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Non-classical effects of androgens on testes from neonatal rats

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The intratesticular testosterone concentration is high during the early postnatal period although the intracellular androgen receptor expression (iAR) is still absent in Sertoli cells (SCs). This study aimed to evaluate the non-classical effects of testosterone and epitestosterone on calcium uptake and the electrophysiological effects of testosterone (1 μM) on SCs from rats on postnatal day (pnd) 3 and 4 with lack of expression of the iAR. In addition, crosstalk on the electrophysiological effects of testosterone and epitestosterone with follicle stimulating hormone (FSH) in SCs from 15-day-old rats was evaluated. The isotope $^{45}Ca^{2+}$ was utilized to evaluate the effects of testosterone and epitestosterone in calcium uptake. The membrane potential of SCs was recorded using a standard single microelectrode technique. No immunoreaction concerning the iAR was observed in SCs on pnd 3 and 4. At this age, both testosterone and epitestosterone increased the $^{45}Ca^{2+}$ uptake. Testosterone promoted membrane potential depolarization of SCs on pnd 4. FSH application followed by testosterone and epitestosterone reduced the depolarization of the two hormones. Application of epitestosterone 5 min after FSH resulted in a delay of epitestosterone-promoted depolarization. The cell resistance was also reduced. Thus, in SCs from neonatal Wistar rats, both testosterone and epitestosterone act through a non-classical mechanism stimulating calcium uptake in whole testes, and testosterone produces a depolarizing effect on SC membranes. Testosterone and epitestosterone stimulates non-classical actions via a membrane mechanism, which is independent of iAR. FSH and testosterone/epitestosterone affect each other's electrophysiological responses suggesting crosstalk between the intracellular signaling pathways.

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1. Introduction

The process of Sertoli cell proliferation, during the immature period, is crucial to a normal fertility in adulthood. In fetal life, androgen action is more important for testicular development than follicle stimulating hormone (FSH) action. However, during the postnatal period, FSH becomes essential to proliferation, determining Sertoli cell numbers [1,2]. Furthermore, in several species such as rat, mice and human, intratesticular testosterone concentration is high in the early postnatal period [3–5], although the intracellular androgen receptor (iAR) expression is absent in these cells [4,6,7]. Thus, Sertoli cells cannot respond to the high testosterone concentration via classical mechanism at this age. In the late neonatal period, testosterone concentration decreases and remains low until puberty when the level of testosterone increases [3–5], as well as iAR expression in Sertoli cell [4,6,7]. It was found that testosterone through its binding to iAR suppresses proliferation at the end of the Sertoli cell proliferative phase and regulates the expression of markers associated with maturation of the Sertoli cell [8]. In adults, testosterone produced in testes greatly exceeds the required concentration for iAR binding in humans and rats (approximately 25–125-fold higher than that present in serum) [9]. The physiological importance of the high concentration of testosterone in the testis, especially in neonatal period when there is no iAR in Sertoli cell, is not fully understood. It may be due to its

Abbreviations: [^{14}C]-MeAIB, α-methylaminoisobutyric acid; ARKO, mice lacking iAR-receptors; ATF-1, activating transcription factor-1; FSHRKO, mice lacking FSH-receptors; iAR, intracellular androgen receptor; GPCR, G protein-coupled receptor; HBSS, Hank's Balanced Salt Solution; PBS, phosphate-buffered saline; RT, room temperature; SCs, Sertoli cells; K^{+}_{ATP}, ATP-sensitive K^+ channel; PIP_2, phosphatidylinositol 4,5 biphosphate; PLC, phospholipase C; Pnd, postnatal day; SCARKO, mice lacking iAR-receptors on Sertoli cells; VDCC, voltage-gated Ca^{2+} channels.

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action through a non-classical mechanism. Therefore, it is necessary to investigate the involvement of a non-classical action of testosterone and the relative contribution of this mechanism of action in the regulation of the testicular tissue development.

Epitestosterone (17alpha-hydroxy-4-androsten-3-one) is the natural 17α-epimer of testosterone. Testicular and adrenal tissues are male sources of epitestosterone [10,11]. As described by Havlíkova et al. [12] and reviewed by Stárka [10], the serum epitestosterone levels, in human, are slightly high than testosterone levels in childhood. Because the epitestosterone/testosterone ratio remains nearly constant in adulthood and epitestosterone concentration is not influenced by exogenous administration of testosterone, this steroid has been used as a natural internal standard for assessing testosterone abuse in sports [10]. Some studies have shown an antiandrogenic action of epitestosterone such as inhibition of 5α-reductase [13], a clear competitive inhibition of the iAR [14], and a decrease in the weight of tissues that increase in response to androgens [15]. However, epitestosterone was not able to inhibit testosterone-stimulated prostatic tumor growth [16].

The nuclear action of testosterone through iAR is known as the classical mechanism of this hormone. It involves dimerization of the hormone-receptor complex, translocation to the nucleus and binding to specific response elements causing DNA transcription and subsequent protein synthesis [17,18]. The non-classical mechanism of testosterone involves a cellular response to the steroid in seconds or in a few minutes. Several authors have been investigating the non-classical mechanism of testosterone action, even though the receptor for this action has not been identified [9,19–22].

Two non-classical signaling pathways have been described for androgens in Sertoli cells [9]. The first non-classical pathway causes the influx of Ca^{2+} through activation of an unidentified receptor. Testosterone (1 μM) stimulates rapid Ca^{2+} influx in several tissues. In osteoblasts, testosterone acts within 5 s to induce Ca^{2+} influx through voltage-gated calcium channels (VDCC) [23]. Cultured Sertoli cells from 17- to 20-day-old rats rapidly increase their intracellular Ca^{2+} in response to testosterone [24]. This steroid increases $^{45}Ca^{2+}$ uptake within 5 min of incubation in isolated Sertoli cells from immature (12- to 15-day-old rats) as well as from rats aged 20, 30, 45 and 60 days [25–27]. Testosterone (0.1–1 μM) induces rapid depolarization of Sertoli cell membranes from immature rats (aged 10–15 days) [25,26]. The testosterone-induced depolarization is produced by a closing of ATP-sensitive K^+ channels (K^+_{ATP}) and subsequent opening of L-type VDCC [26]. This effect seems to involve a membrane receptor that activates phospholipase C (PLC) once U-73122, a PLC inhibitor, blocks the testosterone effect on $^{45}Ca^{2+}$ uptake and in the membrane potential depolarization. The activation of PLC causes hydrolysis of phosphatidylinositol 4,5 biphosphate (PIP_2) [25]. The lack of PIP_2 reduces negative charges near membranes and produces closing of K^+_{ATP} channels [25,28]. These effects of testosterone in the membranes of Sertoli cells from immature rats were reproduced using nandrolone (1 μM), catechin (1 μM) and epitestosterone (0.5–2 μM) [29,30]. These Sertoli cell responses to androgens were specific because neither estradiol nor progesterone produced any effect on Sertoli cell membrane potential [26]. Epitestosterone (1 μM) as well as testosterone were able to stimulate $^{45}Ca^{2+}$ uptake within 5 min and induced depolarization of Sertoli cell membranes from rats aged 15, 21 and 35 days [30]. The effects of both steroids were not affected by flutamide, an iAR antagonist, suggesting that these non-classical actions occur via a receptor other than iAR [29,30]. The other androgen non-classical pathway seems to involve iAR localization near the plasma membrane. The binding of testosterone with iAR near the plasma membrane allows the receptor to interact with activated Src tyrosine kinase, as reviewed in Smith & Walker [9]. This action results in an increase of phosphorylation of ERK and CREB within 1 min [31].

In contrast, FSH inhibits testosterone-mediated ERK phosphorylation in isolated rat Sertoli cells [32], suggesting a crosstalk in the action of testosterone and FSH. Acting through its G protein coupled receptor (GPCR) exclusively expressed in Sertoli cells [33], FSH also produces changes in the membrane potential of Sertoli cells from immature rats. It causes a rapid hyperpolarization (seconds) followed by a prolonged depolarization (6 min) [34]. The hyperpolarization is blocked in the presence of tolbutamide (a K^+_{ATP} channel blocker) [35] and the depolarization is also blocked by verapamil, a VDCC blocker [34]. Because FSH and testosterone have antagonistic actions on K^+_{ATP} channels, it is also possible that crosstalk exists between the actions of these hormones.

The aim of this work is to investigate the non-classical effects of androgens in Sertoli cells from neonatal rat testes in the absence of iAR. In Sprague Dawley rats on postnatal day (pnd) 5, the presence of iAR in Sertoli cells can already be detected in a relatively weak manner. However, in Wistar rats the iAR ontogeny is controversial [36,37]. It was investigated if iAR is present in Sertoli cells of Wistar rats on pnd 3 and 4 and if testosterone and epitestosterone cause changes in the membrane potential and in $^{45}Ca^{2+}$ uptake. In addition, the influence of testosterone or epitestosterone on the FSH response in the membrane potential of Sertoli cells was investigated in rats aged 15 days. In this age, the FSH receptors are exclusively present in Sertoli cell membranes.

2. Material and methods

2.1. Animals

The experimental animals were male Wistar rats on pnd 3, 4 and 15 with the 24 h following the birth defined as pnd 1. The animals were housed under controlled conditions (approximately 24 °C and a 12 h light/dark cycle). A laboratory diet (Nuvilab, Nuvital CR1, Colombo, PR, Brazil) and water were available to the dams *ad libitum*. The suckling rats were kept with their dams until required for the experiments. The rats were sacrificed by cervical dislocation and the testes were immediately removed by abdominal incision, except for immunohistochemical staining as described below. The study and animal care procedures were reviewed and approved by the Ethics Committee for Animal Research at this University (Universidade Federal do Rio Grande do Sul – UFRGS, www.ufrgs.br, protocol number: 22635).

2.2. Chemicals and solutions used

Polyclonal rabbit anti-AR antibody (SC-816) was purchased from Santa Cruz Biotechnology, Inc. AR (Dallas, TX, USA). Anti-IgG (R2004), peroxidase anti-peroxidase soluble complex antibody (P1291), and 3,3′-diaminobenzidine tetrahydrochloride (D5637) were acquired from Sigma–Aldrich Co. (St. Louis, MO, USA). Normal goat serum was purchased from Merck KGaA® (Darmstadt, Germany). Hank's Balanced Salt Solution (HBSS) buffer contained: NaCl (145 mM), KCl (4.6 mM), $NaHCO_3$ (5 mM), $MgCl_2$ (1.6 mM), $CaCl_2{\cdot}2H_2O$ (1.6 mM), glucose (5 mM) and HEPES (10 mM) at pH 7.4. The lanthanum chloride ($LaCl_3$) buffer contained: NaCl (127.5 mM), KCl (4.6 mM), $MgCl_2$ (1.2 mM), glucose (5 mM), HEPES (10 mM) and $LaCl_3$ (10 mM), pH 7.4 at 0 °C. All reagents were purchased from E. Merck (Darmstadt, Alemanha), except HEPES and $LaCl_3$ that were acquired from Sigma–Aldrich Co. (St. Louis, MO, USA). Testosterone and epitestosterone were reconstituted in ethanol and diluted in HBSS buffer. The final solution concentration did not exceed 0.1% of ethanol. FSH was reconstituted in distilled water and diluted in HBSS buffer. Testosterone, epitestosterone and FSH were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). $^{45}Ca^{2+}$ (specific activity 444 GBq/g) was diluted in HBSS buffer at a concentration of 0.2 μCi/sample. The $^{45}Ca^{2+}$ was acquired

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