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Novel molecular events associated with altered steroidogenesis induced by exposure to atrazine in the intact and castrate male rat

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ABSTRACT

Toxicology is increasingly focused on molecular events comprising adverse outcome pathways. Atrazine activates the hypothalamic-pituitary adrenal axis, but relationships to gonadal alterations are unknown. We characterized hormone profiles and adrenal (intact and castrate) and testis (intact) proteomes in rats after 3 days of exposure. The adrenal accounted for most of the serum progesterone and all of the corticosterone increases in intact and castrated males. Serum luteinizing hormone, androstenedione, and testosterone in intact males shared a non-monotonic response suggesting transition from an acute stimulatory to a latent inhibitory response to exposure. Eight adrenal proteins were significantly altered with dose. There were unique proteomic changes between the adrenals of intact and castrated males. Six testis proteins in intact males had non-monotonic responses that significantly correlated with serum testosterone. Different dose–response curves for steroids and proteins in the adrenal and testis reveal novel adverse outcome pathways in intact and castrated male rats.

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Abbreviations: 2D SDS-PAGE, two dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis; ACN, acetonitrile; ACTH, adrenocorticotropic hormone; AMBIC, ammonium bicarbonate; ANDRO, androstenedione; ANOVA, analysis of variance; ATR, atrazine; ATR-d5, deuterated atrazine; BCA, bicinchoninic acid; CORT, corticosterone; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; DACT, diamino-s-chlorotriazine; DEA, deethyl-s-chlorotriazine; DELFIA, dissociation-enhanced lanthanide fluorometric immunoassay; DIA, desisopropyl-s-chlorotriazine; E1, estrone; E2, estradiol; EDTA, Ethylenediaminetetraacetic acid; GLM, general linear model; GnRH, gonadotrophin releasing hormone; HPA, hypothalamic-pituitary-adrenal; IACUC, Institutional Animal Care and Use Committee; LC–MS/MS, liquid chromatography coupled with tandem mass spectroscopy; LH, luteinizing hormone; LOEL, lowest observable effect level; LOQ, limit of quantification; LSM, least square means; MALDI, matrix-assisted laser desorption/ionization; MALDI MS/MS, matrix-assisted laser desorption/ionization coupled mass spectroscopy; MALDI TOF/TOF, matrix-assisted laser desorption/ionization time of flight mass spectroscopy; MSD, mass selective detector; NHEERL, National Health and Environmental Effects Research Laboratory; OBG, octyl-beta-glucopyranoside; ORD, Office of Research and Development; ORISE, Oak Ridge Institute for Science and Education; P4, progesterone; PMSF, phenylmethylsulfonyl fluoride; PPB, parts per billion; RIA, radioimmunoassay; RTP, Research Triangle Park; SEM, standard error of the mean; T, testosterone; TFA, tri-fluoroacetic acid; UBC, ubiquitin C; U.S. EPA, U.S. Environmental Protection Agency.

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1. Introduction

Atrazine (2-chloro-4-ethylamino-6-isoproplyamino-s-triazine; ATR) is a common herbicide used in the United States for agricultural and landscape purposes. An estimated 76.5 million pounds of ATR is used each year mainly within the midwestern "corn belt" and south central regions of the US to control foliage both pre- and post emergently [1]. ATR, along with its metabolites, can persist in soil for months to years depending on soil conditions and the type of bacteria present, and can readily enter surface and ground waters by runoff and soil infiltration [2,3]. The highest concentrations of ATR and its by-products are typically observed in areas of major usage such as the Midwest during the seasonal months of farming, and concentrations above the maximum contaminant level of 3 ppb (parts per billion) set forth by the U.S. Environmental Protection Agency have been reported in community water sources [4,5].

Exposure to ATR is known to produce deleterious effects on the endocrine and reproductive systems as documented in several different species such as fish [6–8], pigs [9,10], Japanese quail [11], and rats [12–14]. Based on a few epidemiological studies, possible adverse effects of ATR on reproduction have been reported in humans as well. Preliminary evidence indicates an association between menstrual cycle irregularity in women and the consumption of drinking water in agricultural communities where ATR is widely used [15]. Previous studies have also suggested a potential link to low semen quality in men exposed to agricultural chemicals (such as ATR) in geographical areas with high ATR use [16,17].

At least some of the adverse reproductive effects produced by ATR administration may be mediated via its effects on the central nervous system. Within the CNS, alterations in neurotransmitter concentrations contribute to observed changes in gonadotropin-releasing hormone (GnRH) [14]. Moreover, ATR exposure in both male and female rats increases adrenocorticotropic hormone (ACTH) which is followed by a dose-dependent release in progesterone (P4) and corticosterone (CORT). Thus, ATR administration produces an activation of the hypothalamic-pituitary-adrenal (HPA) axis resulting in alterations in normal endocrine homeostasis such as the nadir concentrations of CORT and changes in corticosterone binding globulin or CBG [18–20]. Finally, ATR directly influences steroidogenesis in both the adrenal and the gonad. Increases in estradiol (E2), estrone (E1), and P4 production result when human adrenocortical carcinoma and rat granulosa cells are exposed to ATR (10–30 μM) [21,22]. Similarly, increases in testosterone (T) and P4 result when rat Leydig cells are exposed ex vivo and in vitro to ATR (1–50 μM) [23,24].

Therefore, ATR appears to stimulate hormone secretion by both the adrenals and gonad. In the present study we wanted to focus on the male exposed to ATR with and without androgen maintenance in hopes of better defining the relative roles of the adrenal and testis hormones. We used an exposure paradigm (route, duration, doses) that we previously demonstrated to elevate serum CORT. We not only wanted to compare the dose–response of steroid hormones in intact and castrate males, but also wanted to relate the responses of steroid hormones to proteomic alterations in the adrenal and testis.

There is increasing emphasis on identifying the molecular events involved in adverse outcome pathways following toxicant exposure as well as the elucidation of novel biomarkers of effect. Previous studies demonstrated alterations in the transcription of steroidogenic genes in Leydig cell cultures such as StAR, 17BHSD, SF-1, and CYP17A1 following multiple daily exposures to ATR [23,24]. However, to date, no non-targeted, comprehensive proteomic evaluations have been conducted in steroidogenic tissues from ATR exposed animals. Therefore, we evaluated the complete adrenal (intact and castrate Wistar rats) and testis (intact Wistar rats) proteomes following 3 days of exposure to ATR.

Steroid and proteins were evaluated for significant dose–response alterations and significant correlations. This was done to provide additional insight into the relationship between hormonal status and protein expression in both intact and castrate rats exposed to ATR. Proteins that were significantly altered by exposure or correlated with a hormone or another protein were subjected to pathway analysis. Finally, plasma concentrations of ATR and its metabolites were analyzed in order to provide details on any possible alterations in the metabolism of ATR related to the presence or absence of gonadal T.

2. Materials and methods

2.1. Animals

Animal work was completed following review by the U.S. EPA National Health and Environmental Effects Research Laboratory (NHEERL) Institutional Animal Care and Use Committee (IACUC). Adult male Wistar rats (age 45 days upon arrival and 71 days at start of treatment) were obtained from Charles River Laboratories (Raleigh, NC) and maintained on a 12 h light/dark cycle (lights on at 600, lights off at 1800) under controlled conditions [temperature (20–24 °C), humidity (40–50%)]. Animals were given food (Purina Laboratory Chow 5001) and water ad libitum. One week after arriving at the facility, animals were weight-ranked, divided into 5 treatment groups (vehicle control, 5, 25, 75, 200 mg/kg ATR), and further subdivided into 2 groups (castrate or intact). There were 10 castrated and 10 intact animals per treatment that were divided into 2 blocks (total of 100 animals/50 animals per block).

2.2. Castration

Animals were castrated 12 days prior to the start of treatment. In brief, animals were anesthetized and an appropriate plane of anesthesia was maintained with inhalational isoflurane. Each testis was exposed individually via an abdominal incision. The efferent ducts and testicular artery were ligated, fascia between the ligature and testis was cut, and the testis was removed. The abdominal musculature was sutured with 3–0 chromic gut while the skin layer was closed in apposition with Nexaband S/C adhesive (Abbott Laboratories, Abbott Park, IL) and secured with 3 M Precise skin staples (3 M Healthcare, St. Paul, MN). Animals were given 2 mg Rimadyl® MD Tablets for Rodents (MD150-2, BioServ, Frenchtown, NJ) post-operatively for analgesia. Animals were monitored by animal care staff post-operatively.

2.3. Dosing solutions and procedures

ATR (CAS 1912-24-9, 97.1% purity) was a gift from the Syngenta Corporation (Greensboro, NC). All solutions were a suspension of 1% methyl cellulose prepared with distilled water (M7140, Batch#108K0130, Sigma Aldrich, St. Louis, MO). All treatments and decapitation of the animals occurred between 0700 and 0900 h when the circadian fluctuation of CORT was at the nadir. In order to acclimate the animals to the dosing procedure, each rat was administered the vehicle control (1% methylcellulose by oral gavage) for 7 days before the start of ATR exposure. Our previous work demonstrated that animals gavaged for 5–7 days with 1% methyl cellulose maintain basal CORT levels [18,19]. At the onset of ATR treatment, the rats were administered either the test compound (5, 25, 75, 200 mg/kg) or 1% methylcellulose (vehicle control) by oral gavage delivered in a volume of 5.0 ml/kg of body weight for 3 days. Animals were weighed daily and the volume of solution given was adjusted for body weight. After the 3rd day of ATR exposure, the rats were decapitated 30 min after ATR administration, and trunk blood was collected into serum (SST Plus Blood Collection tubes, BD