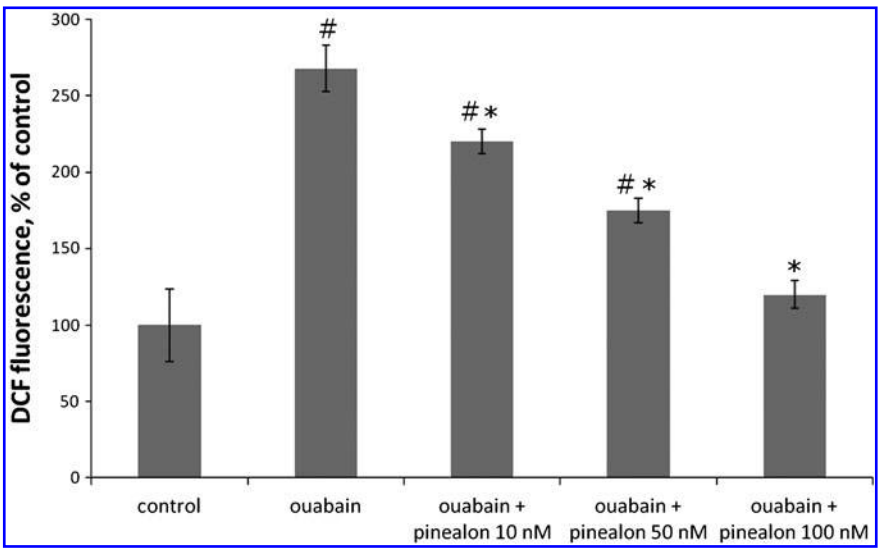

FIG. 1. Pinealon restricts reactive oxygen species (ROS) accumulation in cerebellar granule cells induced by 30 min of incubation of cells with 100 nM ouabain. (#) Significant difference in relation to control ($p < 0.05$; (*) significant difference in relation to samples with ouabain ($p < 0.05$). CDF, 2,7-Dihlorodihydrofluoresceine.

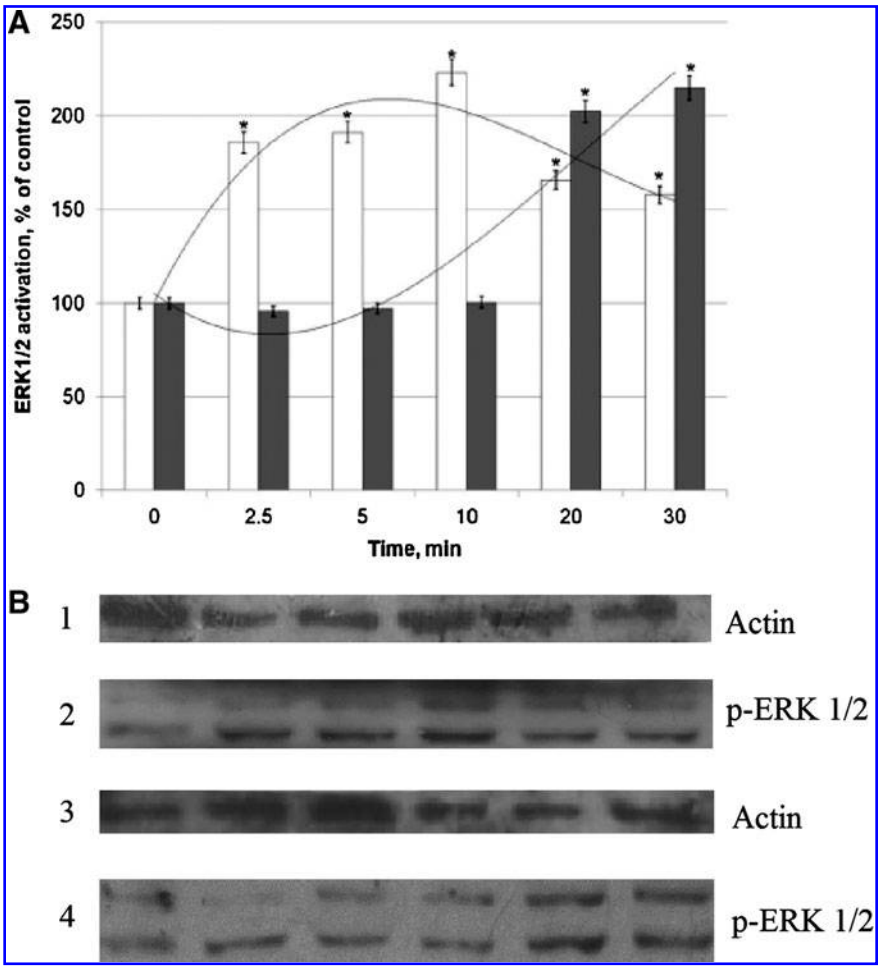

FIG. 2. Effect of pinealon (10 nM) on activation of ERK 1/2 in cerebellar granule cells in the presence of 500 μM homocysteine (HC). **(A)** Results of analysis. **(**White bars) Activation of ERK 1/2 after incubation of cells in the presence of HC; (gray bars) the same in the presence of HC and pinealon. (*) Significant difference between groups with $p < 0.05$. (**B**) Western blots; rows 1 and 2, samples with HC (correspond to white bars); rows 3 and 4, samples with HC and pinealon (correspond to grey bars).

Under these conditions, incubation of cells with 1 mM H_2O_2 resulted in a loss of about half of the entire cell population, while increasing concentrations of pinealon progressively increased the proportion of cells remaining alive, despite the presence of H_2O_2 in the medium (Fig. 5). Thus, pinealon neutralizes effects of toxic compounds that stimulate the development of oxidative stress and protects cells from necrotic death.

It is known that the intracellular ROS level affects distribution of cells between different phases of cell cycle[18]; i.e.,

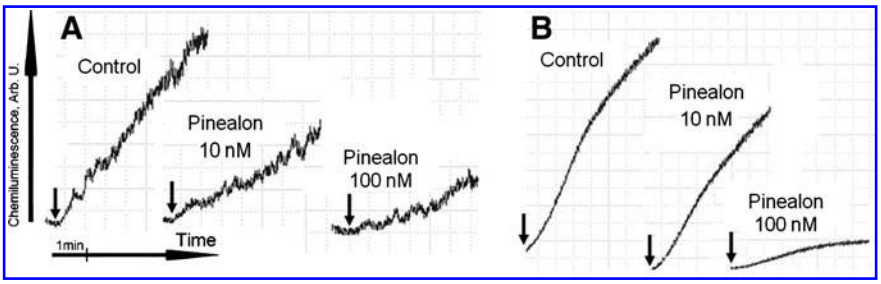

FIG. 3. Effect of pinealon on generation of free radicals by rat neutrophils activated *in vitro* (**A**, arrows correspond to adding zymosan), and *in vivo* (**B**, arrows correspond to start of measurement).

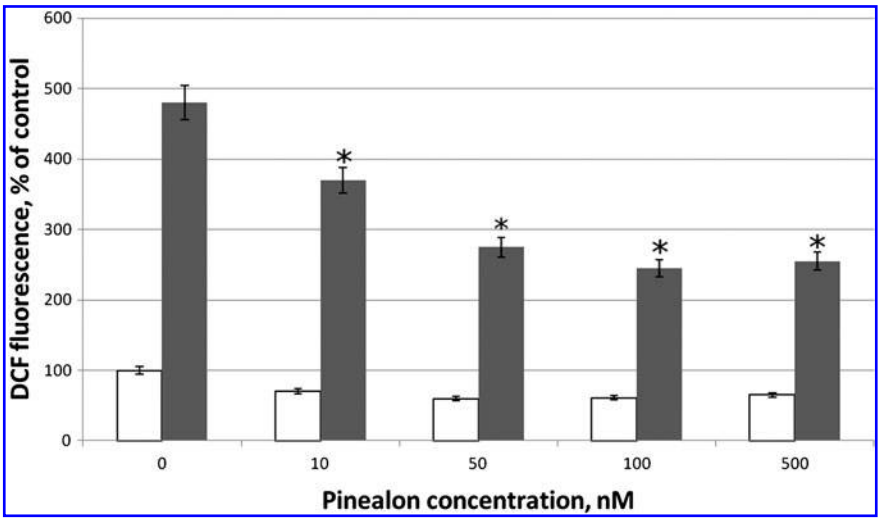

FIG. 4. Effect of pinealon on intracellular reactive oxygen species (ROS) levels in PC12 cells, as measured in the presence or absence of 1 mM hydrogen peroxide (H_2O_2). (White bars) Sample containing only pinealon at various concentrations as indicated; (grey bars) samples containing the same concentrations of pinealon and 1 mM H_2O_2, the incubation time of 60 min. (*) Significant difference from the sample containing H_2O_2. alone ($p < 0.05$). DCF, 2,7-Dihlorodihydrofluoresceine.

ROS are capable of regulating mechanisms that promote the cell cycle from one stage to another.[20] Taking into account the ability of pinealon to reduce intracellular levels of free radicals, we studied its possible effect on the advancement of cells through the cell cycle. Using flow cytometry, we have demonstrated that a 24-hr incubation of PC12 cells in pinealon-containing medium leads to a distinct redistribution of cells through the phases of the cell cycle (Fig. 6A and B). This effect has a clear dose dependence: An increase in the concentration of pinealon from 50 to 500 nM leads to a decrease the number of cells in the G_1 phase and an increase in the number of cells in the G_2 and S phases, indicating the modulating effect of pinealon on the proliferative activity of cells (Fig. 6C).

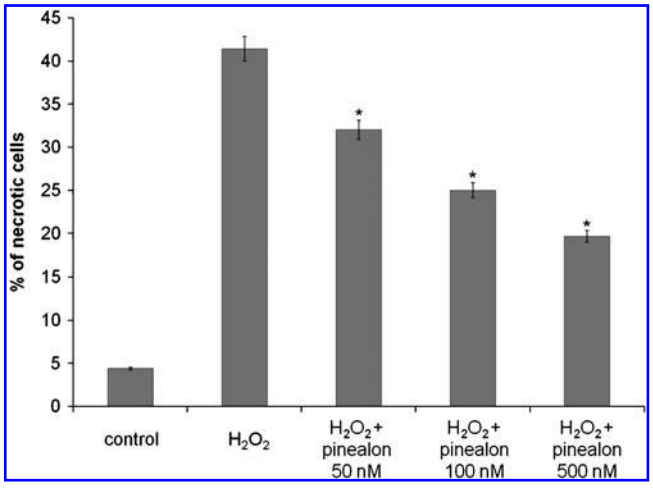

FIG. 5. Influence of pinealon on cellular death of PC12 cells after 30 min of exposure to 1 mM hydrogen peroxide (H_2O_2). (*) Significant decrease from sample with H_2O_2 ($p < 0.05$).

Discussion

It was shown earlier that pinealon has a pronounced antihypoxic effect on neurons that is explained by restriction of excitoxicity of NMDA and by inhibition of ROS accumulation.[7] Pinealon also stimulates the activity of the antioxidative enzymes superoxide dismutase and glutathione peroxidase in the rat brain under hypobaric hypoxia.[19] These data point out the ability of pinealon to diminish oxidative stress.

In this paper, we studied the effects of a synthetic tripeptide pinealon on properties of living cells. Using rat cerebellar granule cells, we have demonstrated the ability of pinealon to reduce stationary ROS levels caused by the action of both receptor-dependent (ouabain, HC) and non-receptor (H_2O_2) activators of oxidative stress. On surviving cultures of neutrophils activated by zymosan, we also showed the ability of pinealon to reduce ROS production during the inflammatory response.

ROS take part in a number of physiological processes, such as inflammation, apoptosis, neoplastic transformation, and aging. In recent years, the amount of evidence regarding the functioning of ROS as second messengers has increased.[21,22] It is known that the redox potential of the cells changes as they move through several phases of cell cycle. For example, in the G_1 phase, ROS control the activity of cyclin-dependent kinases (CDKs) and phosphorylation of the retinoblastoma protein (pRb), thereby adjusting the entrance into the S phase of the cell cycle. Induction of oxidative stress often leads to cell cycle arrest in G_2. Thus, one can assume that adjusting the ROS level within proliferating cells by specific substances can affect the cell function.

Comparison of the effect of pinealon on ROS accumulation and cell death in cultured PC12 cells demonstrated that the increase in survival roughly corresponded to suppression of ROS levels. At the same time, it is seen from comparison of Figs. 4 and 5 that when pinealon concentration reaches from

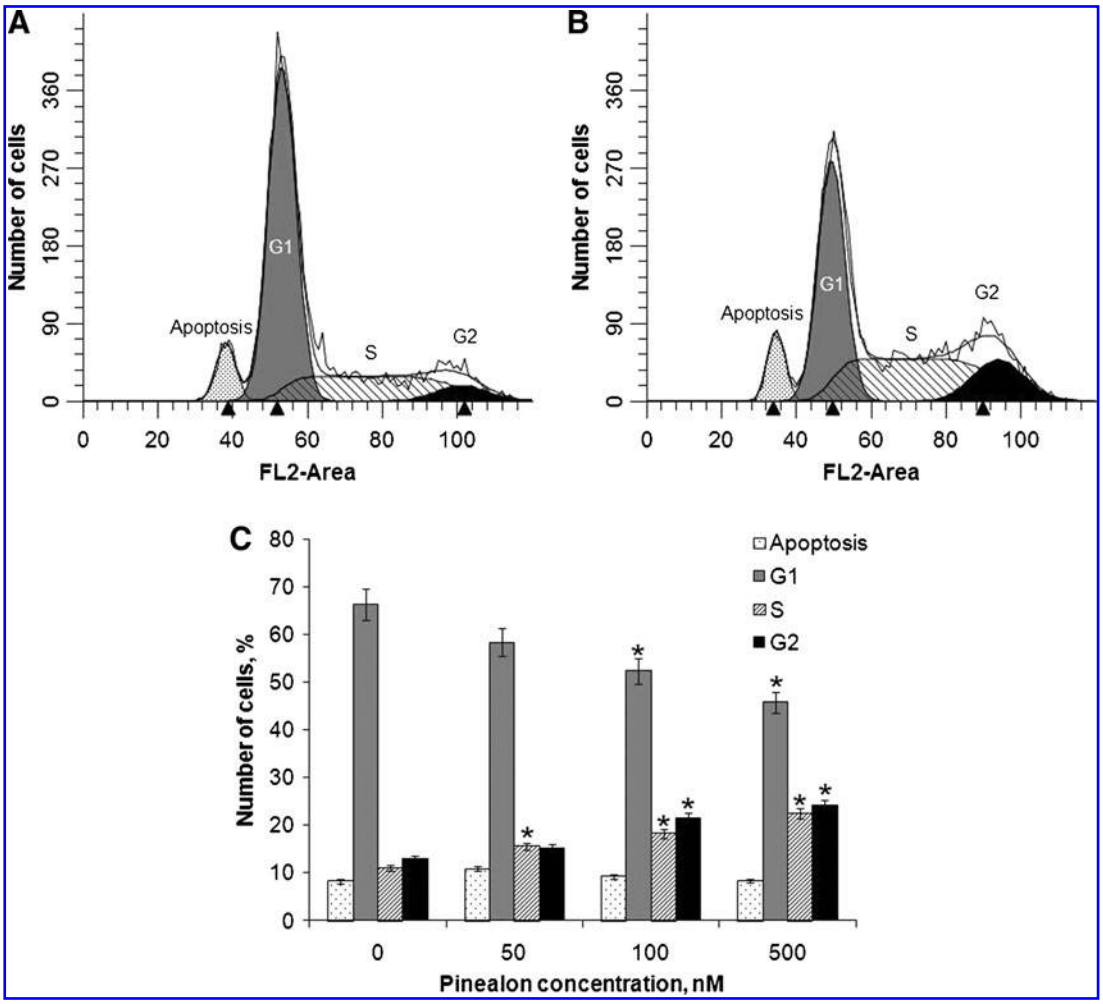

FIG. 6. Effect of pinealon on cell cycle of PC12. (**A**) Distribution of cells between different stages of cell cycle (as indicated) in intact cells. (**B**) Distribution of cells between different stages of cell cycle after 24 hr of incubation in the presence of 100 nM pinealon. (**C**) Effect of different concentrations of pinealon on distribution of PC12 cells by stages of the cell cycle. The data are processed using the program ModFit LTTM. (*) Significant difference from control with $p < 0.05$.

100 nM to 500 nM, the ROS level stops decreasing whereas the number of dead cells continues to decelerate. This observation suggests some additional points regarding the pinealon effect.

We have also showed that simultaneously with suppression of ROS accumulation pinealon delays the time course of ERK 1/2 activation. Such an effect is in a good correlation with its action on cell cycle division, suggesting its possible influence on complex intracellular processes. It is noteworthy that when pinealon concentration increases from 100 nM to 500 nM (which corresponds to saturating area for ROS accumulation) both necrotic death prevention and modulation of cell cycle continue. This observation is in accordance with data concerning the protective action of pinealon on neuronal survival at oxidative stress levels induced by hypoxia[19] and suggests some additional mechanisms of its antioxidant action.

It suggests that, in addition to the antioxidant effect resulting in ROS neutralization, pinealon may act directly with the cell genome and/or gene expression factors. Our paper is the first demonstration that pinealon is able to modulate ERK 1/2 activity and thus activate proliferative processes in PC12 cell culture, thus showing pinealon to be a useful tool for modulating cell metabolism.

Acknowledgments

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Author Disclosure Statement

The authors have no conflict of interests to disclose.

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