Alteration in Sexually Dimorphic Testosterone Biotransformation Profiles as a Biomarker of Chemically Induced Androgen Disruption in Mice

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Assessment of the impact of environmental chemicals on androgen homeostasis in rodent models is confounded by high intra-individual and inter-individual variability in circulating testosterone levels. Our goal was to evaluate changes in testosterone biotransformation processes as a measure of androgen homeostasis and as a biomarker of exposure to androgen-disrupting chemicals. Sex-specific differences in hepatic testosterone biotransformation enzyme activities were identified in CD-1 mice. Gonadectomy followed by replacement of individual steroid hormones identified specific sex differences in biotransformation profiles that were due to the inductive or suppressive effects of testosterone. Notably, significant androgen-dependent differences in testosterone 6α- and 15α-hydroxylase activities were demonstrated, and the ratio of 6α- and 15α-hydroxylase activities proved to be an excellent indicator of the androgen status within the animal. The male or “masculinized” testosterone 6α/15α-hydroxylase ratio was significantly less than the female or “feminized” ratio. Male mice were exposed to both an antiandrogen, vinclozolin, and to a compound that modulates serum androgen levels, indole-3-carbinol, to test the utility of this ratio as a biomarker of androgen disruption. Treatment with the antiandrogen vinclozolin significantly increased the 6α/15α-hydroxylase ratio. Indole-3-carbinol treatment resulted in a dose-dependent, but highly variable, decrease in serum testosterone levels. The 6α/15α-hydroxylase ratio increased as serum testosterone levels decreased in these animals. However, the increase in the ratio was much less variable and more sensitive than serum testosterone levels. These investigations demonstrate that the 6α/15α-hydroxylase ratio is a powerful measure of androgen modulation and a sensitive indicator of exposure to androgen-disrupting chemicals in CD-1 mice.

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Maintenance of steroid hormone homeostasis within an organism is critical to ensuring male and female sexual functions, including sexual differentiation and reproduction (1). Steroid hormones play a decisive role in sexual differentiation of the gonads and accessory reproductive organs occurring during prenatal and neonatal development (2). In addition, steroid hormones influence the acquisition and maintenance of secondary sex characteristics in adults (3). Recently, concern has been raised that both humans and wildlife are being exposed to endocrine-disrupting chemicals in the environment and that these compounds may be eliciting adverse effects on development and fertility along with contributing to the increasing rates of certain types of cancers (4–9).

Recent clinical observations including increased incidence of male genital tract malformations, (10–13), reductions in sperm count (14), and increased incidence of testicular (15,16), prostate (17), and breast cancer (16,18) have been suggested to be linked to exposure to these chemicals. Speculation remains as to whether human health issues are associated with the existence of endocrine-disrupting chemicals in the environment (16,18–20). However, concerns connected with hormone-related health problems in humans along with good evidence in wildlife of endocrine disruption (21,22), a growing knowledge base of hormonally active chemicals (23), and well-controlled experiments demonstrating developmental effects in model organisms consistent with those seen in humans and wildlife (9,24–26) have led Congress to pass two pieces of legislation that mandate the design of testing programs to identify endocrine-disrupting chemicals.

Several studies have suggested that toxicant-induced alterations in steroid hormone levels or metabolism contribute to reproductive impairment and that changes in steroid hormone levels can be indicative of endocrine-disrupting chemical exposure (27–30). However, in many commonly used and widely accepted rodent models, the utility of the measurement of circulating serum hormone levels alone as an indicator of exposure to androgen-disrupting chemicals is limited. In male laboratory mice and males of several other species, including humans, there is striking intra-individual and inter-individual variability in testosterone levels (31–34). Thus, it is extremely difficult to detect statistically significant differences in steroid hormone levels due to exposure to a xenobiotic. Subtle disturbances in homeostatic mechanisms by a xenobiotic may go undetected until more profound irreversible effects are manifested. This toxicity is especially insidious during sex differentiation and development because of the critical role of gonadal steroid hormones in regulating these processes. A more sensitive biomarker of androgen disruption due to chemical exposure is needed.

The hepatic cytochrome P450-dependent monoxygenase system (P450) plays a central role in metabolism. This system is not only important in the detoxification of foreign compounds but also is responsible for the oxidative metabolism of endogenous substrates such as steroid hormones (35–38). Despite their broad substrate specificity, however, P450 isozymes hydroxylate testosterone in a characteristic and unique manner that is both region specific and stereospecific (39). Modulation of individual hepatic steroid hydroxylase activities by hormones (40,41) and sex-dependent expression of hepatic steroid metabolizing enzymes in rat is well-established (42).

In addition to hydroxylation, direct conjugation is a second major route of metabolism (1). Uridine diphosphateglucuronosyltransferase or sulfortransferase enzymes can conjugate glucuronic acid or sulfate, respectively, to testosterone either directly or subsequent to hydroxylation. These reactions generally produce more water-soluble compounds that can then be excreted in urine (43).

Some studies have suggested sexually dimorphic UDP-glucuronosyltransferase activity in humans and in mice, but results from other studies in humans have not supported these observations (49,50). A possible explanation for these conflicting results may be that, because multiple isozymes of UDP-glucuronosyltransferase enzymes exist (51,52), some may be affected by sex while others are not. Sulfortransferase enzymes are also expressed in multiple forms, and there is evidence that their expression can be modulated by steroid hormones (53).

In this study, we hypothesized that changes in androgen-regulated testosterone...
biotransformation processes in CD-1 mice could be used as a sensitive biomarker of exposure to some endocrine-disrupting chemicals. The primary goals of this study were to 1) identify specific sex differences in constitutive steroid biotransformation activities in the CD-1 mouse model; 2) determine the role of androgens in the regulation of these activities; 3) identify an activity that might best serve as a biomarker of exposure to androgen-disrupting chemicals; and 4) validate this biomarker using organisms exposed to xenobiotics that either act as androgen antagonists or modulate endogenous androgen levels.

**Materials and Methods**

**Animals.** Eight-week-old male and female CD-1 mice (sexually intact animals, sham-operated controls, and gonadectomized animals) were purchased from Charles River Laboratories (Raleigh, NC, or Portage, ME). Sham-operated, gonadectomized, and respective control mice designated for hormonal regulation experiments were allowed a 2-week recovery period after surgery at our facility before implantation of hormone pellets. All other mice were held at our animal facility for at least 1 week prior to initiation of treatment. Each chemical and/or surgical treatment, the sex, and the number of mice per treatment used in this study are summarized in Table 1. All animals were treated according to research protocols approved by the North Carolina State University Institutional Animal Care and Use Committee and held under controlled temperature (65–70°F) and light (12-hr diurnal light cycle) conditions.

**Hormone treatments.** To evaluate the potential role of individual steroid hormones in vivo on the expression of testosterone biotransformation activities, individual gonadal steroid hormones were administered to both intact and surgically gonadectomized male and female mice. Testosterone was initially evaluated for its ability to regulate androgen biotransformation activities. Gonadectomized animals (male and female) were implanted with either testosterone (5 mg), or placebo pellets (Innovative Research of America, Sarasota, FL), which corresponds to calculated dosages of 0.24 or 0.48 mg/mouse/day. Sham-operated controls received placebo pellets, whereas gonadally intact controls received no implants. One group of gonadally intact females received testosterone implants. In addition, two groups of intact males each received either estradiol implants (0.5 mg) or progesterone (10 mg) implants, which corresponds to calculated daily dosages of 0.024 or 0.48 mg/mouse/day, respectively. In all cases, dosages of hormone were selected to provide physiologically relevant serum hormone levels. Each treatment group consisted of six mice. Animals receiving implants were anesthetized with a 100-ml subcutaneous injection of a sterile solution of ketamine (1.68 mg)/xylazine (0.16 μg)/NaCl (0.9%). Pellets were implanted subcutaneously below the right scapula using a trochar; the incisions were closed with surgical clamps. On the morning of the twenty-first day after implantation, mice were euthanized by CO2 asphyxiation. Blood was collected and the livers were rapidly excised, quick frozen in liquid nitrogen, and then stored at -80°C until microsomes were prepared. Absence of testes or ovaries in gonadectomized animals was confirmed by visual inspection.

**Table 1. Summary of chemical and surgical treatments**

| Experiment/sex | Animals per treatment (n) | Chemical/surgical treatment | Dosagea | Exposure period (days) |
|---------------|--------------------------|----------------------------|---------|-----------------------|
| Hormone replacement |                         |                            |         |                       |
| Male          | 6                        | Untreated                  | 0       | 21                    |
|               |                          | Placebo/sham-operated     | 0       | 21                    |
|               |                          | Placebo/gonadectomy       | 0       | 21                    |
|               |                          | Testosterone/gonadectomy  | 0.24    | 21                    |
|               |                          | 17β-Estradiol             | 0.024   | 21                    |
| Female        | 6                        | Unreated                  | 0       | 21                    |
|               |                          | Placebo/sham-operated     | 0       | 21                    |
|               |                          | Placebo/gonadectomy       | 0       | 21                    |
|               |                          | Testosterone/sham-operated| 0.24    | 21                    |
|               |                          | Testosterone/gonadectomy  | 0.24    | 21                    |
| Antiandrogen  | Male                     | 6                          | 0       | 21                    |
|               |                          | Vinclozolin               | 160     | 21                    |
| Androgen modulator | 4–8               | Indole-3-carbinol         | 7       | 7                     |
| Male          |                          |                            | 250     | 7                     |
|               |                          |                            | 500     | 7                     |
|               |                          |                            | 750     | 7                     |

*aDosages for hormone treatments are milligrams per animal per day. Dosages for other chemical treatments are milligrams per kilogram body weight per day.

**Vinclozolin treatments.** Male mice were dosed once per day with 160 mg/kg/day vinclozolin (Crescent Chemical Co., Hauppauge, NY) in corn oil via gavage for 3 weeks. This dosing was chosen because previous studies have demonstrated antian- drogenic effects in rodents at similar dosages of vinclozolin (55). Control animals were dosed with the same volume of corn oil vehicle alone. Each of the two groups (treated and control) consisted of six animals housed together. Animals were sacrificed by CO2 asphyxiation 24 hr after the last dose. Livers were immediately excised, rinsed, weighed, and pooled (three sets of two pooled livers each) for microscopic preparation. Pooled livers of vinclozolin-treated and respective control mice were immediately homogenized with a tissue homogenizer and microsomes were prepared by the method described below.

Indole-3-carbinol treatments. Male mice were administered indole-3-carbinol (13C) (Sigma, St. Louis, MO) in their food. 13C was thoroughly mixed into ground standard mouse chow (Agway Prolabs, Creedmoor, NC) with a food processor at concentrations that provided estimated daily 13C dosages of 250, 500, and 750 mg/kg. Food consumption was monitored daily to ensure proper treatment. Control mice were fed the same diet without 13C added. The mice exhibited no observable adverse effects due to these treatment levels. After 7 days of treatment, mice were euthanized. Blood was collected by cardiac puncture. Livers were excised, quick frozen in liquid nitrogen, and stored at -80°C until microsomes were prepared.

**Microsome and cytosol preparation.** Individual livers were thawed and homogenized on ice in chilled buffer (0.1 M HEPES, pH 7.4, 1 mM EDTA, and 10% glycerol) with a Dounce tissue homogenizer (Kontes, Vineland, NJ). Microsomes were prepared by differential centrifugation (56). Cytosolic supernatant was reserved. microsomal pellets were resuspended in buffer (0.1 M potassium phosphate, pH 7.4, 0.1 mM EDTA, and 20% glycerol), and both were stored at -80°C until assays were performed. Protein concentrations were determined using commercially prepared reagents (Biorad, Hercules, CA) and bovine serum albumin (Sigma) as a standard.

**Testosterone hydroxylase assays.** Testosterone hydroxylase activities in mouse livers were assayed as previously described (38,58) using 400 μg microsomal protein and 40 nmol [14C]testosterone as the substrate (1.8 μM Citi/mmol; Dupont NEN, Boston, MA) in 0.1 M potassium phosphate buffer (pH 7.4). Reactions were conducted at 37°C and initiated with 1 mM
NADPH. The total assay volume was 400 µl. The reaction was terminated after 10 min by adding 1 ml ethyl acetate and vortexing. Product formation was linear over this time period. Each tube was vortexed for 1 min and then centrifuged for 10 min to separate the ethyl acetate and aqueous phases. Ethyl acetate fractions were transferred to a fresh tube. The aqueous phase was extracted with ethyl acetate a total of three times (1 ml, 2 ml, 1 ml, respectively) to ensure recovery of all hydroxyl metabolites. Combined ethyl acetate fractions from each aqueous sample were evaporated under a stream of nitrogen to dryness. The residue was resuspended in 70 µl ethyl acetate (5 µl x 2) and metabolites were separated by thin-layer chromatography (TLC). Unmetabolized [14C]testosterone and individual [14C]metabolites were identified and then quantified by liquid scintillation spectroscopy as previously described (58). Specific activity for each metabolite was calculated as picomoles of metabolite produced per minute per milligram of microsomal protein.

**UDP-Glucuronosyltransferase assay.** UDP-glucuronosyltransferase activity toward [14C]testosterone was assayed under conditions previously described (48, 50). Microsomal protein (200 µg) was incubated at 37°C with 40 nmol [14C]testosterone (1.8 µCi/µmol) in 0.1 M potassium phosphate buffer, pH 7.4. Reactions were initiated with 10 µl uridine 5'-diphosphoglucuronic acid in buffer (12.9 mg/ml; Sigma) to give a total assay volume of 400 µl and were terminated after 10 min by adding 2 ml ethyl acetate and vortexing. Product formation was shown to be linear over this time period. Phases were separated by centrifugation, and ethyl acetate fractions were removed. Extraction was repeated a second time with an additional 2 ml of ethyl acetate. Glucuronic acid conjugates were quantified by liquid scintillation counting of a 100-µl aliquot from the postextraction aqueous phase. Samples that consisted of all constituents except NADPH or microsomes were run with each assay. These samples were used to account for any spontaneous conjugation of glucuronic acid to testosterone and any [14C]testosterone that was not extracted from the aqueous phase by the ethyl acetate. Specific activity was calculated as picomoles of conjugate produced per minute per milligram of microsomal protein.

**Sulfotransferase assay.** Sulfotransferase activity was determined as previously described (48). Briefly, 200 µg of cytosolic protein from each sample was combined with 40 nmol [14C]testosterone (1.8 µCi/µmol) in 0.1 M potassium phosphate buffer, pH 6.5. Each reaction was initiated with 10 µl adenosine-3' -phosphate-5' -phosphosulfate in buffer (10 mg/ml; Sigma) for a total assay volume of 400 µl. Assay tubes were covered with paraffilm and incubated in a 37°C water bath for 20 hr. Reactions were shown to be linear over this time period. Reactions were stopped by adding 2 ml ethyl acetate and vortexing the sample tube. Unconjugated testosterone was removed by ethyl acetate extraction (two times, 2 ml each). Sulfate conjugates were quantified by liquid scintillation counting of a 100-µl aliquot from the postextraction aqueous phase. Retention of sulfate conjugates in the aqueous phase averaged 70%. Therefore, sulfate conjugates extracted into the ethyl acetate were separated from the [14C]testosterone by TLC using an 80% methylene chloride: 20% acetone solvent system. Sulfate-conjugated [14C]testosterone was then cut from the TLC plate and was quantified by liquid scintillation spectrometry. Total disintegrations per minute (dpm) associated with sulfate-conjugated [14C]testosterone from each sample were combined to calculate specific activity (picomoles per minute per milligram).

**Serum testosterone measurements.** Blood from each animal was allowed to clot at room temperature for at least 15 min, and serum was obtained by centrifugation at 14,000g for 10 min. Serum was then immediately frozen at -20°C until assayed. Total testosterone was measured within 5 days of serum preparation by solid-phase radioimmunoassay using commercially available reagents and protocols (Diagnostic Products Corp., Los Angeles, CA). The limit for the detection of testosterone in serum with this assay was 0.04 ng/ml.

**Statistical analysis.** Statistical significance (p<0.05) among the various parameters assessed was established by Student's t-test when a single treatment was compared to the control, or by using ANOVA and Dunnett's Multiple Comparison Test when multiple levels of a single chemical treatment were compared to their respective control group. Statistical analyses were performed using JMP (SAS Institute, Cary, NC) statistical software.

**Results**

**Sexually dimorphic testosterone biotransformation in CD-1 mice.** Previous studies in our lab have identified at least seven major mono-hydroxylated testosterone metabolites produced from hepatic microsomes of CD-1 mice, some of which appeared to be generated in a sexually dimorphic manner (48, 58). In untreated animals, total testosterone hydroxylase activity was comparable in both sexes; however, isolation and quantification of individual hydroxylated testosterone metabolites revealed sex-specific differences in the production rates of at least two of these metabolites (48). As illustrated in Figure 1, hepatic testosterone 6α-hydroxylase (6α-OH) activity was consistently higher in female mice than in male mice. Conversely, testosterone 15α-hydroxylase (15α-OH) activity was consistently higher in male mice (Fig. 1). Our results also showed that the constitutive rate of specific activity of UDP-glucuronosyltransferase was higher in hepatic microsomes from male mice, whereas sulfotransferase activity was consistently higher in hepatic cytosol from females (Fig. 1). These consistent differences in testosterone biotransformation between males and females indicate a possible regulatory role for gonadal steroid hormones in one or more of these activities.

**Gonadectomy and hormone replacement experiments: testosterone.** The effects of gonadectomy and subsequent testosterone replacement on 6α- and 15α-hydroxylase activities and on UDP-glucuronosyltransferase and sulfotransferase conjugations are shown in Figures 2 and 3, respectively. There was no statistically significant difference between gonadally intact controls and sham-operated controls with respect to 6α- or 15α-hydroxylase activity in any of the treatments; therefore specific activities for controls and sham-operated mice were combined for all additional statistical comparisons. Gonadectomy and testosterone replacement produced a dramatic effect on
Figure 2. Effect of gonadectomy (gonadex) and testosterone replacement on hepatic testosterone (A) 6α-hydroxylase- and (B) 15α-hydroxylase-specific activities in both adult male and female CD-1 mice. Data are presented as means ± standard deviations (n = 3 sets of two pooled livers each). There was no statistically significant difference between intact controls and sham-operated controls (Student’s t-test, p > 0.05), so specific activities for control and sham-operated controls were combined for all additional statistical comparisons.

*Statistically significant difference when compared to both male controls (p < 0.05).
**Statistically significant difference when compared to both female controls (p < 0.05).

Both 6α- and 15α-hydroxylase activities (Fig. 2). In control animals, as expected, testosterone 6α-OH activity was significantly higher in females, whereas 15α-hydroxylase was higher in males. However, gonadectomy of male mice significantly increased 6α-OH, as compared with male controls, to levels not significantly different from that measured in female controls (Fig. 2A). Replacement of testosterone to gonadectomized males reduced 6α-OH activity to that seen in control males. In female mice, neither testosterone administered to gonadally intact animals nor gonadectomy of female mice significantly lowered 6α-OH activity as compared to female controls. Testosterone treatment of gonadectomized females, however, significantly reduced 6α-OH activity, as compared to female controls, to levels comparable to that of male controls. Taken together, these experiments indicate that testosterone has a suppressive effect on hepatic 6α-OH activity.

Testosterone 15α-OH activity was significantly reduced in gonadectomized males as compared to male controls (Fig. 2B). Replacement of testosterone to gonadectomized males restored 15α-OH activity to control levels. Testosterone administered to intact females significantly increased 15α-OH activity in “masculine” levels. In gonadectomized female mice, 15α-OH activity was similar to that of female controls, but was significantly less than male controls. Administration of testosterone to gonadectomized females significantly increased 15α-OH activity, as compared to gonadectomized females, to levels not significantly different than in male controls. In summary, testosterone has an inductive effect on hepatic 15α-OH activity.

The effects of testosterone on UDP-glucuronosyltransferase and sulfotransferase activities were also investigated in hormonally-manipulated animals (Fig. 3). UDP-Glucuronosyltransferase activity was significantly reduced in gonadectomized males (Fig. 3A). Replacement of testosterone to gonadectomized males restored UDP-glucuronosyltransferase activity to control levels. In female mice, testosterone administered to intact females significantly increased UDP-glucuronosyltransferase activity, as compared to female controls, to levels similar to those seen in male control animals. Gonadectomy of female mice did not significantly alter UDP-glucuronosyltransferase activity as compared to female controls. However, replacement of testosterone to gonadectomized females increased UDP-glucuronosyltransferase activity to “masculine” levels.

Gonadectomy or subsequent testosterone replacement did not significantly affect sulfite conjugation in male mice (Fig. 3B). Testosterone sulfotransferase activity was extremely low in hepatic cytosol from all males irrespective of hormone status. Testosterone administration to intact female mice significantly reduced sulfotransferase activity, as compared to female controls, to levels seen in male control animals. Gonadectomy of female mice did not significantly reduce sulfate conjugation as compared to female controls, whereas testosterone replacement to gonadectomized females successfully “masculinized” sulfotransferase activity. These experiments indicate that the sex-specific differences demonstrated in conjugation of testosterone to glucuronate are due to the inductive effects of testosterone on this activity. Conversely, it appears that testosterone has a suppressive effect on sulfotransferase activity in female CD-1 mice. We elected not to pursue sulfotransferase or UDP-glucuronosyltransferase activities as potential biomarkers of androgen status, however. Sulfotransferase activity was abandoned because factors other than testosterone contribute to its suppression in males. UDP-Glucuronosyltransferase activity was abandoned because it responded similarly to testosterone 15α-hydroxylase activity, and both testosterone 6α-hydroxylase and 15α-hydroxylase can be measured simultaneously in the same assay.

Because 6α-OH activity was suppressed by testosterone and 15α-OH activity was induced by testosterone, we hypothesized that a ratio of 6α-OH to 15α-OH activity would provide an amplified measure of the androgen status of the mice. Table 2 illustrates the 6α/15α-hydroxylase ratios and respective serum testosterone levels of

Table 2. Effect of gonadectomy (gonadex) and testosterone replacement on 6α/15α-hydroxylase ratio and serum testosterone in male and female CD-1 mice

| Treatment                      | 6α/15α Ratio | Serum testosterone (ng/ml) |
|-------------------------------|--------------|----------------------------|
|                               | Mean ± SD    | CV (%)                     |
|                               | Mean ± SD    | CV (%)                     |
| Male                          |              |                            |
| Control                       | 0.55 ± 0.12**| 21.8                      |
| Sham-operated                 | 0.64 ± 0.07**| 10.9                      |
| Gonadex                       | 2.27 ± 0.37**| 16.3                      |
| Gonadex + testosterone        | 0.62 ± 0.05**| 8.0                       |
| Female                        | 1.52 ± 0.29* | 19.1                      |
| Control                       | 0.05 ± 0.04  | 8.0                       |
| Sham-operated                 | 0.09 ± 0.18  | 36.0                      |
| Gonadex                       | 2.15 ± 0.37**| 17.2                      |
| Gonadex + testosterone        | 0.67 ± 0.20**| 29.9                      |

Abbreviations: CV, coefficient of variation; SD, standard deviation; NA, not available. Data are presented as means ± SD of n = 3 sets of two pooled livers each.

*Statistically significant difference when compared to male control (Student’s t-test, p < 0.05).
**Statistically different from female control (Student’s t-test, p < 0.05).
mice from these testosterone modulation experiments. The ratio of 6α/15α-hydroxylase activity was significantly higher in normal adult females than in untreated adult male mice, and better differentiated the masculine and feminine metabolic profile as compared to individual hydroxylase activities. Further, the coefficients of variation for the 6α/15α-hydroxylase ratio were much lower than for serum testosterone levels. These results indicated that the 6α/15α-hydroxylase ratio was a good measure of androgen status and may serve as a more sensitive biomarker of androgen disruption than measurement of serum testosterone levels alone. The role that the other gonadal steroid hormones, 17β-estradiol or progesterone, may play with respect to the 6α/15α-hydroxylase ratio was next evaluated.

Gonadectomy and hormone replacement experiments: 17β-estradiol and progesterone. In the previous experiments, administration of testosterone to intact females altered 6α-OH and 15α-OH activity and the 6α/15α-OH ratio in female mice. To determine whether estrogenic or progestogenic compounds may influence the expression of 6α-OH and 15α-OH activity, 17β-estradiol (E2) or progesterone was administered to gonadally intact males (Table 3). Treatment of male mice with E2 or progesterone did not produce a statistically significant difference in either 6α-OH or 15α-OH activity when compared to control males. There was no statistically significant difference in the 6α/15α-OH ratio when progesterone-treated males were compared to control males. The 6α/15α-OH ratio, however, was significantly increased following E2 treatment of males. This increase corresponds to the suppressive effect of E2 on serum testosterone levels. We concluded from these studies that the sex differences in testosterone 6α-OH and 15α-OH activities is primarily due to the androgen status of the animal.

Vinclozolin treatment. To evaluate the utility of the 6α/15α-OH ratio as a biomarker of androgen disruption, male mice were treated with vinclozolin. Vinclozolin is metabolized in the liver to metabolites with antiandrogenic activity (55). We hypothesized that the antiandrogenic activity of the vinclozolin metabolites should block both the suppressive effect of testosterone on 6α-OH activity and its inductive effect on 15α-OH activity, resulting in an increase in the 6α/15α-OH ratio. Vinclozolin treatment of male mice resulted in the induction of both 6α-OH and 15α-OH, which is consistent with a phenobarbital-type induction of hepatic P450s (Table 4). However, the 6α/15α-OH ratio was significantly increased in the vinclozolin-treated mice, indicative of "demasculinization." Thus, the 6α/15α-OH ratio was successful as a biomarker of exposure to an antiandrogen.

Indole-3-carbinol treatment. I3C proved to be an effective modulator of serum testosterone levels. Treatment of male CD-1 mice with I3C resulted in a dose-dependent, although highly variable, decrease in serum testosterone levels when compared to male controls (Fig. 4A). A statistically significant decrease in serum testosterone, however, was only obtained at the highest dosage of I3C, 750 mg/kg/day. The 6α/15α-OH ratio increased as serum testosterone levels decreased (Fig. 4B), demonstrating the inverse relationship between serum testosterone and the 6α/15α-OH ratio (Fig. 4C). Notably, the 6α/15α-OH ratio at each dosage was much less variable than the serum testosterone levels seen in the animals; a statistically significant increase in the ratio could be detected at a lower dosage (500 mg/kg/day) of I3C (Fig. 4B).

Discussion

Results from this study demonstrate that the testosterone 6α/15α-OH ratio can serve as a sensitive biomarker of androgen disruption in CD-1 mice and is a more powerful measure of androgen status within the animal than direct analysis of serum testosterone levels. Circulating testosterone levels in male

Figure 3. Effect of gonadectomy (gonadex) and testosterone administration on hepatic microsomal (A) UDP-glucuronosyltransferase and (B) sulfotransferase conjugation activities toward [14C]testosterone. Data are presented as mean ± standard deviation (n = 3 sets of two pooled livers each). There is no statistically significant difference between intact controls and sham-operated controls (Student's t-test, p<0.05), so specific activities for control and sham-operated controls were combined for all additional statistical comparisons.

*Statistically significant difference from combined male controls (p<0.05).
**Statistically significant difference when compared to female controls (p<0.05).

Table 3. Effect of 17β-estradiol or progesterone administration on the hepatic testosterone 6α-/15α-hydroxylase activity ratio and serum testosterone levels in CD-1 mouse

| Treatment               | 6α-Hydroxylase activity (pmol/min/mg) | 15α-Hydroxylase activity (pmol/min/mg) | 6α/15α-Hydroxylase activity ratio | Serum testosterone (ng/ml) |
|-------------------------|---------------------------------------|----------------------------------------|----------------------------------|-----------------------------|
| Control male            | 105.85 ± 17.85                         | 212.78 ± 52.63                         | 0.51 ± 0.09                      | 14.93 ± 12.9                |
| Male + 17β-estradiol    | 129.32 ± 46.69                         | 153.45 ± 97.31                         | 0.94 ± 0.26*                     | 0.27 ± 0.18*                |
| Male + progesterone     | 99.97 ± 14.39                          | 150.71 ± 39.03                         | 0.70 ± 0.24                      | 14.30 ± 6.0                 |
| Control female          | 207.73 ± 12.93                         | 117.09 ± 4.60                          | 1.78 ± 0.12*                     | 0.09 ± 0.03*                |

Data are presented as means ± standard deviations (n = 3 sets of two pooled livers each).
*Statistically significant difference when compared to male control (Student's t-test, p<0.05).

Table 4. Effects of vinclozolin treatment of male mice on testosterone 6α-/15α-hydroxylase and 15α-hydroxylase activities and the 6α/15α-hydroxylase ratio

| Treatment          | 6α-Hydroxylase activity (pmol/min/mg) | 15α-Hydroxylase activity (pmol/min/mg) | 6α/15α Ratio |
|--------------------|---------------------------------------|----------------------------------------|--------------|
| Control            | 122.45 ± 26.62                         | 181.81 ± 4.10                          | 0.68 ± 0.04  |
| Vinclozolin        | 453.11 ± 11.19*                        | 387.78 ± 43.17*                        | 1.13 ± 0.09* |

Data are presented as means ± standard deviations (n = 3 sets of two pooled livers each).
*Statistically significant difference when compared to control (p<0.05).
mice are notoriously variable. Mean testosterone levels in control male mice used in this study ranged from 1.15 ng/ml to 14.9 ng/ml, with coefficients of variation (CV) that ranged from 75% to over 150% (Table 2, Table 3, Fig. 4A). Similar findings were demonstrated by Bartke et al. (61), who reported peripheral plasma testosterone concentrations from <0.4 ng/ml to 44.4 ng/ml in adult male mice. Variability of testosterone concentrations in peripheral circulation is evident as early as 20–30 days of age and continues throughout reproductive life (62). Evidence indicates that the variability cannot be eliminated by isolation, housing males either briefly or chronically with other male or female mice, or by administration of human chorionic gonadotropin (hCG) (31,61,63). In addition, the variability in testosterone levels in samples collected from the same animals on different occasions is comparable to the variation between individuals bled on a single occasion (31).

Our results indicate that the testosterone 6α/15α-OH ratio provides a more powerful and sensitive measure of androgen status in CD-1 mice. In contrast to the variability reported in serum testosterone concentrations of control mice, CVs of the 6α/15α-OH ratio ranged from only 1.6% to 31%. When mice were dosed with I3C, a compound that modulated steroid hormone levels, a statistically significant difference in testosterone levels could only be achieved at the highest dosage, 750 mg/kg/day. A significant difference in the 6α/15α-OH ratio could be detected at a lower dosage, 500 mg/kg/day. Therefore, the 6α/15α-OH ratio was not only less variable but also a more sensitive measure of androgen status. The ratio provides insight into the average androgen status of the organisms rather than a snapshot of androgen levels at the time of sampling. The 6α/15α-OH ratio not only detects effects of the chemical on endogenous androgen levels but also responds to antiandrogenicity of a chemical. Antiandrogens would not be expected to lower serum testosterone levels, but would interfere with the functions of testosterone. The use of this biomarker will greatly enhance the ability to detect androgen-disrupting chemicals in assays involving CD-1 mice.

It appears that the 6α/15α-OH ratio cannot be universally applied to all species, however. Little information is available regarding sexual dimorphisms in testosterone biotransformation activity or androgen regulation of these activities in other species. Sexual dimorphisms of testosterone hydroxylase activity are best documented in the rat. For example, hepatic microsomal testosterone 7α-OH activity is expressed in both sexes, but its expression is female-predominant (42). Testosterone 2α-, 2β-, 6β-, and 16α-OH activities have been reported to be higher in male rats than in female rats (64). Sexual dimorphisms in hepatic metabolism of testosterone have been described in hamsters (65), birds (66,67), and fish (68,69), but particular sex-specific differences in hepatic testosterone hydroxylase activities vary with species. Whether testosterone biotransformation profiles could be used as biomarkers of androgen disruption in other species has not been thoroughly explored and would probably require the development of different metabolic ratios applicable to the species of interest.

In humans, epidemiological study of testosterone levels has been limited due to interindividual variability (70,71). While the interindividual variability in humans is not as great as that seen in mice, the normal serum testosterone values in mature adult males range, depending upon the testing method, from a low of 1.8 to a high of 18.4 ng/ml (72). The interindividual variability is confounded by intraindividual variations in serum testosterone values. Both circadian and circannual fluctuations in circulating androgen levels within individuals have been reported (73–76). In addition, several other factors have been isolated that appear to be negatively correlated with serum testosterone levels; these include age (70,77,78), weight (70,79), smoking (70,80), and alcohol ingestion (81,82). Thus, a study using testosterone levels in men as an end point for endocrine disruption would have to control for several confounding factors.

Like male and female CD-1 mice, male and female humans appear to produce the same androgen metabolite profiles (83). Preliminary studies in our lab, however, indicate that sex differences may exist in the rates of production of certain metabolites in humans as well. Characterization of these sex differences may allow for the development of metabolic ratios that could be used as indicators of androgen disruption in humans. Because it has been demonstrated that individual steroid metabolites may be eliminated in and can be isolated from human urine, the potential exists for the development of diagnostic immunoassays for the determination of such metabolites (87). Once a pertinent ratio is identified in humans, androgen disruption can then be assessed noninvasively by assay of urine samples.

**Figure 4.** Effect of indole-3-carbinol (I3C) treatment on serum testosterone and the 6α/15α-hydroxylase ratio in male mice. (A) Serum testosterone levels in male mice treated with 0, 250, 500 or 750 mg/kg/day I3C. (B) The 6α/15α-hydroxylase ratio at each dosage of I3C (n = 4–8). (C) Relationship between mean serum testosterone levels and the 6α/15α-hydroxylase ratio. Statistical analyses were conducted using ANOVA and Dunn’s Multiple Comparison Tests (n = 4–10).

*Statistically significant difference when compared to controls (*P<0.05).**

**Statistically significant difference when compared to controls (**P<0.001).**

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