Characterization of the Hypothalamic-Pituitary-Adrenal Axis Response to Atrazine and Metabolites in the Female Rat

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Atrazine (ATR) has recently been shown to activate the hypothalamic-pituitary-adrenal (HPA) axis in rodents. The current study investigated the effect of ATR and two of its chlorinated metabolites, desisopropylatrazine (DIA) and diamino-s-chlorotriazine (DACT), on the HPA axis in the Long-Evans female rat. A single oral gavage administration of 75 mg/kg ATR or 60.2 mg/kg DIA (a dose equimolar to the applied ATR dose) during the morning of proestrus resulted in significant, acute increases in circulating adrenocorticotropic hormone (ACTH), corticosterone, and progesterone. Oral doses of ATR or DIA were given daily over the course of the 4-day ovarian cycle starting on the day of vaginal estrus, resulting in a similar, dose-responsive activation of the HPA axis. The increase in ACTH, corticosterone, and progesterone by these compounds was of a similar magnitude to that produced by 5-min restraint stress. Single or multiple oral exposures to DACT, on the other hand, did not significantly alter pituitary-adrenal hormone release. These results were observed despite plasma levels of DACT being higher than any other metabolite at the time of hormone measurement. Overall, circulating metabolite concentrations following equimolar dosing were much higher than those observed after ATR administration. Additional studies indicated that the activation of the HPA axis by oral exposure to ATR and DIA was not due simply to the stimulation of gastrointestinal afferents. Similar responses were observed in rats which received an oral dose of ATR following bilateral subdiaphragmatic vagotomy and following intravenous administration of DIA in jugular vein catheterized animals. We conclude that ATR and the metabolite DIA significantly activate the HPA axis following oral exposure in the female rat. Activation of this endocrine axis by these chlorotriazines could contribute to the induced changes of female reproductive function reported previously.

Key Words: atrazine; desisopropylatrazine; diamino-s-chlorotriazine; hypothalamic-pituitary-adrenal axis; ACTH; corticosterone; progesterone.

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine; ATR) is a chlorotriazine herbicide that is used to control broadleaf and grassy weeds around crops of corn, sorghum, and sugarcane. The U.S. Department of Agriculture (1990–1994) estimates that 64 to 75 million pounds of ATR are used annually within the United States, and the herbicide is one of the most widely used agricultural products worldwide (Gressel et al., 1984; Stevens and Sumner, 1991).

ATR metabolism occurs within the environment as well as within mammalian and non-mammalian systems. These processes have been extensively reviewed by Das et al. (2001). The biotransformation of this compound in the environment and metabolism in plants primarily produces hydroxyatrazine and the chlorinated metabolites desisopropylatrazine (DIA), desethylatrazine (DEA), and diamino-s-chlorotriazine (DACT, Fig. 1) (Koskinen and Clay, 1997; Lamoureux et al., 1998). Studies of ATR metabolism within humans and animals indicate the presence of the chlorinated metabolites as well as mercapturated forms (Bakke et al., 1971; Barr et al., 2007; Catenacci et al., 1990; Ikonen et al., 1988).

Human and wildlife contact with this herbicide and its metabolites can result from occupational or environmental exposures (Baranowska et al., 2008; Barr et al., 2007; Catenacci et al., 1993; Gressel et al., 1984; Ikonen et al., 1988). The highest environmental levels ATR and its products are typically observed in the Midwestern states during the seasonal months of farming (Balu et al., 1998). Levels of ATR in river basins and community water supplies of this area have exceeded the maximal contaminant level of 3-ppb set by the U.S. Environmental Protection Agency (U.S. EPA, Kello, 1989; Thurman et al., 2002).

The effects of ATR and its metabolites on mammalian health have been studied extensively, with the most consistent effects being changes within the male and female reproductive axis...
The cellular mechanisms responsible for the disruption of the neuroendocrine control of gonadal function by ATR remain undetermined. However, the disruption of the LH surge following treatment with the chlorotriazines appears to result from hypothalamic perturbation as opposed to a direct influence on the ovary or anterior pituitary. In the ovariectomized female, for example, ATR diminishes the pulsatile release of LH (indicative of hypothalamic gonadotropin release hormone [GnRH] neuronal activity) (Cooper et al., 2007) and attenuates the LH surge (Cooper et al., 2000). In the ovary-intact female, ATR treatment increased proestrous GnRH content in the hypothalamus, indicating that release of this peptide was reduced (Cooper et al., 2007). Direct ATR effects on the pituitary are also unlikely since the compound had no effect on in vitro LH secretion (Cooper et al., 2000).

Relevant to these observations is the recent demonstration that ATR alters the activity of another important neuroendocrine axis—the hypothalamic-pituitary-adrenal (HPA) axis. Studies in the mouse (Pruett et al., 2003; Schwab et al., 2005) and rat (Laws et al., 2009; Modic, 2004) indicate that ATR exposure activates the HPA axis, thereby increasing circulating concentrations of adrenocorticotropic hormone (ACTH) and corticosterone. These observations are particularly important because activation of the adrenal axis by ATR may directly or indirectly influence the central control of the gonadal axis in these or other species. The inhibition of reproductive indices and outcomes by various stimuli that activate the HPA axis has been extensively studied in many species, including humans (Rabin et al., 1988; Rivier and Rivest, 1991). The complex mechanism by which the HPA axis modifies the function of the gonadal axis is still under investigation and may be stimulus and/or species specific. There is significant evidence that a direct or indirect interaction exists between the primary mediator of the central stress response, corticotropin-releasing factor (CRF), and GnRH neurons (Rivest and Rivier, 1995). In addition, steroid hormone feedback from the adrenal (i.e., corticosteroids and progesterone) can alter the secretion of GnRH and LH (Ringstrom and Schwartz, 1984, 1985; Suter and Schwartz, 1985; Wagenmaker et al., 2009) as well as pituitary responsiveness to GnRH (Breen and Karsch, 2004; Kamel and Kubjak, 1987; Rosen et al., 1991; Suter et al., 1988). Thus, there is ample evidence to hypothesize that if the chlorotriazines activate the adrenal axis, the response could contribute to the well-characterized attenuation of LH secretion following exposure to these herbicides.

The purpose of the present studies was to determine the extent to which ATR and two of the chlorotriazine metabolites, DIA and DACT, increase circulating levels of pituitary ACTH, adrenal corticosterone, and progesterone in the female rat. Better characterization of the HPA axis activation by these compounds will provide the information needed to further evaluate the potential impact that changes in adrenal function may have on the animal’s reproductive physiology, in particular, and overall health, in general. Plasma levels of ATR and metabolites at the time of hormone measurement were determined to better understand the concentration of these compounds within the blood both acutely and following multiple oral exposures. Finally, a bilateral subdiaphragmatic vagotomized rat model as well as a catheterized jugular vein rat model were utilized to determine if the HPA axis response to oral chlorotriazines was an artifact of gastrointestinal discomfort and subsequent vagus nerve activation.
MATERIALS AND METHODS

These experiments were approved by the U.S. EPA, National Health and Environmental Effects Research Laboratory’s Institutional Animal Care and Use Committee and were conducted in accordance with National Institute of Health standards for laboratory animal research.

Study 1

Animals

Female Long-Evans Hooded rats (60 days old) were purchased from Charles River Laboratories (Raleigh, NC) and housed singly in a room maintained at 22°C ± 2°C with a 14:10 h light:dark schedule (lights on at 0500 h: lights out at 1900 h). Food and water were provided ad libitum. Beginning at 80 days of age, the ovarian cycle of each female was monitored for a period of 2 weeks by taking daily vaginal lavages as described in detail elsewhere (Goldman et al., 2007). Only females displaying 4-day estrous cycles were included in the experiments. All animals were acclimated to oral gavage dosing with 1% methyl cellulose vehicle once per day for at least 4 days. Pilot data indicated that animals gavaged for multiple days with methyl cellulose maintained baseline ACTH and corticosterone levels (data not shown). Animals assigned to receive four daily doses of oral chlorotriazine were ranked by body weight and placed into treatment groups such that the mean body weights for all groups were similar (Table 2, 4× exposure). Treatment groups for this study were equally represented across two blocks of the study. All other experiments (1× exposure or acute restraint experiments) were conducted in separate blocks, and females displaying 4-day estrous cycles were randomly assigned to treatment groups without weight ranking. All hormone measurements were made in rats sacrificed between 0900 and 1000 h on proestrus to avoid hormone variability in basal (Atkinson and Waddell, 1997) and stress-induced (Viau and Meaney, 1991) levels observed over the course of the estrous cycle.

Dosing Solutions and Procedures

ATR (purity 97.1%), DIA (purity 94.5%), and DACT (purity 96.8%) were gifts from Syngenta Crop Protection, Inc. (Greensboro, NC). All the chemicals were administered by oral gavage in a suspension of 1% methyl cellulose/distilled water (M-7140; Sigma Chemical Co., St Louis, MO) in a volume of 5.0-ml dosing solution/kg body weight. As in previous studies (Laws et al., 2003; Stoker et al., 2002), the metabolite doses administered are the molar equivalent doses of each ATR dose (Table 1) and were selected based on previous studies of ATR effects on reproductive end points (Cooper et al., 2007). Control animals received 1% methyl cellulose vehicle (5.0 ml/kg body weight).

Experimental Procedures

1× females-chlorotriazine exposure. To evaluate the acute effects of ATR, DIA, or DACT on HPA axis hormone secretion, females were gavaged once at 0900 h on the day of proestrus with vehicle, 75 mg/kg ATR, or the equimolar dose of DIA (60.2 mg/kg) or DACT (50.6 mg/kg). Animals were euthanized in a separate room exactly 15 min postgavage. Trunk blood was collected in 100-mm K3 EDTA blood collection tubes (Becton-Dickinson, Rutherford, NJ) and centrifuged at 3000 rpm for 30 min at 4°C. Plasma and serum were subsequently divided into two aliquots and stored at −80°C. Separate plasma aliquots were used for analytical chemistry analysis and ACTH measurements. Serum aliquots were assayed for corticosterone and progesterone. Vaginal proestrus was confirmed by vaginal smear and uterine weight at the time of necropsy. The whole pituitary was removed, weighed, and snap frozen for future analysis.

4× females-chlorotriazine exposure. To evaluate HPA axis hormone response to ATR, DIA, or DACT exposure over the course of the estrous cycle, females were gavaged once per day at 0900 h beginning on the day of vaginal estrus and ending on the day of proestrus. Groups of rats received vehicle, 12.5 or 75 mg/kg ATR, or equimolar dosings of DIA (10 or 60.2 mg/kg) or DACT (8.4 or 50.6 mg/kg). Animals were euthanized in a separate room exactly 15 min postgavage. Trunk blood and pituitaries were collected and processed as described above. Vaginal proestrus was confirmed by vaginal smear and uterine weight at the time of necropsy.

1× females-restraint stress. Females displaying regular 4-day estrous cycles were restrained in a cylindrical plastic restrainer (Braintree Scientific, Braintree, MA) for 5 min at 0900 h on the day of proestrus. Following the restraint, the animal was returned to its home cage. Fifteen minutes after the start of the restraint, the animals were euthanized in a separate room. Trunk blood and pituitaries were processed as described above. Vaginal proestrus was confirmed by vaginal smear and uterine weight at the time of necropsy.

4× females-restraint stress. Females displaying regular, 4-day estrous cycles were restrained in a cylindrical plastic restrainer for 5 min at 0900 h once per day starting on the day of vaginal estrus and ending on the day of proestrus. Following the restraint, the animal was returned to its home cage. Fifteen minutes after the start of the restraint on proestrus, the animals were euthanized in a separate room. Trunk blood and pituitaries were collected and processed as described above. Vaginal proestrus was confirmed by vaginal smear and uterine weight at the time of necropsy.

Study 2

Animals

In all, 10 male Wistar rats (40 days old) that had undergone bilateral subdiaphragmatic vagotomy and 10 intact, male Wistar rats were purchased from Charles River Laboratories. Animals were housed singly in a room maintained at 22°C ± 2°C with a 12:12 h light:dark schedule (lights on at 0800 h: lights out at 2000 h). Vagotomized animals were maintained on a liquid diet consisting of 50% sweetened condensed milk and 0.12% Poly-Vi-Sol (vitamin supplement, Enfamil, Mead Johnson & Company) diluted in water as suggested by Garcia-Medina et al. (2007). Purina 5001 rat chow and water were provided ad libitum to intact rats.

Experimental Procedures

Experimental procedures began ~3 weeks after vagotomy. All animals were acclimated to oral gavage dosing with 1% methyl cellulose vehicle once per day for 4 days. The day of the experiment, rats were dosed by oral gavage at 0100 h with 100 mg/kg ATR or vehicle. Animals were then euthanized in a separate room exactly 15 min postgavage. No more than 15 s transpired from the time the animal was removed from its home cage to the time of euthanasia. Trunk blood was collected in 100-mm K3 EDTA blood collection tubes

<p>| Table 1 |
| Doses (mg/kg) of Chemicals as Compared to Molar Equivalents of ATR |</p>
<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>ATR (FW 215.72a)</th>
<th>DIA (FW 173.0)</th>
<th>DACT (FW 145.5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.058</td>
<td>12.5</td>
<td>10b</td>
<td>8.4</td>
</tr>
<tr>
<td>0.348</td>
<td>75</td>
<td>60.2</td>
<td>50.6</td>
</tr>
</tbody>
</table>

Note. FW, formula weight. Atrazine equimolar dose in milligrams per kilogram.
Radioimmunoassays atrazine (ATR-d5; Cambridge Isotope Laboratories, Andover, MA), was added with anhydrous sodium sulfate using mortar and pestle. After the DEA, DIA, and DACT, were based on methods described by Brzezicki of 1 ml 7.8 mM DIA or 19% DMSO/saline vehicle over the course of containing 0.05M EDTA, centrifuged at 3000 rpm for 30 min at 4°C. Heparinized saline. Blood samples were collected in microcentrifuge tubes stored at 4°C until hormone analysis. Food and water were provided ad libitum.

Experimental Procedures
Due to the limited solubility of chlorotriazines in water (ATR 33 mg/l, DIA 670 mg/l) (Mills and Thurman, 2002), a 7.8mM solution of DIA in 19% dimethy sulfoxide (DMSO; Sigma-Aldrich, St Louis, MI) in saline was prepared for iv infusion. Preliminary studies indicated that infusion of 1 mL of this solution yielded 15-min plasma levels of DIA that were similar to those achieved by oral gavage of 10 mg/kg DIA (2272 ± 989 ng/ml). Animals were connected to the tether assembly (Instech Solomon) the afternoon prior to experimentation to allow for acclimation to the system. Experiments were performed during the nadir of HPA axis activity (between 0700 and 1000 h) the following day. Baseline blood samples (300µl) were drawn prior to iv infusion of 1 mL 7.8 mM DIA or 19% DMSO/saline vehicle over the course of approximately 3 min. Fifteen minutes after compound administration, another 300-µl blood sample was drawn and volume replaced with 10 IU/ml heparinized saline. Blood samples were collected in microcentrifuge tubes containing 0.05M EDTA, centrifuged at 3000 rpm for 30 min at 4°C, and stored at −80°C until assayed for ACTH and corticosterone.

Radioimmunoassays
Plasma ACTH (MP Biomedical, Orangeburg, NY) and serum corticosterone and progesterone radioimmunoassays (Coat-A-Count; Siemens Healthcare Diagnostic, Deerfield, IL) were performed according to the manufacturer’s instructions.

Analytical Chemistry Extraction and analysis of rat plasma samples for ATR and metabolites (DEA, DIA, and DACT, Fig. 1) were based on methods described by Brzezicki et al. (2003) with slight modifications. The samples were homogenized to a fine powder with anhydrous sodium sulfate using mortar and pestle. After the addition of diethyl ether, an appropriate amount of internal standard, deuterated atrazine (ATR-d5; Cambridge Isotope Laboratories, Andover, MA), was added to each sample. The extraction, derivitization, and analytical procedures were the same as described in detail by Brzezicki et al. (2003). The final extract volume was adjusted to 500 µl with hexane. For calibration standards, ATR, DACT, DIA, and ATR-d5 were derivatized individually. Known amounts of these methylated standards were combined to prepare a working stock solution. From this stock solution, calibration standard solutions at three concentrations were prepared, and a known amount of ATR-d5 was added to each calibration standard. Preliminary analysis was performed on Agilent GC 6890 with 5973N mass selective detector (MSD) equipped with a DB-17MS analytical column (J&W Scientific, Folsom, CA) in the selected ion monitoring mode. Subsequent analysis was carried out on a Hewlett Packard 5890 with 5971A MSD. Quantification was performed using the internal standard method. Procedural blanks and spiked samples were taken through the entire analytical procedure for quality assurance. Procedural blanks were free of analytes and spike recoveries were 87% for ATR and 117 to 128% for DACT. The method detection limit was estimated to be 4 ng/ml (ppb) for all analytes.

Results
Study 1: Oral Chlorotriazine Exposure and Restraint Stress
Body weight as well as whole pituitary weight and proestrus uterine weight relative to body weight were unaffected by multiple days of oral chlorotriazine exposure (Table 2). Proestrus uterine weight of rats acutely restrained for 4 days were slightly, but significantly, elevated compared to vehicle-treated rats.

The effects of single or multiple ATR, DIA, or DACT exposures on pituitary and adrenal hormone release are shown in Figures 2 and 3. A single dose (1×) of 75 mg/kg ATR or an equimolar dose of DIA on proestrus significantly increased circulating levels of ACTH, corticosterone, and progesterone 15 min following oral gavage (Fig. 2). In contrast, a 1× DACT exposure had no effect on any of these hormones.

Similar results were observed on proestrus, 15 min after a fourth daily oral exposure to 12.5 or 75 mg/kg ATR or equimolar doses of DIA or DACT (Fig. 3). Dose-dependent increases in circulating hormones were observed following exposure to ATR or DIA. Both doses of ATR or DIA increased serum corticosterone and progesterone levels. Plasma ACTH concentrations were also increased in these groups; however, secretion in response to 12.5 mg/kg ATR did not reach statistical significance. Again, neither dose of DACT altered the release of any measured hormone.

The ACTH and corticosterone responses of single or multiple exposures to 75 mg/kg ATR, 60.2 mg/kg DIA, or 50.6 mg/kg DACT from Figures 2 and 3 are redrawn in Figure 4 to facilitate a comparison of hormone secretion between these two exposure paradigms. Analysis of these data reveals that pituitary ACTH secretion is significantly attenuated following multiple exposures to ATR or DIA as compared to a single exposure to these compounds. Interestingly, the corticosterone response to these compounds is similar between dosing paradigms despite the aforementioned attenuation of ACTH release in rats of the 4× dosing regimen.

To better understand the ATR/metabolite-induced HPA axis response compared to a well-known stressor, the 1× and 4× experimental paradigms were repeated with rats undergoing a 5-min restraint instead of oral gavage of chlorotriazines. Single or multiple acute restraint stress significantly increased ACTH, corticosterone, and progesterone release compared to vehicle-treated groups (Fig. 5). With the exception of ACTH,
these increases were of a similar magnitude to that induced by ATR or DIA (1× and 4× 60.2 mg/kg DIA data are shown for comparison purposes).

**Plasma metabolite measurement.** Plasma collected from animals gavaged with methyl cellulose vehicle did not contain detectable levels of ATR or metabolites (data not shown). Significant, dose-related levels of ATR and the chlorinated metabolites were detected in plasma 15 min after 1× or 4× oral gavage exposure (Table 3). Low, but detectable, plasma levels of the parent molecule were observed following single or multiple oral dosing of ATR. DACT (5–31μM) was the most abundant plasma metabolite measured following any dose of ATR; however, significant levels of DIA and DEA were also detected (5 and 0.5μM, respectively). Oral administration of equimolar doses of DIA or DACT did not yield plasma metabolite concentrations comparable to those observed following oral gavage with ATR. Plasma DIA concentrations were approximately 10 times higher following a single oral dose of 60.2 mg/kg DIA compared to an equimolar dose of 75 mg/kg ATR (52 versus 5.2μM). Similarly, a single 50.6 mg/kg DACT dose yielded approximately 14 times higher plasma DACT concentrations than an equimolar ATR dose (86 versus 6.3μM).

**Study 2: Vagotomy**

Oral exposure to 100 mg/kg ATR increased circulating levels of ACTH, corticosterone, and progesterone in both intact and vagotomized rats as compared to their respective methyl cellulose controls (Fig. 6). Secretion of ACTH and progesterone was augmented in ATR-treated vagotomized rats compared to intact controls (p < 0.01, treatment × vagus status interaction).

**Study 3: iv DIA Exposure**

Iv infusion of DMSO/saline vehicle had no effect on circulating levels of ACTH but significantly increased corticosterone (p < 0.05) (Fig. 7). Iv DIA administration, however, increased the secretion of both hormones by 15 min, and these levels were significantly higher than those observed following vehicle infusion (significant treatment × time interaction).

**DISCUSSION**

The chlorotriazine herbicides have been implicated in the disruption of a number of endocrine-related processes including ovulation, puberty, and pregnancy (Cooper *et al.*, 2007). The mechanism underlying ATR alteration of these reproductive processes remains to be fully characterized but is thought to be mediated through the hypothalamus (Cooper *et al.*, 2007). The current study demonstrates that, in addition to the HPG axis, HPA axis function is also altered by the chlorotriazines in the female rat. Oral exposures to ATR or DIA acutely increased circulating levels of pituitary ACTH, adrenal corticosterone, and progesterone in a dose-dependent manner. These hormonal responses were similar to those generated by acute restraint—a well-characterized stimulator of the adrenal axis. The results also agree with previous studies investigating the effects of chlorotriazines on the HPA axis in rodents. In female mice, a single ip (100–300 mg/kg) or oral

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**TABLE 2**

**Body Weight, Whole Pituitary (PIT) Weight, and Uterine Weight Data**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>n</th>
<th>Body weight (g)</th>
<th>PIT weight (mg)</th>
<th>PIT weight (mg)/100 g body weight</th>
<th>Uterine weight (mg)</th>
<th>Uterine weight (g)/100 g body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1× exposure</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>4</td>
<td>300.0 ± 14.0*</td>
<td>14.0 ± 0.4</td>
<td>4.7 ± 0.3</td>
<td>724 ± 102</td>
<td>0.247 ± 0.046</td>
</tr>
<tr>
<td>75 mg/kg ATR</td>
<td>6</td>
<td>293.8 ± 10.4</td>
<td>11.8 ± 0.7*</td>
<td>3.9 ± 0.3</td>
<td>569.3 ± 49.9</td>
<td>0.183 ± 0.018</td>
</tr>
<tr>
<td>60.2 mg/kg DIA</td>
<td>7</td>
<td>324.9 ± 7.0</td>
<td>13.4 ± 0.8</td>
<td>4.1 ± 0.3</td>
<td>567.3 ± 30.5</td>
<td>0.175 ± 0.024</td>
</tr>
<tr>
<td>50.6 mg/kg DACT</td>
<td>6</td>
<td>296.2 ± 10.8</td>
<td>13.1 ± 1.0</td>
<td>4.4 ± 0.2</td>
<td>482.7 ± 41.8*</td>
<td>0.164 ± 0.015</td>
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<tr>
<td>5-min restraint</td>
<td>9</td>
<td></td>
<td>13.3 ± 0.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>4× exposure</strong></td>
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<td></td>
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<tr>
<td>Vehicle</td>
<td>11</td>
<td>294.5 ± 6.0</td>
<td>12.6 ± 0.4</td>
<td>4.3 ± 0.1</td>
<td>513 ± 30.9</td>
<td>0.175 ± 0.011</td>
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<tr>
<td>12.5 mg/kg ATR</td>
<td>11</td>
<td>286.0 ± 7.5</td>
<td>12.5 ± 0.7</td>
<td>4.4 ± 0.3</td>
<td>501.7 ± 28.6</td>
<td>0.176 ± 0.010</td>
</tr>
<tr>
<td>75 mg/kg ATR</td>
<td>10</td>
<td>285.5 ± 4.8</td>
<td>12.3 ± 0.4</td>
<td>4.3 ± 0.2</td>
<td>529.8 ± 26.4</td>
<td>0.186 ± 0.009</td>
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<tr>
<td>10 mg/kg DIA</td>
<td>9</td>
<td>298.6 ± 7.1</td>
<td>11.7 ± 0.3</td>
<td>3.9 ± 0.1</td>
<td>495.3 ± 18.7</td>
<td>0.166 ± 0.005</td>
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<td>60.2 mg/kg DIA</td>
<td>11</td>
<td>282.6 ± 6.7</td>
<td>11.8 ± 0.5</td>
<td>4.2 ± 0.2</td>
<td>493.8 ± 24.5</td>
<td>0.175 ± 0.008</td>
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<tr>
<td>8.4 mg/kg DACT</td>
<td>10</td>
<td>291.9 ± 8.0</td>
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<td>4.5 ± 0.2</td>
<td>571.8 ± 43.2</td>
<td>0.195 ± 0.013</td>
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<tr>
<td>50.6 mg/kg DACT</td>
<td>9</td>
<td>286.4 ± 6.9</td>
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<td>4.5 ± 0.3</td>
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<td>0.190 ± 0.015</td>
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<td>5-min restraint</td>
<td>14</td>
<td>286.6 ± 5.3</td>
<td>12.6 ± 0.4</td>
<td>4.4 ± 0.2</td>
<td>616.1 ± 28.7*</td>
<td>0.212 ± 0.010*</td>
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</tbody>
</table>

*Mean ± SE.

aData not collected.

*p < 0.05 versus vehicle.
A single oral dose of ATR increased circulating concentrations of corticosterone (Pruett et al., 2003). Similarly, a single oral dose of ATR, DIA, or DEA stimulated dose- and time-dependent increases in circulating ACTH, corticosterone, and progesterone in male rats (Laws et al., 2009; Modic, 2004). Although the effects of DEA were not investigated in this study, one would predict that this metabolite would also activate the HPA axis in the female rat. In contrast, the terminal chlorinated metabolite, DACT, does not appear to significantly activate this endocrine axis, despite its high concentration in the circulation. These results agree with those observed in male rats where an oral dose of 135 mg/kg DACT (equimolar to 200 mg/kg ATR) evoked only small increases in corticosterone over 60 min (Laws et al., 2009).
ATR-induced alteration of reproductive system function is thought to be centrally mediated through the hypothalamus or a hypothalamic-projecting region. Oral exposure to ATR increases median eminence concentrations of GnRH peptide on proestrus (indicating decreased secretion) and attenuates the LH surge (Cooper et al., 2007). These effects are correlated with increased dopamine concentrations within the ME and medial preoptic area—hypothalamic regions important to the regulation of GnRH secretion (Cooper et al., 2007). The robust increases in ACTH observed in our single (1×) exposure experiments suggest that a similar hypothalamic mechanism may be involved in acute ATR- and DIA-induced activation of the HPA axis. Just as GnRH stimulates LH release from the anterior pituitary, the hypothalamic neuropeptides CRF and arginine vasopressin (AVP) act at the pituitary to release ACTH. Given the importance of catecholamine signaling for the secretion of these releasing hormones (see Ziegler and Herman [2002] for review), it could be hypothesized that the dysregulation of this system by ATR may be responsible for acute increases in ACTH release by CRF or AVP.

In addition to chlorotriazine action at the level of the central nervous system, in vitro studies suggest that ATR also has a direct effect on steroidogenesis. Twenty-four hour treatment with 10–30 μM ATR increases media levels of cortisol and progesterone in human adrenocortical carcinoma cells (H295R cells, unpublished observations). In addition, treatment of adrenal tissue in perifusion with 50μM DIA produces a greater than twofold increase from basal progesterone release over a 90-min period (Hotchkiss et al., 2009). Sanderson et al. (2001, 2000) also demonstrated that ATR and the mono-dealkylated metabolites, but not DACT, induce aromatase activity in H295R and JEG-3 placental choriocarcinoma cells. In the current study, differences in the HPA hormone responses between single and multiple daily exposures provide the first
**TABLE 3**

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Dose (mg/kg)</th>
<th>N</th>
<th>ATR</th>
<th>DEA</th>
<th>DIA</th>
<th>DACT</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATR</td>
<td>1×</td>
<td>75</td>
<td>2</td>
<td>12±(0.056μM)</td>
<td>74 (0.4μM)</td>
<td>896 (5.2μM)</td>
</tr>
<tr>
<td></td>
<td>4×</td>
<td>12.5</td>
<td>4</td>
<td>7 ± 4° (0.03 ±0.02μM)</td>
<td>43 ± 17 (0.2 ± 0.09μM)</td>
<td>374 ± 234 (2.2 ± 1.4μM)</td>
</tr>
<tr>
<td></td>
<td>4×</td>
<td>75</td>
<td>5</td>
<td>11 ± 3 (0.05 ±0.01μM)</td>
<td>97 ± 53 (0.52 ± 0.3μM)</td>
<td>2630 ± 408 (15 ± 2.4μM)</td>
</tr>
</tbody>
</table>

DIA

| 1×       | 60.2±        | 3   | ND             | ND             | 9016 ± 2055 (52 ±12μM) | 3557 ± 695 (24 ± 4.7μM) | ND              | ND              |
| 4×       | 10±          | 4   | ND             | ND             | 2630 ± 408 (15 ± 2.4μM) | 2351 ± 262 (16 ± 1.8μM) | ND              | ND              |
| 4×       | 60.2±        | 4   | ND             | ND             | 5217 ± 1665 (30 ± 9.6μM) | 11305 ± 2925 (78 ± 20μM) | ND              | ND              |

DACT

| 1×       | 50.6±        | 2   | ND             | ND             | ND              | 12563±(86μM)     |
| 4×       | 8.4±         | 5   | ND             | ND             | ND              | 5459 ± 2705 (38 ± 19μM) | ND              | ND              |
| 4×       | 50.6±        | 5   | ND             | ND             | ND              | 13739 ± 2730 (94 ± 19μM) |

**Note.** ND, not detected.

1. Plasma concentrations, ng/ml is equivalent to ppb. Value in parenthesis is μM equivalent.
2. Mean (n = 2).
3. Mean ± SD.
4. Doses of DIA and DACT are equimolar to that used for ATR (75 and 12.5 mg/kg).

in vivo evidence for this mechanism of action. ACTH secretion in response to multiple exposures to ATR or DIA is significantly attenuated compared to a single exposure to these compounds. In contrast, adrenal corticosterone responses to single or multiple exposures to ATR or DIA are similar in magnitude (Fig. 4). Indeed, significant elevations in corticosterone are still observed after 21-day ATR oral exposure in the male rat (Modic, 2004) and 28-day ip (150 mg/kg) exposure in rone are still observed after 21-day ATR oral exposure in the magnitude (Fig. 4). Indeed, significant elevations in corticosterone responses to ATR or DIA are similar in magnitude (Fig. 4). Indeed, significant elevations in corticosterone are still observed after 21-day ATR oral exposure in the male rat (Modic, 2004) and 28-day ip (150 mg/kg) exposure in the female mouse (Pruett et al., 2009). These observations could be a result of increased adrenal sensitivity to ACTH (i.e., lower circulating ACTH concentration activates a similar level of adrenal steroidogenesis) or the corticosterone response could be maximal for the amount of ACTH released by the pituitary. However, the combined in vitro and in vivo data lend support the hypothesis that long-term exposure to ATR or DIA directly activates corticosterone steroidogenesis in the adrenal. This hypothesis should be tested in future studies.

While these compounds clearly induce increases in circulating progesterone, the site at which ATR and DIA stimulate release of this hormone is unclear due to the presence of the ovaries. Tinio and Laws (2009) recently demonstrated that ATR increases progesterone levels in rat primary granulosa cell cultures. However, progesterone is also released from the adrenal in response to ACTH (Resko, 1969). Therefore, it is likely that ATR- or DIA-induced alterations of progesterone steroidogenesis occur both at the ovary and the adrenal. Further studies using ovariectomized animals could provide an answer to this question.

Recent in vitro studies examining the mechanism by which ATR alters cellular function may help to provide an explanation for the effects observed on multiple target tissues. Sanderson et al. (2002) demonstrated that 30μM ATR induced small increases in intracellular cyclic adenosine monophosphate levels in H295R cells. Similar results were observed in JEG-3 cells (Suzawa and Ingraham, 2008). Additional studies indicate that ATR inhibits phosphodiesterase (Roberge et al., 2004, 2006); however, other signaling cascades, such as PI3K, are also altered (Suzawa and Ingraham, 2008). The ubiquitous nature and important function of these cellular signaling mechanisms throughout the body, including in GnRH neurons (Ojeda et al., 1988; Paruthiyil et al., 2002) and steroidogenic cells (Free and Paik, 1977), suggest that chlorotriazine-induced alterations of these intracellular pathways might influence a number of processes within multiple target tissues.

Regardless of the mechanisms by which ATR increases hormonal secretion of the HPA axis, activation of this neuroendocrine system could contribute to the observed alterations in reproductive function caused by this herbicide. The interaction between stress and reproductive function was first reported in the early 20th century by Selye (1939). Since then, a number of studies have demonstrated that acute (Donadio et al., 2007) and prolonged stresses of different types (Collu et al., 1979; Hagino, 1968; Rivier et al., 1986; Yonetani et al., 1974) inhibit LH release. Although the mechanism by which the HPA axis modifies the function of the gonadal axis is not completely understood, stress hormones can act at all levels of the gonadal axis. For instance, the central administration of the neurotransmitter/neuromodulator CRF potently inhibits hypothalamic GnRH (Petraglia et al., 1987) and pituitary LH secretion in the intact (Petraglia et al., 1987) and ovariectomized (Petraglia et al., 1987; Rivier and Vale, 1984) rat. Adrenal steroids also alter the secretion of GnRH (Ringstrom and Schwartz, 1984, 1985; Suter and Schwartz, 1985; Wagenmaker et al., 2009) as well as pituitary responsiveness to GnRH (Breen and Karsch, 2004; Kamel and Kubajak, 1987; Rosen et al., 1991; Suter et al., 1988).
Additional studies are needed to determine if a cause-and-effect relationship exists between ATR- and DIA-induced activation of the HPA axis and the observed attenuation of the LH surge by these compounds.

While most studies investigating the effects of ATR on different physiological systems administer the compound through oral gavage, none have determined if this route of exposure alters gastrointestinal function or signaling. As gastrointestinal discomfort due to oral compound administration might be a source of HPA axis activation, we utilized two different rat models to better understand this issue. First, we used a bilateral subdiaphragmatic vagotomized rat model in which a small section of each branch of the vagus nerve below the diaphragm is cut out to block transmission of gastrointestinal afferent information to the brain. This model has been used to investigate conditioned taste aversion to many substances including arsenic (Garcia-Medina et al., 2007) and cytokines (Goehler et al., 1995). In the current study, vagotomized and vagus-intact rats exposed to 100 mg/kg ATR via oral gavage responded with similar increases in ACTH and corticosterone, suggesting that HPA axis hormone release is not the result of afferent signaling from the gastrointestinal tract. To further investigate this issue, we used a chronically catheterized jugular vein rat model to facilitate iv compound administration—thereby bypassing the gastrointestinal system. Iv infusion of DIA stimulated increases in both ACTH and corticosterone at 15 min that were significantly above baseline (0 min) levels and greater than the response of vehicle-treated animals. Based on the results of these two experiments, we believe that activation of the HPA axis by oral chlorotriazine exposure initiated somewhere other than the gastrointestinal tract.

In addition to characterizing chlorotriazine effects on the HPA axis, this study measured plasma ATR metabolite concentrations at the time of hormone measurement. The pattern of ATR metabolism observed in this study is similar to that described by our group as well as other investigators (Laws et al., 2009; McMullin et al., 2003, 2007; Ross and Filipov, 2006; Ross et al., 2009). Fifteen minutes after a single or fourth dose of 75 mg/kg ATR, the parent molecule and all three chlorinated metabolites are present, with DACT, DIA, and DEA being of much higher concentration than ATR. This rapid
absorption and metabolism of ATR and ready absorption of metabolites agree with the physiologically based pharmacokinetic (PBPK) model proposed by McMullin et al. (2007). Measurement of ATR and metabolite plasma concentrations at only one time point after exposure (15 min) limit the conclusions that can be made about which metabolites are biologically important to the activation of the HPA axis. Plasma levels of the parent molecule are very low compared to the chlorinated metabolites and are markedly lower than what has been used in the cell culture experiments described above. The potency with which oral doses of DIA increased circulating ACTH, corticosterone, and progesterone suggest that this metabolite is actively stimulating this endocrine axis. The other monodealkylated metabolite, DEA, is also likely “active” in this nature since oral dosing with this compound robustly increased HPA axis hormone secretion in male rats (Laws et al., 2009). In contrast, DACT had little effect on HPA axis hormone stimulation, despite high circulating concentrations. In agreement with our results, several studies have reported DACT as the major, persistent plasma metabolite following a single oral dose of ATR (Laws et al., 2009; McMullin et al. 2003, 2007; Ross and Filipov, 2006; Ross et al., 2009) with elimination occurring over a period as long as 70 h (McMullin et al., 2007; Ross et al., 2009). Based on our current results and those of Laws et al., 2009, we conclude that this final metabolite has no acute effect on HPA axis activity.

In conclusion, similar to that observed in the male rat, oral exposure to ATR and the intermediate metabolite DIA, but not DACT, increase circulating levels of ACTH, corticosterone, and progesterone in the female rat. This response does not appear to result from generalized gastrointestinal discomfort via vagal afferent signaling, but it is likely that acute exposure to ATR and DIA activate the HPA axis through a central mechanism, similar to that described for ATR effects on GnRH and LH secretion. In addition, combined in vitro and in vivo data support the hypothesis that long-term exposure to ATR or DIA may increase steroidogenesis directly at the adrenal and/or gonads. Future studies should examine the relationship between chlorotriazine-induced alterations of the HPA and HPG axes as a potential mechanism for altered reproductive function by these compounds in the female rat.

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