



Neurosteroids enhance the viability of staurosporine and doxorubicin treated differentiated human neuroblastoma SH-SY5Y cells

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Abstract:

Previously, we found that neurosteroids inhibited hydrogen peroxide- and staurosporine-induced damage of undifferentiated human neuroblastoma SH-SY5Y cells. However, differentiated neuroblastoma cells morphologically and functionally resemble neuronal cells, and are thus considered to be a model system for studying neuronal apoptotic processes. In the present study, we examined the effects of dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS), and pregnenolone (PGL) on the viability of retinoic acid-differentiated human neuroblastoma SH-SY5Y cells. Mitochondrial and extracellular apoptotic processes in these cells were induced by staurosporine and doxorubicin, respectively. Calcein viability assays showed that doxorubicin (0.5 μM for 24 h) decreased cell viability by ca. 20% as compared to control cultures. DHEA and DHEAS at 0.1 and 1 μM concentrations, respectively, significantly inhibited the doxorubicin toxicity. PGL showed a neuroprotective effect only at 0.1 μM , whereas it was inactive at a higher concentration (1 μM). Staurosporine (1 μM for 24 h) decreased SH-SY5Y cell viability by ca. 50%. DHEA (0.1 and 1 μM) and DHEAS (0.1 and 1 μM) significantly antagonized the toxic effects of staurosporine, whereas these compounds showed no activity at the lowest concentration (0.01 μM). PGL inhibited the staurosporine-induced decrease in cell viability only at the concentration of 0.1 μM . Since staurosporine generated a stronger detrimental effect on SH-SY5Y cell viability than doxorubicin, we studied the mechanisms of neurosteroid action only in the former model. Staurosporine (1 μM for 24 h) enhanced lactate dehydrogenase (LDH) release by ca. 40% and this effect was inhibited by DHEA (0.01, 0.1, and 1 μM), DHEAS (0.1 and 1 μM) and PGL (0.01 and 0.1 μM). In order to verify an involvement of phosphatidylinositol-3-kinase (PI3-K) in the antiapoptotic action of neurosteroids, a specific inhibitor of this protein kinase (LY 294002 at 10 μM) was used. Pretreatment of the cells with LY 294002 antagonized the ameliorating effects of DHEA, DHEAS, and PGL on staurosporine-induced LDH release.

These data indicated that at physiological concentrations, DHEA, DHEAS, and PGL prevented RA-differentiated SH-SY5Y cell damage produced by activation of both mitochondrial and extracellular apoptotic pathways. Furthermore, this study confirmed that the neuroprotective effects of neurosteroids in a staurosporine model of cytotoxicity appeared to be dependent upon PI3-K activity.

Key words:

dehydroepiandrosterone, pregnenolone, staurosporine, doxorubicin, calcein, lactate dehydrogenase, phosphatidylinositol-3-kinase, differentiated SH-SY5Y cells

Introduction

The precursors of steroid hormones can serve as excitatory neurosteroids, which are synthesized *de novo* in brain tissue mainly from cholesterol, e.g. pregnenolone (PGL) or dehydroepiandrosterone (DHEA) [17]. These compounds accumulate in the brain and modulate its function predominantly in non-genomic ways by allosteric modulation of GABA_A receptors, NMDA receptors, σ receptors, and voltage-dependent calcium channels [7, 13–15]. Pharmacological studies have demonstrated that excitatory neurosteroids enhance cognitive processes and facilitate memory, but some may provoke seizures at high doses [20]. Under some conditions, excitatory neurosteroids, especially PGLS, aggravate glutamate- and NMDA-induced neurotoxicity in cortical and hippocampal neurons [23, 25]. Moreover, proapoptotic effects of dehydroepiandrosterone sulfate and pregnenolone sulfate in neural precursor cells have also been demonstrated [27]. On the other hand, despite the ability to enhance NMDA receptor activity, prevailing evidence indicates that excitatory neurosteroids protect neurons against a variety of damaging agents in both *in vivo* and *in vitro* models. Accordingly, DHEA was shown to ameliorate hippocampal and cortical neuronal damage induced by glutamate receptor agonists, free radicals, glucocorticoids, or hypoxia [1, 9, 10]. In addition, PGL was shown to attenuate glutamate and β -amyloid-mediated toxicity in primary hippocampal cell cultures, and to facilitate recovery after spinal cord injury [4, 5]. The mechanisms responsible for the neuroprotective effects of excitatory neurosteroids are only partially known. However, accumulating evidence suggests that these compounds may not only interfere with necrotic, but also with apoptotic cell death. Recently, we have found that DHEA, DHEAS, and PGL protect undifferentiated human neuroblastoma SH-SY5Y cells against hydrogen peroxide- and staurosporine-induced toxicity. In addition, excitatory neurosteroids may affect the toxicity evoked by inducers of the death receptor signaling pathway such as doxorubicin [2, 6, 18]. Moreover, differentiated neuroblastoma cells are considered to be a better model for studying the mechanisms of neuronal degeneration than undifferentiated ones because they morphologically and functionally resemble primary neuronal cultures [19]. Therefore, in the present study we examined the effects of DHEA, DHEAS, and PGL on staurosporine-

and doxorubicin-evoked decreases in the viability of retinoic acid differentiated human neuroblastoma SH-SY5Y cells. Additionally, since previous reports have indicated an involvement of phosphatidylinositol-3-kinase (PI3/K) in the antiapoptotic action of neurosteroids, the effect of a specific inhibitor of this protein kinase (LY 294002) on DHEA-, DHEAS-, and PGL-treated cells undergoing staurosporine-induced changes in lactate dehydrogenase (LDH) release was evaluated.

Materials and Methods

Cell culture

The SH-SY5Y neuroblastoma cell line was obtained from the American Type Culture Corporation (ATCC). Cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Germany) supplemented with 10% fetal bovine serum (FBS, Gibco-BRL), 100 units/ml of penicillin, and 100 μ g/ml of streptomycin (Sigma Co., St. Louis, MO, USA), and kept in a humidified atmosphere of 5% CO₂/95% O₂ at 37°C. Cells were differentiated by adding retinoic acid (RA) to the culture medium to a final concentration of 10 μ M. Cells were differentiated for 7 days and the medium was changed every 3 days.

Treatment of cells

The RA-differentiated SH-SY5Y cells were treated with dehydroepiandrosterone, dehydroepiandrosterone sulfate, or pregnenolone (Sigma Co.) at concentrations from 0.01 to 1 μ M. One hour later, staurosporine (1 μ M; Sigma Co.) or doxorubicin (0.5 μ M) was added and the cells were cultured for 24 h.

Subsequently, cells were treated with LY 294002 (10 μ M; Sigma Co.), an inhibitor of PI3-K. Thirty minutes after addition of the protein kinase inhibitor, cultures were treated with neurosteroids. One hour later, staurosporine (1 μ M) was added and cells were cultured for 24 h.

LY 294002 and staurosporine were dissolved in DMSO (the final concentration of DMSO was 0.5%), neurosteroids in ethanol/water mixture, and staurosporine in water. The control cultures were treated with the appropriate amount of vehicle.

Lactate dehydrogenase (LDH) assay

Toxicity was quantified by measuring the efflux of lactate dehydrogenase (LDH) into the culture media 24 h after staurosporine treatment. LDH activity was determined in the medium using a colorimetric method (Cytotoxicity Detection Kit, Roche Diagnostic GmbH). In brief, the amount of colored hydrazone formed by the reaction of pyruvic acid with 2,4-dinitrophenylhydrazine was inversely proportional to the LDH activity in the sample and could be quantified by measuring the absorbance at 400–550 nm.

Calcein test for cell viability

After a 24 h treatment with staurosporine or doxorubicin, the cells were incubated with calcein AM (Trevigen, USA) at 37°C for 30 min. Next, the red fluorescence was measured with a fluorescence plate reader at 490 nm (excitation) and 520 nm (emission) wavelengths. Calcein AM is a non-fluorescent compound that easily enters live cells and is hydrolyzed by endogenous intracellular esterases to calcein, a fluorescent compound. In this method, the fluorescence intensity is proportional to the number of viable cells.

Statistical analysis

The data are presented as the means \pm SD of three independent experiments, each performed in triplicate wells. The significance of the differences among the means was evaluated by the Duncan's test followed by an ANOVA analysis.

Results

Effects of neurosteroids on staurosporine-induced decreases in the viability of RA-differentiated SH-SY5Y cells

As evaluated by the calcein assay, staurosporine (1 μ M for 24 h) decreased the cell viability by ca. 50%. However, neurosteroids given alone did not affect the cell viability (data not shown). DHEA (0.1 and 1 μ M) and DHEAS (0.1 and 1 μ M) significantly attenuated the toxic effects of staurosporine, whereas the lowest concentrations used (0.01 μ M) had no effect. PGL

showed a protective effect only at the 0.1 μ M concentration (Fig. 1).

Effects of neurosteroids on doxorubicin-induced decreases in the viability of RA-differentiated SH-SY5Y cells

Treatment of the cells with doxorubicin (0.5 μ M for 24 h) decreased the cell viability by ca. 20%. DHEA,

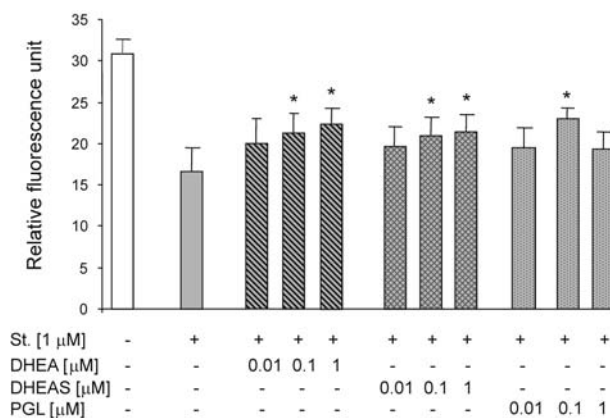


Fig. 1. The effect of dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS), and pregnenolone (PGL) on staurosporine (St.)-induced decreases in the viability of differentiated SH-SY5Y cells. The data are shown as the calcein fluorescence in viable cells (the mean \pm SD) and the significant differences among the means were evaluated by the Duncan's test following ANOVA (* $p < 0.05$ vs. cells treated with St.); $n = 9$

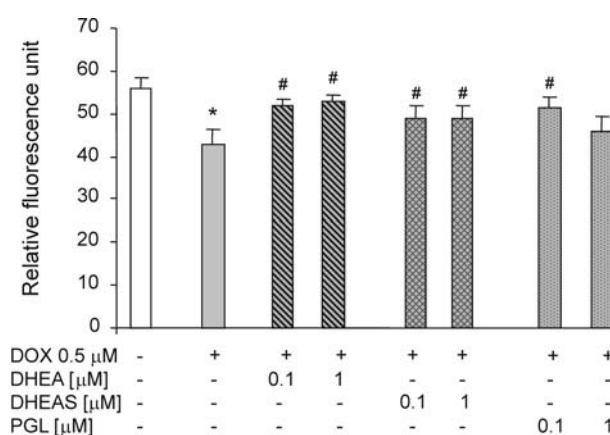


Fig. 2. The effect of dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS), and pregnenolone (PGL) on doxorubicin (DOX)-induced decreases in the viability of differentiated SH-SY5Y cells. Results are shown as the mean \pm SD, and significant differences among the means were evaluated by the Duncan's test following ANOVA (* $p < 0.05$ vs. control culture; # $p < 0.05$ vs. cells treated with DOX); $n = 9$

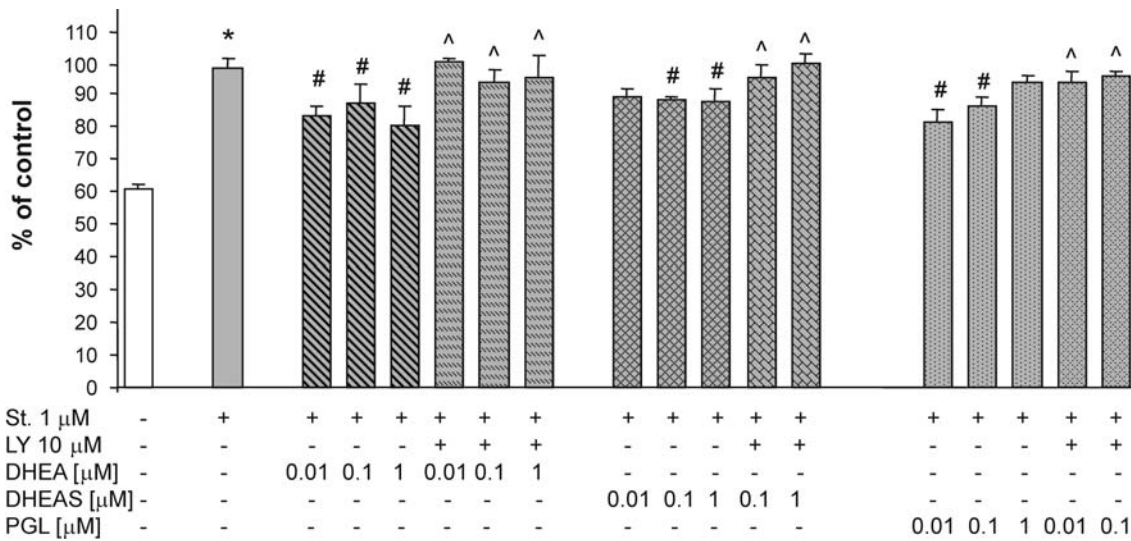


Fig. 3. The effect of LY294002 (PI3-K inhibitor) on the protective effect of dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS), and pregnenolone (PGL) against staurosporine (St.)-induced LDH release in differentiated SH-SY5Y cells. Results are shown as the mean \pm SD and significant differences among the means were evaluated by the Duncan's test following ANOVA (* $p < 0.05$ vs. control culture; # $p < 0.05$ vs. cells treated with St.; ^ $p < 0.05$ vs. cells treated with neurosteroids and St.); $n = 9$

DHEAS, and PGL at 0.1 and 1 μ M concentrations had no effect on the viability of control cells (data not shown). DHEA (0.1 and 1 μ M), DHEAS (0.1 and 1 μ M), and PGL (0.1 but not 1 μ M) significantly attenuated the doxorubicin-induced decreases in cell viability (Fig. 2).

Effects of neurosteroids and LY 294002 on staurosporine-induced lactate dehydrogenase (LDH) efflux from RA-differentiated SH-SY5Y cells

A 24 h incubation of cells with 1 μ M staurosporine increased LDH efflux by ca. 40%. None of the studied neurosteroids given alone affected the basal level of LDH in the control cultures (data not shown). Staurosporine-induced LDH release was significantly inhibited by DHEA (0.01, 0.1 and 1 μ M), DHEAS (0.1 and 1 μ M), and PGL (0.01 and 0.1 μ M). In order to verify the involvement of phosphatidylinositol-3-kinase (PI3-K) in the neuroprotective effect of neurosteroids, a specific inhibitor of this protein kinase (LY 294002 at 10 μ M) was applied. Pretreatment of the cells with LY 294002 antagonized the neuroprotective effects of DHEA, DHEAS, and PGL on staurosporine-induced LDH release (Fig. 3).

Discussion

The present study showed that excitatory neurosteroids ameliorated both staurosporine and doxorubicin evoked decreases in SH-SY5Y cell viability. Staurosporine is a nonspecific protein kinase inhibitor that is widely used for inducing mitochondrial apoptotic cell death, whereas doxorubicin (adriamycin) at a low concentration is an activator of the death receptor signaling pathway [2, 6, 18]. This indicates that excitatory neurosteroids at physiological concentrations, like those used in our study, may attenuate both intra- and extracellular apoptotic processes. The antiapoptotic effect of DHEA in our conditions is consistent with the results of a study by Zhang et al. [27], performed on neural precursor cell cultures, and with our previous data obtained in undifferentiated SH-SY5Y cells [11] and primary cortical neuronal cells (unpublished). Despite some differences in the pharmacological profile of DHEA and its sulfate form (DHEAS), we observed that both of these compounds had similar cytoprotective effects. Further evidence for the antiapoptotic properties of DHEAS came from a study by Kaasik et al. [8], who found that this neurosteroid inhibited apoptosis induced by oxygen-glucose deprivation in cerebellar granule cell cultures. Regarding

non-neuronal cells, DHEAS has also been shown to ameliorate hydrogen peroxide-induced apoptosis in a retinal pigment endothelial cell culture [3] and staurosporine evoked apoptosis in human peripheral blood lymphocytes [24]. In contrast to our present and previous data, Zhang et al. [27] observed that DHEAS aggravated staurosporine evoked apoptosis. The difference between our studies and Zhang's work may stem from the various cellular models and probably not from the concentrations of DHEAS utilized, since in both studies low physiological concentrations were used. It should also be mentioned that Zhang et al. [27] used proliferating precursor cells and suggested an effect of DHEAS on neurogenesis. Another finding of the present study was that, in contrast to DHEA and DHEAS that protected cells at 0.1 and 1 μM , PGL was only active at 0.1 μM . The lack of a neuroprotective effect of PGL at higher concentrations may be explained by its specific membrane receptor profile which differs from that of DHEA or DHEAS, especially regarding their action on σ -1 and NMDA receptors [16]. Thus far, the effect of PGL on the apoptotic processes in neuronal cultures has not been evaluated by other investigators though this neurosteroid has been reported to exert neuroprotective effects during spinal cord injury in an *in vivo* study, and on glutamate and β -amyloid-induced toxicity in hippocampal neurons under *in vitro* conditions [4, 5].

With respect to the intracellular mechanisms of neurosteroid actions, the present data confirm the hypothesis that activation of the PI3-K/Akt pathway is important for the neuroprotective effects of these agents. Thus, we have shown that the selective inhibitor of PI3-K (LY 294002) inhibited the action of neurosteroids on staurosporine-induced LDH release. Inhibition of PI3-K was previously demonstrated by our group and others to inhibit the neuroprotective actions of neurosteroids, vitamin D₃, and insulin-growth factor 1 on staurosporine-induced apoptosis in undifferentiated SH-SY5Y cells [11, 21, 22]. It should be stressed that the PI3-K/Akt signaling pathway activated mainly by insulin and growth factors has been identified as a major cell survival mechanism. Thus, Akt has been shown to participate in the regulation of cell growth, glucose uptake, and metabolism. Akt activates some antiapoptotic agents and inactivates proapoptotic factors such as GSK-3 β . Although our data suggest a critical role for the PI3-K/Akt pathway in the neuroprotective effects of neurosteroids, their mechanisms of action may also involve the activation

of NF- κ B, antioxidant effects, inhibition of glucose-6 phosphate dehydrogenase, and activation of extracellular signal-regulated mitogen-activated protein kinase (ERK-MAPK) [12, 26].

In summary, these data indicated that the examined excitatory neurosteroids prevented the damage in differentiated SH-SY5Y cells related to the activation of both mitochondrial and extracellular apoptotic pathways. Furthermore, this study confirmed that the neuroprotective effects of these neurosteroids appeared to depend on the PI3-K/Akt signaling pathway.

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