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Regulation of apoptotic signaling pathways by 5α-dihydrotestosterone and 17β-estradiol in immature rat Sertoli cells

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ABSTRACT

Apoptosis is an important regulatory event in testicular homeostasis and optimization of sperm production. Sertoli cells (SCs) form the blood–testis barrier creating a special microenvironment where germ cells develop and are under strict hormonal control. Estrogens and androgens are known to play critical roles in SCs functioning, improving their *in vitro* survival by preventing apoptotic progression. Herein, we studied the influence of 17β-estradiol (E2) and 5α-dihydrotestosterone (DHT) on the apoptotic signaling pathways of immature rat cultured SCs. For that we chose key points of the apoptotic pathway that interact with the mitochondria and evaluated the mRNA expression and/or protein levels of several apoptotic markers such as p53, the anti-apoptotic protein Bcl2, the pro-apoptotic Bcl2 family member Bax, the apoptosis-inducing factor (AIF) and caspase-3 and 9. Caspase-3 activity and DNA fragmentation were also evaluated as endpoint markers of apoptosis. E2 and DHT down-regulated the mRNA transcript levels of p53, Bax, caspase-9 and caspase-3. The protein levels of AIF were reduced after DHT treatment while E2-treated cells presented decreased levels of cleaved caspase-9 protein. Moreover, Bax/Bcl2 ratio was significantly decreased in E2-treated cells. The apoptotic endpoints caspase-3 activity and DNA fragmentation presented significant decreased levels after hormonal treatment. Taken together, these results show that E2 and DHT act as apoptotic signaling modulators in *in vitro* immature rat SCs suggesting that androgens and estrogens may be capable of modulating independent pathways of the apoptotic event by regulating different pro-apoptotic factors.

1. Introduction

The whole spermatogenesis process is dependent on the delivery of specific products that are produced by Sertoli cells (SCs) and are necessary for germ cell survival. SCs are responsible for creating a unique and essential environment in the adluminal compartment, in order to provide structural support, nutrition and immunological protection to the progression of spermatogenesis [1–3]. The number of germ cells that are produced during spermatogenesis is directly related to the number of functional SCs [4], as one single SC can only support 30–50 germ cells at different stages [5].

Although it is commonly accepted that SCs regulate apoptosis of developing germ cells in a complex process controlled by a network of endocrine and other regulatory factors, particularly through the FasL/Fas signal transduction system [6], the mechanisms by which SCs proliferation and death is controlled have been poorly investigated. It has been reported that SCs may have protective mechanisms that render them resistant to apoptosis derived from a variety of insults when compared to germ cells [7–9]. Nevertheless, studies on infertile men demonstrated significantly increased apoptotic levels in SCs of those patients [10]. It has been reported that a combination of peptide, steroid hormone and growth factors are able to modulate SCs apoptosis and survival [11] but the mechanisms have not been explored. Furthermore, it is known that androgens and estrogens play an important role in the regulation of growth, development, homeostasis and programmed cell death in testicular cells [12–14].

The process of apoptosis is a fundamental biochemical cell death pathway characterized by several morphologic changes [15] and plays a fundamental role in the maintenance of tissue homeostasis in the adult organism, turned on in response to environment

Abbreviations: AIF, apoptosis-inducing factor; Apaf-1, apoptotic protease activating factor 1; AN, accession number; β2MG, β-2-microglobulin; BSA, bovine serum albumin; E2, 17β-estradiol; DHT, 5α-dihydrotestosterone; cCasp9, cleaved caspase 9; DAB, 3,3′-diaminobenzidine hydrochloride; DMEM:Ham's F12, Dulbecco's modified Eagle's medium:Ham's nutrient mixture F12; dNTPs, deoxynucleotide triphosphates; EDTA, ethylene diamine tetra acetic acid; FBS, fetal bovine serum; GPER, G protein coupled estrogen receptor; HBSS, Hank's balanced salts solution; ITS, insulin–transferrin–sodium selenite; M-MLV RT, moloney murine leukemia virus reverse transcriptase; RT-PCR, reverse transcriptase polymerase chain reaction; SCs, Sertoli cells; TdT, terminal deoxynucleotidyl transferase; TUNEL, TdT-mediated dUTP nick end labeling.

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signals or triggered by intrinsic factors [16]. These signals can be observed in several situations during cell development, homeostatic regulation of cell populations and aging [17]. There are two main pathways involved in the apoptotic process: the extrinsic or death receptor pathway, occurring in response to activated death receptors present in the cell surface, and the intrinsic or mitochondrial pathway occurring in response to signals originated from inside the cell [16]. The intrinsic pathway can be triggered due to various signals as DNA damage, cytotoxic stress and growth factors deprivation [18] and involves the action of mitochondria, which stimulates and transduce signals to execute apoptosis *via* a distinctive set of molecules [17–19]. The Bcl-2 family consists of anti- and pro-apoptotic members such as Bcl-2 and Bax respectively and the Bax/Bcl-2 ratio is often used as biomarker for apoptosis unbalance in several pathological conditions [20]. Moreover, the up-regulation or activation of pro-apoptotic molecules, such as Bax, leads to its translocation to the outer membrane of mitochondria [21] and consequent permeabilization of the outer mitochondrial membrane, which is followed by the release of many apoptosis promoting proteins that reside in the mitochondrial intermembrane space, including cytochrome c, apoptosis-inducing factor (AIF) and some pro-caspases [21]. This permeabilization can lead to sequential activation of effector caspases (such as caspase-3) and when the caspases cascade is initiated, the process of cell death cannot be reversed [17]. Additionally, it can also lead to the release of other pro-apoptotic factors (such as the flavoprotein AIF) that, ensuing translocation to the nucleus, contribute to chromatin condensation and chromatinolysis, resulting in apoptosis [22].

Several pathological conditions known to cause subfertility and infertility are characterized by severe hormonal deregulation [23,24]. We have previously shown that 17β-estradiol (E2) and 5α-dihydrotestosterone (DHT) are modulators of SCs metabolism [25,26] and there is a close relationship between metabolism and apoptosis, being the mitochondria the central organelle [27], thus we hypothesized that sex steroid hormones could have a role on the regulation of mitochondria related pro-apoptotic factors. So, the aim of our work was to study the influence of E2 and DHT on the apoptotic signaling pathways in immature rat SCs. Therefore, we chose key points of the apoptotic pathway that interact with the mitochondria and evaluated mRNA expression of p53, the pro-apoptotic Bcl2 family member Bax, caspase-3 and caspase-9. The protein expression of caspase-9, Bax, the anti-apoptotic Bcl2, and AIF was also determined. Finally, Caspase-3 activity and DNA fragmentation (TUNEL assay) were evaluated as endpoint markers of apoptosis.

2. Materials and methods

2.1. Chemicals

Hank's balanced salts solution (HBSS), Dulbecco's modified Eagle's medium:Ham's nutrient mixture F12 (DMEM:Ham's F12), ethylene diamine tetra acetic (EDTA) acid, soybean trypsin inhibitor, DNAse, Collagenase type I, 17β-estradiol (E2), 5α-dihydrotestosterone (DHT), bovine serum albumin (BSA), ExtrAvidin-Peroxidase Staining Kit, 3,3′-diaminobenzidine hydrochloride (DAB), trypsin–EDTA, insulin–transferrin–sodium selenite supplement (ITS supplement), TRI reagent and other drugs were obtained from Sigma Aldrich. (St. Louis, MO, USA). Fetal Bovine Serum (FBS) was obtained from Biochrom AG (Germany). Moloney murine leukemia virus reverse transcriptase (M-MLV RT) and random hexamer primers were obtained from Invitrogen (CA, USA). dNTPs were obtained from GE Healthcare (Buckinghamshire, UK). 1× Buffer and Taq DNA polymerase were obtained from Fermentas Life Sciences (Ontario, Canada). Polyclonal antibodies were obtained from Santa Cruz Biotechnology (Heidelberg, Germany) and Cell Signaling (Massachusetts, USA). TdT-FragEL DNA fragmentation Detection kit was obtained from Calbiochem (Darmstadt, Germany).

2.2. Animals

Wistar male rats (*Rattus norvegicus*) were obtained from Charles River (Barcelona, Spain) and housed under a 12 h light-12 h darkness cycle, with food and water available *ad libitum*. Housing, maintenance and handling of animals comply with the "Guide for the Care and Use of Laboratory Animals"; published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the rules for the care and handling of laboratory animals (Directive 86/609/EEC).

2.3. Sertoli cell culture

Ten male Wistar rats (20-day old) were sacrificed by cervical dislocation, the testis were immediately excised in aseptic conditions and washed two times in a 50 mL conical tube in 30 mL of ice cold HBSS containing 10,000 U/mL of penicillin, 10 mg/mL streptomycin and 25 μg/ml amphotericin B (pH 7.4). SCs were isolated and cultured (in phenol-red free media) using a previously described method by Meroni and collaborators [28], adapted by Oliveira and collaborators [29]. Briefly, tissue from decapsulated testes was placed in a Petri dish containing glycine medium (HBSS plus 1 M glycine, 2 mM EDTA, 0.002% (w/v) soybean trypsin inhibitor; pH 7.2). The tubular pellet was digested with Collagenase type I and DNAse in HBSS for 15–20 min at room temperature. The sertoli cell suspension, was collected by centrifugation (300 × *g* for 3 min), washed in HBSS and resuspended in Sertoli culture medium which consisted of a 1:1 mixture of DMEM:Ham's F12 (pH 7.2–7.4) supplemented with 15 mM HEPES, 50 U/mL penicillin and 50 mg/mL streptomycin sulfate, 0.5 mg/mL fungizone, 50 μg/mL gentamicin and 10% heat inactivated FBS. This cellular suspension was then forced through a 20 G needle, in order to disaggregate large Sertoli clusters. The cellular suspension was plated on culture flasks (Cell+; Sarstedt), and incubated at 33 °C in an atmosphere of 5% CO_2, 95% O_2 in Sertoli culture medium. SC culture purity was assessed by the immunoperoxidase detection of specific markers, anti-Mullerian hormone and Vimentin as described elsewhere [30]. Briefly, cells were grown on 6 well culture plates, incubated overnight at 4 °C with primary polyclonal antibody and labeled streptavidin–biotin method using an ExtrAvidin-Peroxidase Staining Kit, giving a brown coloration to the SCs after reaction with diaminobenzidine. The cell nucleus was then stained with haematoxylin. Negative-control incubations were executed using PBS instead of primary antibody. Cultures were examined by phase contrast microscopy and selected if cells contaminants were below 5% after 96 h.

2.4. Experimental groups

SCs were allowed to grow until reach 90–95% confluence, and then washed thoroughly and the medium replaced by serum and phenol-red free media (DMEM:F12, 1:1, with ITS supplement, pH 7.4). To evaluate the effects of sex hormones on mRNA and protein expression, SCs were treated during 50 h with 100 nM of E2 or 100 nM of DHT. DHT was chosen as a androgen family representative because it is not conversable to E2 by the cells [31]. The concentrations of the sex steroid hormones were chosen based on available data which reported that intratesticular interstitial fluid concentrations of those hormones are particularly higher than those of circulating plasma, reaching values up to 200 nanomolar [32,33]. Working stock solutions of DHT