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Atrazine activates multiple signaling pathways enhancing the rapid hCG-induced androgenesis in rat Leydig cells

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ABSTRACT

Atrazine (ATR) is an endocrine disruptor that affects steroidogenic process, resulting in disruption of reproductive function of the male and female gonads. In this study, we used the primary culture of peripubertal Leydig cells to investigate the effect of ATR on the rapid androgen production stimulated by human chorionic gonadotropin (hCG). We demonstrated that ATR activated multiple signaling pathways enhancing the rapid hCG-stimulated androgen biosynthesis in Leydig cells. Low hCG concentration (0.25 ng/mL) caused cAMP-independent, but ERK1/2-dependent increase in androgen production after 60 min of incubation. Co-treatment with ATR for 60 min enhanced the cAMP production in hCG-stimulated cells. Accumulation of androgens was prevented by addition of U0126, *N*-acetyl-L-cysteine and AG1478. Co-treatment with hCG and ATR for 60 min did not alter steroidogenic acute regulatory protein (*Star*) mRNA level in Leydig cells. After 120 min, hCG further increased androgenesis in Leydig cells that was sensitive to inhibition of the cAMP/PKA, ERK1/2 and ROS signaling pathways. Co-treatment with ATR for 120 min further enhanced the hCG-induced androgen production, which was prevented by inhibition of the calcium, PKC and EGFR signaling cascades. After 120 min, ATR enhanced the expression of *Star* mRNA in hCG-stimulated Leydig cells through activation of the PKA and PKC pathway. Collectively, these data suggest that exposure to ATR caused perturbations in multiple signaling pathways, thus enhancing the rapid hCG-dependent androgen biosynthesis in peripubertal Leydig cells.

1. Introduction

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine; ATR) is a broad-spectrum herbicide primarily used in crop production to control broadleaf and some grassy weeds. ATR was banned in the EU countries in 2004 (Sass and Colangelo, 2006); however, it is still applied in the United States for agricultural and residential purposes. Due to its extensive use and mobility, ATR is frequently detected in streams, rivers and groundwater (Barr et al., 2007). Data obtained from the area of the greatest ATR use in North America revealed that environmental concentrations of ATR usually do not exceed 0.02 mg/L, except following storm runoff, when concentrations in streams and rivers can reach maximal values of approximately 0.1 mg/L (Solomon et al., 1996). In the United States, maximum contaminant level in drinking water is currently set at 0.003 mg/L (Sass and Colangelo, 2006).

ATR causes a wide range of reproductive and developmental toxicity in fish, amphibians and mammals (Cooper et al., 2000; Hayes et al., 2011; Hayes et al., 2002). In male rats, oral administration of ATR resulted in decreased weight of the ventral prostate, seminal vesicles and epididymis, and also caused delayed puberty, whereas testicular sperm number increased due to reduced sperm motility (Kniewald et al., 2000; Stoker et al., 2000). Gestational exposure to ATR delayed preputial separation and increased the weight of the prostate (Rayner et al., 2004). In female rats, gestational exposure to high doses of ATR reduced the body weight of the offspring and delayed vaginal opening. High doses of

Abbreviations: 22-OH cholesterol, 22(*R*)-hydroxycholesterol; cAMP, 3′-5′-cyclic adenosine monophosphate; CYP11A1, cytochrome P450C11A1; CYP17A1, cytochrome P450C17A1; ATR, atrazine; EGFR, epidermal growth factor receptor; hCG, human chorionic gonadotropin; HSD17β, 17β-hydroxysteroid dehydrogenase; HSD3β, 3β-hydroxysteroid dehydrogenase; LH, luteinizing hormone; LHR, luteinizing hormone receptor; NAC, *N*-acetyl-L-cysteine; PDE, phosphodiesterase; PKA, protein kinase A; PKC, protein kinase C; ROS, reactive oxygen species; SCARB1, scavenger receptor class B, member 1; StAR, steroidogenic acute regulatory protein; T+DHT, testosterone + dihydrotestosterone; TSPO, translocator protein.

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ATR administered orally to adult female rats have been reported to reduce the luteinizing hormone (LH) surge and estrous cycle, as well as decrease the number of corpora lutea and oocytes released from the ovaries (Foradori et al., 2009; Samardzija et al., 2016).

Steroidogenesis is one of the primary targets of the ATR's action in both male and female reproductive system. ATR disrupts estradiol and progesterone biosynthesis in the ovarian granulosa cells (Fa et al., 2013; Pogrmic-Majkic et al., 2014; Samardzija et al., 2016; Tinfo et al., 2011) and alters testosterone level in Leydig cells (Friedmann 2002; Kucka et al., 2012; Pogrmic et al., 2009; Pogrmic-Majkic et al., 2010). Effect of ATR on the steroid-producing reproductive cells is often mediated through alterations in the expression of key proteins involved in steroid biosynthesis, such as aromatase (Fa et al., 2013), steroidogenic acute regulatory protein (StAR), cytochrom P450 11a1 (CYP11A1), 3 beta-hydroxysteroid dehydrogenase (3βHSD) (Abarikwu et al., 2011), cytochrom P450 17a1 (CYP17A1) and 17 beta-hydroxysteroid dehydrogenase (17βHSD) (Pogrmic et al., 2009; Pogrmic-Majkic et al., 2010). We have previously shown that ATR-dependent increase in steroid production in the Leydig and granulosa cells is most likely mediated by inhibition of the specific cyclic adenosine monophopshate (cAMP) phosphodiesterase (cPDE) (Kucka et al., 2012; Pogrmic-Majkic et al., 2014). On the other hand, several studies have demonstrated that ATR-altered androgen production in Leydig cells was not cAMP-dependent (Abarikwu et al., 2013; Karmaus and Zacharewski 2015). Hormone-stimulated regulation of androgen production in Leydig cells requires activation of multiple signaling pathways and is not solely dependent on increase in the cAMP level. While cAMP is the main transmitter of the human chorionic gonadotropin (hCG) action on steroidogenesis in Leydig cells, involvement of the epidermal growth factor receptor/extracellular signal-regulated kinase 1/2 (EGFR/ERK1/2) (Carbajal et al., 2011; Evaul and Hammes 2008) and protein kinase C (PKC) (Manna et al., 2009) pathways has been documented as well. Moreover, ATR can transmit its toxic effect through multiple signaling cascades, such as ERK1/2 in Leydig cells and granulosa cells (Fa et al., 2013; Karmaus and Zacharewski 2015), AKT in granulosa cells (Pogrmic-Majkic et al., 2014) and human choriocarcinoma cell line (JEG3) (Suzawa and Ingraham, 2008), G protein estrogen receptor in different cancer cells (Albanito et al., 2008), and nuclear receptor 5A subfamily in JEG3 cells (Suzawa and Ingraham, 2008).

In this study, we examined the involvement of multiple signaling cascades in ATR's effect on the rapid hCG-induced steroidogenesis. We used a model of the primary culture of Leydig cells isolated from peripubertal rats. Previous results from our laboratory demonstrated that the primary culture of Leydig cells responded to hCG with an increase in androgen production, which was sensitive to ATR exposure (Kucka et al., 2012; Pogrmic et al., 2009; Pogrmic-Majkic et al., 2010). Here, we report that ATR-induced enhancement of the rapid hCG-induced androgenesis depends on the interplay between several different signaling pathways, including the cAMP/PKA, ERK1/2, ROS, calcium, PKC and EGFR signaling.

2. Material and methods

2.1. Chemicals

Atrazine, medium 199 containing Earle's salt and L-glutamine (M199), Hanks' M199 containing 25 mM HEPES, Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham with L-glutamine, 15 mM HEPES (DMEM/F12), HEPES, percoll, bovine serum albumin (BSA) fraction V, collagenase type IA, testosterone, trypan blue, dimethyl sulfoxide, cholesterol, 22(*R*)-hydroxycholesterol, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM), ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), U0126, *N*-acetyl-L-cysteine (NAC), PKI 14–22 amide myristoylated (PKI 14–22), GF109203X and TRIzol Reagent were obtained from Sigma-Aldrich (Steinheim, Germany); AG1478 was from Calbiochem (San Diego, CA, USA); hCG (Pregnyl, 3000 IU/mg) was from Organon (West Orange, NJ, USA).The cAMP EIA Kit was purchased from Cayman Chemicals (Ann Arbor, MI, USA).

2.2. Leydig cell culture and treatments

The primary culture of Leydig cells was obtained from the peripubertal 51 day-old Wistar rats raised in the animal facility of the Department of Biology and Ecology (University of Novi Sad) under controlled environmental conditions (temperature 22 ± 2 °C and 14 h light/10 h dark) with food and water *ad libitum*. Experiments were approved by the Ethics Committee for Protection and Welfare of Experimental Animals of the University of Novi Sad. Isolation of the testicular interstitial cells and purification of the Leydig cells were performed as previously described (Pogrmic-Majkic et al., 2010). In each experiment, the cells obtained from 8 animals were pooled and used for subsequent analyses. For the initial experiments, Leydig cells were allowed for 3 h to attach to 24-well plates (300 000 cells/0.5 mL DMEM/F12/well) before applying ATR (50 μM). This dose of ATR was chosen because it inhibits cAMP-specific PDE4 and increases androgenesis in the hCG-stimulated Leydig cells (Kucka et al., 2012). ATR was added for 30, 60 and 120 min in the presence or absence of two low sub-saturated doses of hCG (0.1 ng/mL and 0.25 ng/mL) in DMEM/F12-0.1% BSA. In the subsequent experiments, the cells were cultured in DMEM/F12-0.1% BSA with a selected low sub-saturated dose of hCG (0.25 ng/mL) for 30, 60 and 120 min in the presence or absence of ATR (50 μM). Leydig cells were pretreated with the following inhibitors: PKI 14–22 (50 μM), U0126 (10 μM), NAC (5 mM), AG1478 (1 μM), BAPTA-AM (20 μM) + EGTA (1 mM) or GF109203X (20 μM) for 60 min and then stimulated with hCG (0.25 ng/mL) in the presence of 50 μM ATR for 60 and 120 min. In some experiments, the cells were treated with the same concentration of ATR during 60 and 120 min in the presence of hCG (0.25 ng/mL) and the two testosterone precursors: cholesterol (20 μM) or 22(*R*)-hydroxycholesterol (20 μM).

2.3. Hormone and cAMP assays

Androgen levels in the incubation medium were estimated by radioimmunoassay using [1,2,6,7^{3}H(*N*)]-labeled testosterone from the New England Nuclear (Brussels, Belgium) and anti-testosterone serum no. 250 provided by Dr. G.D. Niswender (Colorado State University, Fort Collins, CO, USA) that has high cross-reactivity with dihydrotestosterone (DHT), so the results were expressed as T + DHT values. The amount of cAMP accumulated in the culture medium was measured using the cAMP EIA kit, which typically displays an IC_{50} value of approximately 0.5 pmol/mL and a detection limit of 0.1 pmol/mL (at 80% B/B_0) for acetylated cAMP samples.

2.4. Quantitative RT-PCR analysis

Total RNA from Leydig cells was extracted using the TRIzol reagent, followed by the DNAse I treatment (Applied Biosystems, Foster City, CA, USA). Total RNA was transcribed into cDNA and quantitative RT-PCR (qRT-PCR) was performed using the SYBR Green PCR kit as previously described (Pogrmic-Majkic et al., 2010). Gene-specific primer pairs shown in Table 1 were designed using the Primer Express 3.0 software (Applied Biosystems). Data obtained from the qRT-PCR reaction were analyzed using the