Upregulation of Peripubertal Rat Leydig Cell Steroidogenesis Following 24 h In Vitro and In Vivo Exposure to Atrazine

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Atrazine is currently one of the most widely used herbicides in the United States and elsewhere. Here we examined 24 h in vitro and in vivo effects of atrazine on androgen production and on expression and activity of steroidogenic enzymes and regulatory proteins involved in cyclic adenosine monophosphate (cAMP)–signaling pathway in peripubertal rat Leydig cells. When in vitro added, 1–50 μM atrazine increased basal and human chorion gonadotropin-stimulated testosterone production and accumulation of cAMP in the medium of treated cells. The stimulatory action of atrazine on androgen production but not on cAMP accumulation was abolished in cells with inhibited protein kinase A. Atrazine also stimulated the expression of mRNA transcripts for steroidogenic factor-1, steroidogenic acute regulatory protein, cytochrome P450 (CYP)17A1, and 17β-hydroxysteroid dehydrogenase (HSD), as well as the activity of CYP17A1 and 17BHSD. The stimulatory effects of atrazine on cAMP accumulation and androgen production were also observed during the first 3 days of in vivo treatment (200 mg/kg body weight, by gavage) followed by a decline during further treatment. These results indicate that atrazine has a transient stimulatory action on cAMP signaling pathway in Leydig cells, leading to facilitated androgenesis.

Key Words: atrazine; LC steroidogenesis; gene expression.

Atrazine (chloro-s-triazine herbicide) is one of the most commonly used herbicides in the United States (for reference, see Barr et al., 2007) and Serbia (Gasic et al., 2002). Atrazine and its degradates are also the most commonly detected herbicides in ground and surface waters (for references, see Barr et al., 2007; Ochoa-Acuña et al., 2009). It also acts as an endocrine disruptor (Cooper et al., 2000; Laws et al., 2003; Stoker et al., 2000a) and may be associated with risk of mammary cancer (Birnbaum and Fenton, 2003; Cooper et al., 2007).

There is a great number of studies suggesting that atrazine can alter reproductive function in different species: amphibians (Hayes et al., 2003, 2010), young fish (Spano et al., 2004), developing alligators (Crain et al., 1997), and peripubertal rats (Friedmann, 2002; Stoker et al., 2000a; Trentacoste et al., 2001). It affects the reproductive function of both females and males (Cooper et al., 2007; Stoker et al., 2000b). In male rats, the exposure to atrazine from postnatal day (pnd) 21 until 53 caused a significant decrease in serum and testicular testosterone levels when administered at doses of 100–200 mg/kg body weight (bw) (Stoker et al., 2000a). Trentacoste et al. (2001) also suggested that atrazine inhibits luteinizing hormone (LH) and testosterone production when applied at concentrations at or above 100 mg/kg per day and that reduction is secondary to weight loss. Friedmann (2002) further showed that atrazine applied by gavage in the dose of 50 mg/kg bw/dy reduced significantly the serum and intratesticular testosterone levels, both acutely (from pnd 46 to 48) and chronically (from pnd 22 to 48).

In line with these studies, we (Pogrmic et al., 2009) have shown previously that in vivo exposure to atrazine, from pnd 23 to 50, dramatically downregulated Leydig cell steroidogenesis via suppression of luteinizing hormone receptor (LHR) gene expression, inhibition of cyclic adenosine monophosphate (cAMP) production, followed by severe decline in mRNA transcripts of several genes responsible for steroidogenesis: LHR, scavenger receptor-B1 (SR-B1), steroidogenic acute regulatory protein (STAR), translocator protein (TSPO), steroidogenic factor-1 (SF-1), phosphodiesterase 4B (PDE4B), cytochrome P450 (CYP)17A1 and 17β-hydroxysteroid dehydrogenase (HSD), as well as basal and human chorion gonadotropin hormone (hCG)–stimulated androgen production.

In the present study, we investigated concentration dependence of atrazine on in vitro steroidogenesis by Leydig cells from peripubertal rats (pnd 51). Contrary to the long-term in vivo studies, these short-term (24 h) treatments caused facilitation of cAMP signaling and increased steroidogenic capacity of Leydig cells. To address these two opposite effects of atrazine, we also performed in vivo experiments in which groups of rats were treated for 1, 3, or 6 days with atrazine. These experiments confirmed the stimulatory but transient action of atrazine on cAMP signaling and androgenesis.
MATERIALS AND METHODS

Chemicals. Atrazine (98%) obtained from Supelco was used for in vitro experiments, whereas technical grade atrazine (98%) that was used for oral application was a generous gift from Dr Sanja Lazic (Department for Environmental and Plant Protection, Faculty of Agriculture, University of Novi Sad, Serbia; Pogrmic et al., 2009). The RNAlater-4PCR Kit for total RNA isolation was obtained from Ambion, chemicals for cDNA preparation (High Capacity cDNA Reverse Transcription Kit with RNase inhibitor), and Power SYBR Green PCR Master Mix was purchased from the Applied Biosystems. Primers for real-time PCR were obtained from the Integrated DNA Technologies. The anti-testosterone-11-BSA serum no.250 and anti-progesterone-11-BSA serum no.337 were kindly supplied by Dr Niswender (Colorado State University, Fort Collins, CO). The cAMP enzyme immunoassay (EIA) Kit was purchased from the Cayman Chemicals (Ann Arbor, MI). The [1,2,6,7(H)] labeled testosterone and [1,2,6,7(H)]-progesterone were obtained from the New England Nuclear (Brussels, Belgium). Medium 199 containing Earle’s salt and l-glutamine (M199), Dulbecco’s modified Eagle’s medium (DMEM)/Nutrient Mixture F-12 Ham (DMEM/F12) with l-glutamine and 15mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), HEPES, Percoll, bovine serum albumin (BSA) fraction V, collagenase type IA, progesterone, Δ4-androstenedione, testosterone, tranpy, blue, dimethyl sulfoxide (DMSO), and N-[(p-bromo-cinnamylamino)ethyl]-5-isoquinalinium-sulfonamide dihydrochloride hydrate (H89) were obtained from Sigma (Steinheim, Germany), whereas hCG (Pregnyl, 3000 IU/mg) was obtained from Organon (West Orange, NJ). All other reagents were of analytical grade.

Animals. All experiments were performed with peripubertal male Wistar rats raised in animal facility at the Department of Biology and Ecology under controlled environmental conditions (temperature 22°C ± 2°C and 14 h light/10 h dark) with food and water ad libitum. For in vitro experiments, animals were sacrificed by decapitation on pnd 51, and Leydig cells were isolated and purified by Percoll gradient. Groups of rats were treated with atrazine by gavage on pnd 50 at dose of 200 mg/kg bw/day and experiments were repeated three times. Another groups of rats were treated with atrazine by gavage on pnd 46, and 48 until pnd 50 at dose of 200 mg/kg bw/day, together with group of rats treated only on pnd 50. Treated rats were sacrificed at the same day 24 h after last exposure on pnd 51. These experiments were repeated twice. Atrazine was dissolved in olive oil to desired concentrations. The control groups of rats received olive oil. All experiments were approved by the Local Animal Ethical Committee of the University of Novi Sad and were conducted in accordance with the principles and procedures of the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Leydig cell purification and incubation procedure. Isolation and purification of Leydig cells has been done according to Leckie et al. (1998) with some modifications as previously described (Andric et al., 2009). Purified Leydig cells were prepared from suspensions of testicular interstitial cells by centrifugation on a Percoll gradient consisting of four 2-mL layers of Percoll with densities of 1.090, 1.080, 1.065, and 1.045 g/mL, and fractions containing Leydig cells were collected from the 1.080/1.065 g/mL and 1.065/1.045 g/mL interfaces. The proportion of Leydig cells present in the culture was determined by staining for 3β-HSD activity (Payne et al., 1980) and was typically more than 95%. For in vitro experiment, Leydig cells were pooled and allowed 3 h to attach to 55-mm Petri dishes (1 × 10^6 cells/2 ml culture medium/dish) and then treated with atrazine for 24 h with the following concentrations (0.001, 1, 10, 20, and 50μM). At the end of the incubation period, culture medium was collected and cells were treated with different concentrations of hCG or different steroid substrates: progesterone and Δ4-androstenedione for 2 h. After the treatment, cell-free media were collected and stored at −70°C prior to measurement of cAMP, androgen, and progesterone levels, whereas cell lysates were used for real-time PCR analysis. In case of 3-h exposure, cells (1 × 10^6/2 ml culture medium/dish) were incubated in the presence of atrazine (232μM) and in the presence or absence of hCG (0.25 ng/ml and 10 ng/ml culture medium). Atrazine was dissolved in DMSO, which final concentration did not exceed 0.1% in the culture medium, except in the case of 232μM when DMSO final concentration was 0.4%. Corresponding control with 0.4% DMSO was included in the experiment.

For each in vivo experiment, Leydig cells obtained individually from four to five rats were pooled and each pool/group was cultured in four to eight replicates per experiments. Cells were allowed 3 h to attach to 55-mm Petri dishes (2.5 × 10^6 cells/2 ml culture medium/dish) and then cultured in M199-0.1% BSA without or with saturated dose of hCG (10 ng/ml) for 2 h. MTT assay. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay is based on the measurement of conversion of the yellow thiazolyl blue tetrazolium bromide to a purple formazan derivative by mitochondrial dehydrogenase in viable cells. The assay was conducted in purified rat Leydig cells, seeded in 96-well plate at the density 50,000 cells/well in DMEM/F12, and 3 h after seeding, exposed in multiplicates to different doses of atrazine. Control and blank wells were included in each plate. After 24 h of incubation, the medium was removed and cells were incubated with 3 h with 0.05 mg/0.1 ml/well MTT dissolved in DMEM/F12. Formazan salts were dissolved in 0.1 ml/well of 0.04M HCl in isopropanol, and light absorption was measured using a plate reader (ThermoLabsystems) on 540 nm, with reference wavelength 690 nm. In a case of 3-h incubation with 232μM of atrazine, parallel 3-h incubation according to the above described procedure were done for permuting MTT assay.

Hormone and cAMP assays. Androgen and progesterone levels in the incubation medium were estimated by radioimmunoassay according to the procedure described in Andric et al. (2000). Each experiment was run in a single assay. The precision of androgen assay was 6 pg/tube; intra- and interassay variation coefficients were 5.8 and 7.5%, respectively. Because the anti-testosterone serum used in our assay has a high cross-reactivity with dihydrotestosterone, assay values are referred to as testosterone + dihydrotestosterone levels (T + DHT). Progesterone measurements were also done in one assay (sensitivity 6 pg/tube; intra-assay variation coefficient 6-8%). The amounts of cAMP accumulated into the culture medium were measured by the cAMP EIA Kit, which typically displays an IC50 value of approximately 0.5 pmol/ml and a detection limit of 0.1 pmol/ml (at 80% B/B0) for acetylated cAMP samples.

Reverse transcription PCR. Total RNAs from rat Leydig cells were isolated using RNAlater-4PCR Kit and following the protocol recommended by the supplier. Concentrations and purity of RNA were determined spectrophotometrically. To eliminate residual genomic DNA, RNA samples were treated with 1 μl DNase-1 (2 IU/μl in 60 μl RNA samples). Following DNase-1 treatment, up to 2 μg of the total RNA from each sample were reversely transcribed into cDNA in a 20-μl reaction mixture containing random primers and MultiScribe reverse transcriptase (Applied Biosystems) according to the supplier’s instructions. Reverse transcription was estimated at 25°C for 10 min, at 37°C for 120 min, and then at 85°C for 5 s in the Veriti Thermal Cycler (Applied Biosystems). Real-time PCR was performed with the 7900HT Fast Real-Time PCR system (Applied Biosystems). The SYBR Green PCR Master Mix reagent kits were used according to the supplier’s instructions for quantification of gene expression. Rat-specific primers were designed for the relevant genes: LHR, SR-B1, StAR, TSPO, SF-1, PDE4B, 3βHSD, CYP17A1, 17βHSD, and CYP19A1. The housekeeping gene β-actin was used as an endogenous control. Primers were designed by using software Primer Express 3.0 (Applied Biosystems) and full gene sequences from NCBI Entrez Nucleotide Database (http://www.ncbi.nlm.nih.gov/sites/entrez). Cycling conditions, determined in ABI Prism 7900HT Sequence Detection System (SDS) protocol, were as follows: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 94°C for 15 s and 60°C for 1 min, in the presence of 200nM of specific primers and 5 μl of product from reverse transcription reaction. Amplification efficiencies were determined to be 100% for the target genes and β-actin (data not shown). After real-time PCR, a melting curve analysis was conducted to determine the specificity of the PCR product, which was displayed by a single peak for all investigated genes (data not shown). The calculation of the relative expression levels of each
RESULTS

Effects of Atrazine on cAMP Accumulation and Gonadal Steroidogenesis

In order to examine direct effects of atrazine on the steroidogenic pathway in Leydig cells, we investigated in vitro basal and hCG-supported cAMP, androgen, and progesterone production by Leydig cells. Figure 1, left, shows that atrazine significantly stimulated basal cAMP accumulation in medium during 24-h incubation in both (1 and 20 μM) concentrations. Atrazine in 20 μM concentrations also significantly elevated hCG-stimulated cAMP accumulation during 2-h incubation (Fig. 1, right).

In parallel experiments, we examined concentration-dependent effects of atrazine on basal and hCG-stimulated steroidogenesis. Table 1 summarizes effects of atrazine on basal steroidogenesis. Basal progesterone production was bidirectionally regulated by atrazine, facilitated in the presence of smaller, and inhibited in the presence of higher concentrations of atrazine. In contrast, basal androgen production was facilitated by atrazine in a concentration-dependent manner (Table 1). Similar pattern of changes was observed in hCG-supported androgen and progesterone production. Figure 2 summarizes concentration-dependent effects of atrazine on testosterone (left panels) and dihydrotestosterone (right panels) production.

The ability of atrazine to stimulate both cAMP and androgen production is consistent with the cAMP-protein kinase A (PKA) dependence of androgenesis (Payne and Youngblood, 1995). To test this hypothesis more directly, effects of H89, a PKA inhibitor, on atrazine-induced cAMP accumulation and androgen production were tested. Initially, Leydig cells were treated from 24 h with/without 20 μM atrazine, followed by the washout and additional 2-h incubation in 10 ng/ml hCG-containing medium in the presence and absence of 10 μM H89. Atrazine-induced cAMP accumulation was not affected by H89, in contrast to...
atrazine-induced androgen production (Fig. 3). These results indicate that atrazine stimulates cAMP accumulation, which in turn facilitates testicular androgenesis.

We also studied effects of higher (232 µM) concentration of atrazine on Leydig cell androgenesis during 3-h incubation period. These experiments revealed that stimulatory effect of atrazine on Leydig cell androgenesis depends on hCG concentration. In two experiments with cells bathed in medium containing 0.25 ng/ml hCG, atrazine increased androgen production for 126 and 142%. In contrast, in three experiments with cells stimulated with 10 ng/ml hCG, atrazine stimulated androgen production for only 10, 34, and 12% (Table 2).

Atrazine in concentrations of 10, 50, and 232 µM was tested for possible toxic effect on Leydig cells used in in vitro studies. Obtained results on mitochondrial dehydrogenase activity in atrazine-treated cells compared with control values indicated that applied atrazine concentrations had no toxic effect on viability of peripubertal Leydig cells.

**Steroidogenic Gene Expression Profile**

We further determined the status of mRNA transcripts of genes involved in LHR action, cholesterol transport, and steroidogenesis (Fig. 4). These experiments showed that expression of gene for SF-1 increased 141 and 191% after exposure to 1 and 20 µM of atrazine, respectively. Also, expression of STAR gene involved in cholesterol transport into mitochondria increased 162% in Leydig cells treated with higher dose of atrazine. In response to application of 20 µM of atrazine, there was 247% increase in the level of CYP17A1 transcript and 153% increase in the level of 17βHSD transcript (Fig. 4). In contrast, the transcript levels for LHR, SR-B1, and TSPO were not affected by atrazine treatment. The expression of 3βHSD genes, the enzyme that catalyses the conversion of pregnenolone to progesterone, was also not affected. The expression of gene for PDE4B, which is a cAMP-specific form of PDE4, was also not affected. The same result was obtained for CYP19A1, an enzyme that catalyses the conversion of androgen to estrogens (data not shown).

**Progesterone- and Androstenedione-Supported Testosterone Production**

The results of gene expression demonstrated that CYP17A1 and 17βHSD were affected by 24 h in vitro atrazine exposure. To estimate whether the protein level of these enzymes was also affected during this period, we measured the conversion of progesterone to testosterone, which reflected the activity of CYP17A1 and/or 17βHSD. We also measured the conversion of Δ4-androstenedione to testosterone, which reflected the activity of 17βHSD. Leydig cells were exposed to atrazine for 24 h and then challenged with different steroid substrates: progesterone and Δ4-androstenedione in a concentration-dependent manner. Conversion of progesterone to testosterone
...gene was based on the cycle threshold (Ct) of specific primers. Calculation of the relative expression levels of each target time PCR reactions were performed by SYBR Green technology in the presence of atrazine for 24 h. After that, medium was replaced and cells were incubated in the absence of atrazine for additional 2 h. Real-time PCR reactions were performed by SYBR Green technology in the presence of specific primers. Calculation of the relative expression levels of each target gene was based on the cycle threshold (Ct) method with controls expressed as 1. In these experiments, β-actin was used as an endogenous control. Columns represent mean ± SEM values of three independent experiments. Statistical significance: *p < 0.05 versus corresponding controls (0).

was significantly stimulated when Leydig cells were challenged with higher concentrations of atrazine (20 and 50μM) for 24 h and incubated in the presence of progesterone and atrazine for additional 2 h. The increase of conversion of progesterone to testosterone in the presence of these two concentrations of atrazine was similar regardless of concentrations of progesterone added as a substrate (average value 227%). Conversion of Δ4-androstenedione to testosterone was also stimulated in the presence of atrazine but to a lesser extent than conversion of progesterone to testosterone. In the presence of 0.25μM of Δ4-androstenedione as a substrate, all concentrations of atrazine, except the smallest one, induce significant increase of substrate conversion to testosterone, whereas in the presence of higher concentration of Δ4-androstenedione, only 20μM atrazine induced significant increase in androgen level. Moreover, the increase in androgen production in the presence of all three concentrations of Δ4-androstenedione was similar, when Leydig cells were challenged with 20μM atrazine (average value 170%) (Fig. 5). These results indicate that there is a parallelism in the action of atrazine on mRNA expression and the activity of these two enzymes.

Ex Vivo Androgenesis

In parallel with in vitro experiments, we conducted in vitro experiments where the peripubertal male rats were exposed to atrazine (200 mg/kg bw by gavage) from pnd 45 (6 applications), pnd 48 (3 applications), or pnd 50 (single application) until pnd 50. Twenty-four hours later, Leydig cells were isolated and levels of cAMP and testosterone were determined after 2-h incubation in basal and in hCG-stimulated conditions. These experiments revealed similar pattern of changes in basal and hCG-stimulated cAMP accumulation and androgen production: a significant increase after single injection, peak amplitude in response after 3-day treatment, and smaller responses after 6-day treatment (Fig. 6).

DISCUSSION

Testosterone is synthesized in Leydig cells from precursor cholesterol through a multistep enzymatic cascade, including CYP11A1, 3βHSD, CYP17A1, and 17βHSD. Conversion of cholesterol to pregnenolone is controlled by CYP11A1, pregnenolone is further metabolized by the action of 3βHSD to progesterone, conversion of progesterone to androstenedione is catalyzed by CYP17A1, and 17βHSD converts androstenedione to testosterone (Payne and Youngblood, 1995). The main control of this process occurs through periodic release of LH from the pituitary gland and activation of LHR expressed in Leydig cells. These receptors signal through heterotrimeric G proteins, leading to stimulation of adenylyl cyclases and increase in cAMP intra- and extracellular levels. In addition to cAMP, several other signaling pathways are activated by these and other receptors in Leydig cells (Manna et al., 2006, 2007).

As stated in the “Introduction” section, prolonged in vivo treatment with atrazine causes downregulation of testicular androgenesis (Friedmann, 2002; Laws et al., 2000; Stoker et al., 2000a; Trentacoste et al., 2001). Our previous studies also demonstrated that oral application of atrazine to peripubertal male rats for 28 days downregulated the expression of genes for steroidogenic enzymes and regulatory proteins involved in the control of testicular steroidogenesis (Pogrmic et al., 2009). However, there are only limited informations about direct influence of atrazine on Leydig cells, showing that 232μM of atrazine reduced LH-stimulated testosterone production in Leydig cells after 3-h incubation period (Friedmann, 2002).

The objective of the current study was to investigate direct effects of atrazine on Leydig cell steroidogenesis and the possible mechanisms of actions. Specifically, we analyzed the effect of atrazine on hormone production, cAMP accumulation, and expression of steroidogenesis-related genes in Leydig cell in vitro. Our experiments showed that atrazine increased steroidogenic capacity of Leydig cells in a concentration-dependent manner when applied in 1–50μM. Such stimulatory effect was present in both basal and unsaturated/saturated hCG conditions. At higher concentrations (232μM), the stimulatory effect of atrazine on testicular steroidogenesis was still present when Leydig cells were challenged with smaller concentration of hCG but practically abolished in the presence of saturated...
concentration of hCG. Such atypical concentration response probably reflects different sensitivity of multiple effectors in these cells.

We have also progressed in understanding the mechanism of stimulatory action of atrazine on androgenesis. Our results clearly showed significant increase in cAMP levels in bath medium collected from atrazine-treated cells, compared with untreated cells. It is well established that elevation in intracellular cAMP levels leads to proportional increase in extracellular cAMP, presumably transported by multidrug resistance proteins (Andric et al., 2006b). Thus, the measurement of bath cAMP provides a sensitive test about the status of cAMP signaling in cells.

In general, increase in cAMP levels reflect increase in cAMP production and/or decrease in cAMP metabolism by phosphodiesterases. Our results do not provide a clue, which of these two pathways is affected. However, others have shown that atrazine inhibits phosphodiesterases from bovine heart (Roberge et al., 2004) and swine lung, brain, and heart, but not liver and kidney (Roberge et al., 2006).

Our results also indicate that effects of atrazine on androgenesis depend on elevated cAMP levels and activation of PKA because combined exposure to atrazine and PKA inhibitor, H89, abolished atrazine-induced upregulation of androgenesis. This in turn suggests that PKA stimulates the expression of enzymes controlling steroidogenesis. Consistent with this hypothesis, here we show that atrazine stimulates the expression of several genes responsible for steroidogenesis: SF-1, StAR, CYP17A1, and 17βHSD.

One of the major mechanisms by which cAMP/PKA pathway promotes steroidogenesis is upregulation of the expression of genes for StAR (Rao et al., 2003; Stocco et al., 2005), SR-B1 (Azhar and Reaven, 2002; Rao et al., 2003), and SF-1 (Lehmann et al., 2005; Urs et al., 2007). Thus, it can be predicted that increased levels of cAMP could lead to increased expression of those genes. Our results on upregulation of gene expression for SF-1 and StAR, in the environment of elevated cAMP levels in Leydig cells treated with atrazine, confirmed such relations. However, the transcript level for SR-B1, involved in cholesterol transporting, was not disturbed in Leydig cells treated with atrazine.

In addition to the StAR protein, the mitochondrial high-affinity cholesterol binding protein TSPO works in a coordinated
manner with StAR to transfer cholesterol into mitochondria. It is shown that TSPO functions as a channel specific for cholesterol (reviewed by Papadopoulos et al., 2007). Our results suggested that higher concentrations of atrazine increased transcript level for StAR, whereas they had no effect in the case of TSPO.

Furthermore, it is known that SF-1 controls the expression of several genes involved in steroidogenesis, including genes for CYP17A1 (Li et al., 2007; Sewer and Jagarlapudi, 2009) and StAR (Suzawa and Ingraham, 2008). The results of our study indicate that direct exposure of Leydig cells to atrazine markedly increased the level of transcript for CYP17A1. Namely, de novo synthesis of CYP17A1 stops in the absence of cAMP, and expression of this enzyme depends on SF-1 (Payne, 2007). The results of gene expression also showed that 17βHSD is affected by 24 h in vitro atrazine exposure. Moreover, the measurement of activities of these enzymes revealed that increased conversion of progesterone to testosterone and conversion of Δ5-androstenedione to testosterone in Leydig cells challenged with atrazine could be attributed to the increased enzyme activity in that part of steroidogenic pathways, that is, to upregulated expression of CYP17A1 and 17βHSD.

Our previous study on prolonged in vivo oral exposure to atrazine from pnd 23 to 50 indicated downregulation of Leydig cell steroidogenesis via the suppression of cAMP production and inhibition of steroidogenic gene expression (Pogrmic et al., 2009). This contrast the present data showing that 24-h incubation of peripubertal rat Leydig cells in the presence of atrazine increased cAMP production and steroidogenic capacity of those cells. To understand and clarify these two opposite effects of atrazine and possible impact of short versus prolonged exposure, we performed in vivo experiments in which groups of rats were treated for 1–6 days with 200 mg/kg atrazine until pnd 50. The stimulatory effects of atrazine on cAMP accumulation and androgen production were also observed in vivo during the first 3 days of treatment followed by a decline during further treatment. These results indicate that atrazine has a transient stimulatory action on cAMP signaling pathway and steroidogenesis. Further study would clarify duration of treatment when stimulatory atrazine action turns to inhibition.

Taking into account the results from our previous study (Pogrmic et al., 2009), we could suggest that oral application of atrazine induced biphasic response of androgen production in ex vivo conditions, stimulation after relatively short exposure, followed by strong inhibition of androgenesis after 28 days of exposure. Similar response was observed in the study of Andric et al. (2006a) after oral daily application of technical mixture of polychlorinated biphenyls, named pyralene, to adult male rats, but switch to inhibition was observed as early as 24 h.

Finally, here we show that hCG-stimulated progesterone production was stimulated in cells bathed in medium containing low concentrations of atrazine but inhibited in the presence of higher doses of atrazine. We could speculate that such bidirectional action of atrazine on progesterone accumulation reflects increased CYP17A1 and 17βHSD enzyme activity in atrazine-treated cells, causing transformation of progesterone to androgens and presumably to estrogens, which were not measured in our experiments. In other studies, it has been shown that atrazine elicits estrogen action by upregulating aromatase activity in human adrenocortical carcinoma H295R cells (Henewer et al., 2004; Sanderson et al., 2000, 2001). Analogous induction profiles were observed for human placental carcinoma cell line JEG-3 (Sanderson et al., 2002). However, atrazine exposure did not produce similar increases in either the human breast cancer cell line MCF-7 (Sanderson et al., 2002), the rat Leydig cell carcinoma R2C (Henewer et al., 2004), or the human ovarian granulose-like tumor KGN cell line (Morinaga et al., 2004).

It is also important to stress that in addition to its direct action on testicular steroidogenesis, atrazine may also affect steroidogenesis by altering the hypothalamo-pituitary functions. It has been recently shown that atrazine and its metabolites activate hypothalamo-pituitary-adrenal axis in male rats and that elevation in adrenocorticotropic hormone release accounts for increase in adrenal steroidogenesis (Fraites et al., 2009; Laws et al., 2009). In our study, we did not measure LH release, and thus, we cannot exclude the possibility that elevation in this pituitary hormone content did not contribute to changes in basal ex vivo steroidogenesis. For this reason, we also performed all experiments with hCG to evaluate more precisely direct effects of atrazine in the presence of fixed levels of gonadotropins.

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