Molecular and Cellular Endocrinology 336 (2011) 149–155

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Direct effect of dehydroepiandrosterone sulfate (DHEAS) on PC-12 cell differentiation processes

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ARTICLE INFO

Article history:
Received 24 August 2010
Received in revised form 3 December 2010
Accepted 21 December 2010

Keywords:
Dehydroepiandrosterone-sulfate
Steroid sulfatase
Estrone sulfamate

ABSTRACT

Dehydroepiandrosterone sulfate is classically seen as an inactive reservoir for the production of dehydroepiandrosterone. Steroid sulfatase is the enzyme that catalyzes the hydrolysis of dehydroepiandrosterone sulfate to dehydroepiandrosterone, which can then be further metabolized to other steroid hormones. Recent studies, however, indicate that dehydroepiandrosterone sulfate can mediate biological effects without being converted to dehydroepiandrosterone.

This study aims to evaluate whether dehydroepiandrosterone sulfate itself influences the differentiation of PC-12 cells or if its desulfation to dehydroepiandrosterone is required. dehydroepiandrosterone and dehydroepiandrosterone sulfate both influence the differentiation of chromaffin PC-12 cells. Blocking steroid sulfatase activity and thereby the conversion of dehydroepiandrosterone sulfate to dehydroepiandrosterone by the enzyme blocker estrone sulfamate showed that the effect of dehydroepiandrosterone sulfate is independent of its conversion to dehydroepiandrosterone. Dehydroepiandrosterone sulfate, similar to dehydroepiandrosterone, reduced nerve growth factor-induced neurite outgrowth of PC-12 cells and the expression of synaptosomal-associated membrane protein of 25 kDa, increased the expression of chromogranin A and significantly increased dopamine release of PC-12 cells. In addition, dehydroepiandrosterone sulfate, dehydroepiandrosterone and membrane impermeable dehydroepiandrosterone-BSA all significantly reduced NGF-induced MAPK ERK1/2 signaling after 5 min.

In summary, this study provides evidence that dehydroepiandrosterone sulfate, independent of its conversion to dehydroepiandrosterone, directs PC-12 cells' differentiation to a neuroendocrine direction. Furthermore, employing membrane-impermeable dehydroepiandrosterone-BSA indicates the involvement of plasma-membrane bound receptors.

1. Introduction

Dehydroepiandrosterone (DHEA) and its sulfate ester DHEA-sulfate (DHEAS) are the major androgens produced by the human adrenal cortex. More than 99% (3.5–20 mg/daily) of DHEA is sulfated to DHEAS prior to secretion (Burger, 2002). The normal range of DHEA plasma levels in humans is 7–31 nm. DHEAS ranges from 1.3 to 6.8 μm (Chen et al., 2005) representing the primary circulating form of DHEA with concentrations more than 100-fold higher than any other steroid hormone. Steroid sulfatase (STS) is the enzyme that catalyzes the hydrolysis of steroid sulfates to their unconjugated, biologically active forms (Stanway et al., 2007). In the periphery, STS converts DHEAS to DHEA, which can then be further metabolized to other steroid hormones; conversely, DHEA can be inactivated via sulfonation to DHEAS by cytosolic hydroxysteroid sulfotransferase SA1 (SULT2A1) (Hammer et al., 2005). Due to its rapid metabolic clearance rate, DHEA has a rather short half-life of 1–3 h, while the half-life of DHEAS is 10–20 h (Rosenfeld et al., 1975).

DHEA and DHEAS are widely distributed throughout the body and DHEAS has long been seen merely as a circulating storage pool for DHEA regeneration (Chen et al., 2005). Numerous recent studies, however, have demonstrated that DHEAS indeed can have a direct impact on various cell systems. DHEAS can be synthesized *de novo* in the CNS and in brain glial cells from cholesterol or from steroid precursors through mechanisms partly independent of peripheral steroidogenic endocrine glands, gonads or adrenals and was found to directly increase the length of neurites (Compagnone and Mellon, 1998). Among other major biological actions of DHEAS are those associated with neuroprotection, neu-

Abbreviations: DHEAS, dehydroepiandrosterone sulfate; DHEA, dehydroepiandrosterone; STS, steroid sulfatase; EMATE, enzyme blocker estrone sulfamate; NFG, nerve growth factor; SNAP-25, synaptosomal-associated membrane protein of 25 kDa; CGA, chromogranin A.

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doi:10.1016/j.mce.2010.12.031

ronal survival, apoptosis, catecholamine synthesis and secretion, as well as antioxidant, anti-inflammatory and anti-glucocorticoid effects (for review Maninger et al., 2009). Furthermore, DHEAS was found to inhibit vascular smooth muscle cell (VSMC) proliferation (Ii et al., 2009) and to directly increase superoxide formation in neutrophils (Radford et al., 2010). Our own recent data show that DHEA and DHEAS differentially modulate growth factor-induced proliferation of bovine adrenomedullary chromaffin cells in an age-dependent manner. While DHEA reduced the proliferation of adrenal cells from young and adult animals, DHEAS exclusively increased the proliferation in adult-cell cultures. These effects were not mediated by androgen or estrogen receptors (Sicard et al., 2007). Our studies on PC-12 cells further demonstrated that DHEA (Ziegler et al., 2008) and DHEAS (Krug et al., 2009) promote a neuroendocrine phenotype of these cells.

The present study aims to clarify whether the effects of DHEAS on PC-12 cell differentiation are indeed mediated directly by DHEAS or are exerted by DHEA after conversion by STS. By blocking this enzyme we were able to pin down direct effects of DHEAS on chromaffin PC-12 cells.

2. Materials and methods

2.1. Cell culture

Rat PC-12 cells were sustained in Kaighn's modified Ham's F-12 medium (ATCC, Manassas, USA) with 2 mmol/l l-glutamine and 1500 mg/l sodium bicarbonate supplemented with 15% horse serum, 2.5% fetal calf serum (FCS) and 20 U/ml penicillin–streptomycin (Gibco, Gaithersburg MD, USA) in a humidified 5% CO_2/95% O_2 atmosphere at 37 °C. The culture medium was changed every other day. Cells were grown in this culture medium for 72 h, and left in serum-free medium overnight prior to the experiments. DHEA and DHEA-BSA were dissolved in ethanol, DHEAS and NGF were dissolved in DMSO and 10 mmol/l sodium acetate (pH 5), respectively. The final volume of ethanol or DMSO in each well, including controls, was 0.01% for all assays performed. The solvents had no effect in control experiments.

2.2. RNA isolation and RT-PCR

RNA was isolated using the Quiagen RNeasy Plus kit, according to the manufacturer's instructions (Quiagen, Germany). This kit includes DNA binding columns to exclude genomic DNA. Furthermore, for avoidance of genomic DNA amplification we used the exon-spanning primers for STS detection.

For reverse transcriptase (RT)-PCR, 500 ng RNA from each sample was reverse transcribed into cDNA by Moloney murine leukemia (MML) virus reverse transcriptase using random primers (Promega) in a final volume of 20 μl. One μl of cDNA was amplified in a 10 μl solution containing 1.5 mM $MgCl_2$, 1× PCR buffer (Invitrogen, CA, USA), 0.25 mM of each deoxynucleotide (Promega), 1 unit of Taq DNA polymerase (Invitrogen, CA, USA) and 5 pmol of each of the different primers. Samples were denatured for 5 min at 94 °C, then subjected to 15 s 94 °C, 15 s 65 °C, 15 s 72 °C and 1 min 72 °C for 40 cycles altogether. After agarose gel electrophoresis and ethidium bromide staining, bands were visualized under UV light.

Exon-spanning primer were found through data base search and checked for right length and position using BLAST (basic local alignment search tool). Primer sequences (5′–3′): ACTCCAACTCAGCTACAACCTGGTG and CACAGAGAAAGTTGAGCTGGGAGAG.

2.3. Neurite outgrowth

Single cell suspensions were plated in 200 mm^2 wells at a density of 2×10^4 cells per well in serum-free culture medium, then treated with NGF (20 ng/ml) and DHEAS or DHEA (10^{-6} mol/l), alone and in combination as well as w/wo EMATE (10^{-6} mol/l). After 24 h incubation, the percentage of cells showing neurite outgrowth was determined by light microscopy. Cells with one or more neurites whose lengths were at least twice the diameter of the cell body were scored as positive. Neurite outgrowth was determined from at least three different regions of interest (ROI) in three independent experiments (Kim et al., 2004; Xiao et al., 2002; Ziegler et al., 2008).

2.4. High-performance liquid chromatography (HPLC)

Dopamine concentrations in culture medium were determined as previously described (Ziegler et al., 2008). Briefly, catecholamines were extracted by solid-phase extraction after 24 h stimulation of cells with NGF (20 ng/ml) and DHEA as well as DHEAS (10^{-6} mol/l), alone and in combination as well as w/wo EMATE (10^{-6} mol/l). The cell medium samples were processed according to the sample preparation protocol for urine (Chromsystems, Munich, Germany). 6 ml neutralization buffer and 100 μl internal standard (3,4-dihydroxy-benzylamine) were added to 3 ml cell medium. This mixture was run on a sample clean-up column by vacuum. The eluate was discarded. Subsequently, the solid-phase extraction column was washed twice with pure water (10 ml) and eluted with 6 ml elution buffer by vacuum. Finally, the eluate was collected and acidified with 180 μl 5N HCl. An aliquot of the purified eluate was applied to a HPLC reverse-phase column, and dopamine concentrations were measured using an electrochemical detector (Bio-Rad, Munich, Germany). Dopamine measurements were quantified in correlation to the added internal standard.

2.5. Western blot analysis

Western blot analysis was performed as previously described (Krug et al., 2003). PC-12 cells were washed twice in ice-cold phosphate-buffered saline (PBS) and lysed in ice-cold Cell Lytic Mammalian Cell Lysis/Extraction reagent (Sigma) containing 1% protease inhibitor cocktail (Sigma). Cell lysates were matched for protein content. Protein concentrations were determined by BCA assay (PIERCE, Rockford, USA). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane. After blocking, membranes were immunostained using the following antibodies: rabbit anti-phospho-ERK1/2 (1:1000, Cell Signaling, Danvers, USA), rabbit anti-SNAP-25 (1:5000, Sigma–Aldrich, Steinheim, Germany), goat anti-chromogranin A (CgA) (1:500, Santa Cruz, Heidelberg, Germany) and rabbit anti-β-actin (1:500, Cell Signaling, Danvers, USA). The bands were visualized using peroxide solution and luminol solution (PIERCE, Rockford, USA). Chemoluminescence signals were read with the GeneGnome Chemoluminescence detector (Syngene, Frederick, USA). As a loading control we used the house-keeping gene β-actin.

2.6. Quantification of ERK1/2 phosphorylation by ELISA

We performed in situ cell-based ELISA as described previously, in a slightly modified way (Krug et al., 2009). Cells were seeded in 96-well plates at a concentration of 20,000 cells per 0.32 cm^2 and after reaching approximately 80% confluence, were serum-starved for 24 h. Subsequently, cells were equilibrated in 1 × HEPES-Ringer solution (130.0 mmol/l NaCl, 5.4 mmol/l KCl, 1.0 mmol/l $CaCl_2$, 1.0 $MgCl_2$, 1.0 mmol/l NaH_2PO_4, 10 mmol/l HEPES, and 5 mmol/l glucose; pH 7.4) at 37 °C for 30 min, and then stimulated in the same buffer w/wo the hormones and growth factors of interest. After fixation of cells with 8% formaldehyde in PBS, endogenous peroxidases were quenched with freshly prepared 1% H_2O_2 and 0.1% azide. Cells were washed again three times in the same buffer, blocked by 5% FCS in PBS/Triton for 1 h and, finally, incubated overnight with the primary antibody (pERK, Cell Signaling, 1:1000) in PBS/Triton containing 5% bovine serum albumin at 4 °C. The next day, cells were washed three times with PBS/Triton and incubated with the secondary antibody (anti-rabbit HRP-linked IgG, Cell Signaling, 1:3000) in PBS/Triton containing 5% bovine serum albumin for 1 h at room temperature. After one washing step, chemoluminescence signals were read using a Mithras multi-well reader (Berthold Technologies, Bad Wildberg, Germany). After two washing steps, air-dried wells were stained with 100 μl of crystal violet solution (0.25% in PBS) for 30 min at room temperature. Following three PBS washing steps, 100 μl of 1% SDS solution was added and the plate was incubated on a shaker for 1 h at room temperature. Finally, absorbance was measured at 595 nm. Chemoluminescence signals were normalized to the protein content in each well as determined by crystal violet staining. This step allows normalizing ERK1/2 phosphorylation to total protein content per well.

2.7. Statistical analysis

In all experiments, statistical differences between experimental groups relative to appropriate controls were determined by analysis of variance (ANOVA). Data are presented as means ± S.E.M. Significance of differences was tested by analysis of variance with Bonferroni's as a secondary test. Differences were considered significant at values of $p < 0.05$. Cells from at least two different passages were used for each experimental series; n represents the number of cells or tissue culture dishes investigated.

3. Results

The prerequisite for the conversion of DHEAS to DHEA is the expression of the enzyme steroid sulfatase which catalyzes the conversion of DHEAS to DHEA. RT-PCR analysis revealed the expression of STS in PC-12 cells (Fig. 1).

To identify possible direct effects of DHEAS, intracellular STS activity was blocked by addition of the enzyme blocker estrone sulfamate (EMATE). The effects of both DHEA and DHEAS were evaluated in the presence and absence of EMATE. We focused on various neuronal and neuroendocrine differentiation parameters in accor-