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Effects of dehydroepiandrosterone on proliferation and differentiation of chromaffin progenitor cells

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ABSTRACT

Dehydroepiandrosterone producing adrenocortical zona reticularis and the adrenal medulla are in direct contact and are highly intermingled in many species. This results in potentially strong paracrine influences of high local dehydroepiandrosterone concentrations on the adrenal medulla. Dehydroepiandrosterone has neuroprotective properties and increases neural stem cell proliferation and neurogenesis. Therefore, we aimed to establish its effects on chromaffin progenitor cell proliferation and differentiation. Previously, we successfully isolated chromaffin progenitors from bovine adrenal medulla in spherical cultures, so-called chromospheres. Seven days treatment of chromospheres with dehydroepiandrosterone at high concentrations (100 μM) hampered proliferation of chromaffin progenitors. Under differentiation conditions, dehydroepiandrosterone in the presence of retinoic acid, increased tyrosine hydroxylase and decreased dopamine-β-hydroxylase mRNA expression. In addition, there was a tendency to increase dopamine contents. Dehydroepiandrosterone/retinoic acid is therefore suggested to induce dopaminergic differentiation from chromaffin progenitor cells. Furthermore, the high dehydroepiandrosterone concentrations present in the fetal and adult adrenal may play an important role in adrenomedullary cell proliferation and differentiation.

1. Introduction

Dehydroepiandrosterone (DHEA) and DHEA sulfate (DHEAS) are the most abundant circulating adrenal steroids in the human body. Plasma concentrations of DHEAS in young human males are 6–9 μM, and DHEA concentrations are approximately 1/300 of DHEAS (Rodriguez et al., 2007; Woolcott et al., 2010). The desulfated form DHEA has biological activity and can be converted into downstream sex hormones including testosterone, estrone and estradiol. Plasma concentrations of DHEA and DHEAS vary between genders and are age-dependent. Their concentrations reach a first peak in neonates after birth, decrease during the first year of life, remain low in childhood, increase during puberty, reach a second peak in 20–30 years olds, and then gradually decrease (de Peretti and Forest, 1976; Kroboth et al., 1999; Labrie et al., 1997). Thus, they are considered as "youth hormones" and possible markers for physiologic aging despite the fact that their function and mechanisms of action have not been clarified yet.

DHEA is mainly synthesized and secreted from the zona reticularis, the innermost layer of the adrenal cortex. The close contact and the intermingled structure between the zona reticularis and the medulla suggest physiological interactions or communication between the two endocrine tissues (Bornstein et al., 1991; Ehrhart-Bornstein et al., 1998). Furthermore, these close contacts most likely result in high local concentrations of DHEA in the adrenal medulla. Indeed, it has been shown that DHEA and DHEAS influence catecholamines synthesis (Charalampopoulos et al., 2005; Liu et al., 1996), and DHEA has an anti-apoptotic effect on PC12 cells, a rat pheochromocytoma cell line which is a widely accepted model for chromaffin cells with some properties of progenitor cells (Charalampopoulos et al., 2004). Previous research from our lab proves that these androgens influence the proliferation and differentiation of PC12 cells (Krug et al., 2009; Ziegler et al., 2008) and primary bovine adrenal chromaffin cells (Sicard et al., 2007).

Abbreviations: bFGF, basic fibroblast growth factor; BMP4, bone morphogenesis protein 4; DBH, dopamine β-hydroxylase; DDC, DOPA decarboxylase; Dex, dexdexamethasone; DHEA(S), dehydroepiandrosterone (sulfate); DMEM/F12, Dulbecco's Modified Eagle's Medium/Ham's F-12 Nutrient Mixture; EGF, epidermal growth factor; HPLC, high-performance liquid chromatography; ITS-X, insulin–transferrin–selenium-X; LIF, leukemia inhibitory factor; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; NGF, nerve growth factor; PNMT, phenylethanolamine N-methyl transferase; RA, retinoic acid; TH, tyrosine hydroxylase.

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DHEA is a neurosteroid whose *de novo* synthesis is also found in the central nervous system (Majewska et al., 1990). It has protective properties against neurotoxicity and increases neurogenesis in the adult rodent hippocampus *in vivo* (Karishma and Herbert, 2002; Kimonides et al., 1998). Furthermore, DHEA *in vitro* has been shown to increase stem cell proliferation and neurogenesis from human neural stem cells and murine mesenchymal stem cells (Shiri et al., 2009; Suzuki et al., 2004). DHEA also has the potential to promote dopaminergic neuron differentiation from both mouse embryonal carcinoma cells and human neural progenitors (Azizi et al., 2010). Previous studies from our group indicate that proliferation of chromaffin cells from young and old cattle is differentially influenced by these androgens (Sicard et al., 2007). This age-dependent sensitivity suggests the possible influence of DHEA/S also on undifferentiated chromaffin progenitor cells.

The adrenal medulla, due to its capability to proliferate and adapt to physiological needs during the whole life span, may contain neural crest derived progenitor cells. Recently, we established a method to isolate and culture chromaffin progenitor cells from adult bovine adrenal medulla (Chung et al., 2009). They grow in spherical clusters, named chromospheres, and show self-renewal and differentiation ability *in vitro*. These cells provide an applicable tool to understand the development of the sympathoadrenal system and the differentiation of chromaffin cells especially in the adult adrenal. In addition, these cells may bear potential in the treatment of neurodegenerative diseases. As the primary source of peripheral catecholamine secretion, the adrenal chromaffin cell was early on considered a candidate for autologous intrabrain transplantation to replace the deficiency of dopaminergic neurons (Madrazo et al., 1987). Consequently, more than 300 Parkinson patients were transplanted from 1988 to 2001 with some improvement of the clinical symptoms (for review: Drucker-Colin and Verdugo-Diaz, 2004). However, the survival rate of the grafted adult chromaffin cells was disappointing and clinical improvements disappeared after 1–2 years (Quinn, 1990; Fernandez-Espejo et al., 2005). The potential of chromaffin progenitor cells, however, to differentiate into dopaminergic neurons suggests that these cells might be an expandable source of progenitor cells suitable for the treatment of neurodegenerative diseases, *e.g.* Parkinson's disease (Ehrhart-Bornstein et al., 2009, 2010).

The development of chromaffin cells highly depends on factors present in their local environment, *e.g.* glucocorticoids secreted from adrenal cortex (Unsicker et al., 2005, 1978). Furthermore, the adrenal medulla has an astonishing capacity to respond and adapt to external stimuli (Kvetnansky et al., 2009), probably including the proliferation and differentiation of chromaffin progenitor cells. Due to the local secretion of DHEA from the adrenal cortex, chromaffin progenitor cells in the medulla are in a milieu of high DHEA concentrations, suggesting an influence of this hormone on chromaffin cell proliferation and/or differentiation. Therefore, we aimed to elucidate the effects of DHEA on the proliferation and differentiation of chromaffin progenitor cells. In the present study, differentiation of chromaffin progenitor cells was induced by retinoic acid (RA) and bone morphogenesis protein 4 (BMP4). RA has a profound effect to launch neuronal differentiation from an embryonal carcinoma cell line P19 (Jones-Villeneuve et al., 1982). In human neuroblastoma cells, DHEA acted as an enhancer of the effect of RA; both factors synergistically induced neuronal differentiation in these cells (Silvagno et al., 2002). RA and DHEA together successfully induced dopaminergic neuron differentiation from P19 and human neural progenitor cells (Azizi et al., 2010). Since the chromaffin progenitors possess the potential to differentiate into neuron, RA is used in this study to reveal its possible synergistic effects with DHEA to induce dopaminergic neuron differentiation. On another hand, BMP4 plays a crucial role in the migration and induction of sympathoadrenal progenitor cells which give rise to sympathetic neurons and chromaffin cells (Reissmann et al., 1996). We recently showed that BMP4 decreases dopamine β-hydroxylase (DBH) mRNA expression suggesting its potential to induce the differentiation into dopamine-secreting cells (Chung et al., 2009). Therefore, a possible synergistic effect of RA, BMP4 and DHEA on chromaffin progenitor cells differentiation was investigated.

2. Materials and methods

2.1. Culture of chromaffin progenitor cells

The method of chromaffin progenitor cells isolation from bovine adrenal medulla has been established, and the characterization of these cells has been described previously (Chung et al., 2009). Briefly, cells from adrenal medulla were obtained by enzymatic (collagenase/DNase) and mechanical dissociation followed by centrifugation. Non-chromaffin cells were removed by differential plating. The isolated cells were then cultured in low-attachment plates (Corning, Lowell, MA, USA) with 10% steroid-free FBS (Hyclone, South Logan, UT, USA) and 1% antibiotic–antimycotic solution (Gibco, Invitrogen, Carlsbad, CA, USA) in Dulbecco's Modified Eagle's Medium/Ham's F-12 Nutrient Mixture (DMEM/F12, Gibco) for 7 days. Under these conditions, cells grew as spherical structures, named chromospheres. Chromaffin progenitor cells in these chromospheres expressed several progenitor markers and showed self-renewal ability (Chung et al., 2009; Ehrhart-Bornstein et al., 2010). Seven days old chromospheres were collected for subsequent analysis by 200 × *g* centrifugation for 8 min. All cells were cultivated at 37 °C in a humidified atmosphere with 95% O_2 and 5% CO_2.

2.2. Reagents

All reagents used, if not otherwise indicated, were purchased from Sigma–Aldrich (Saint Louis, MO, USA). DHEA, RA and dexamethasone (Dex) were dissolved in ethanol and BMP4 was dissolved in PBS.

2.3. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay

Chromospheres were dissociated by Accumax (PAA Laboratories, Pasching, Austria) treatment for 8 min at 37 °C. During and after incubation, cells were mechanically dissociated by repeated pipetting. To analyze the effects of DHEA on chromaffin progenitor cells' proliferation, cells dissociated from chromospheres, after washing with PBS, were treated with different concentrations of DHEA (0.1, 1, 10 and 100 μM) in serum-free medium containing 20 ng/ml epidermal growth factor (EGF), 20 ng/ml basic fibroblast growth factor (bFGF), 10 ng/ml leukemia inhibitory factor (LIF), 1% insulin–transferrin–selenium-X (ITS-X, Gibco), and 1% antibiotic–antimycotic solution in DMEM/F12. Controls (0 μM) contained 0.1% ethanol, the solvent of DHEA, in the same medium. The cells were then cultured in 96-well ultra low-attachment plates as triplicates for each treatment. Twenty-five μl fresh medium containing the different concentrations of DHEA were added every second day; viable cell density was analyzed using the MTS assay (CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay, Promega, Madison, WI, USA) on the 7th day.

2.4. Cell differentiation induction

Dissociated cells from chromospheres were plated on poly-D-lysine and laminin coated plates or slides and cultured with 1 μM DHEA, 5 μM RA, 20 ng/ml BMP4 and 10 μM Dex alone or in the following combinations: DHEA/RA, DHEA/BMP4 and DHEA/Dex in serum-free medium containing 20 ng/ml EGF, 20 ng/ml bFGF, 1% ITS-X, and 1% antibiotic–antimycotic solution in DMEM/F12. Half of the medium was replaced by fresh medium containing respective factors every second day up to 7 days after differentiation induction.

2.5. Quantification of differentiated neuron-like cells

Differentiated neuronal progeny can be distinguished by its characteristic morphology and neurite extrusions. To understand the potential of the factors investigated to induce neuron-like cell differentiation, the percentage of cells showing neurite outgrowth was determined by microscopy. Cells with one or more neurites whose lengths were at least twice the diameter of the cell body were scored as positive. For each experiment, singularized cells from one chromosphere isolation were seeded onto 6 slides and treated with different factors, respectively. After 7 days induction, 4–7 fields from each slide were randomly examined at 100× magnification to quantify differentiated neuron-like cells as percentage of total cell number. Three experiments were repeated on 3 independent chromosphere isolations.