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The non-aromatizable androgen, dihydrotestosterone, induces antiestrogenic responses in the rainbow trout

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Abstract

In order to satisfy government mandates, numerous studies have been performed categorizing potential endocrine disrupting chemicals as (anti)estrogens or (anti)androgens. We report here that dihydrotestosterone (DHT), a potent, non-aromatizable androgen receptor agonist, induces antiestrogenic responses through direct and/or indirect modulation of vitellogenin (Vg), steroid hormone and total cytochrome P450 levels. DHT and two weak, aromatizable androgens, DHEA and androstenedione (0.05–50 mg/kg per day), were fed to juvenile trout for 2 weeks. DHEA and androstenedione significantly increased blood plasma Vg by up to 30- and 45-fold, respectively ($P < 0.05$, t-test). 17β-Estradiol (E_2) increases were also observed with both androgens, albeit with lower sensitivity. DHT markedly decreased Vg and E_2 levels, suggesting that DHEA and androstenedione increased Vg and E_2 via conversion to E_2 and not by estrogen receptor agonism. DHEA and androstenedione had no effect on total cytochrome P450 content, while DHT significantly decreased P450 content in a dose dependent fashion. These results indicate that alterations in metabolism mediated by androgen receptor binding may be responsible for the Vg and E_2 decreases by DHT. In an attempt to decipher between receptor and non-receptor androgenic mechanisms of the observed DHT effects, DHT (0, 50 or 100 mg/kg per day) and flutamide (0-1250 mg/kg per day), an androgen receptor antagonist, were fed to juvenile rainbow trout for 2 weeks. Flutamide alone was as effective as DHT in decreasing E_2 and Vg levels in males but did not significantly reverse DHT induced Vg decreases in either sex ($P > 0.05$, F-test). DHT decreases in total P450 content were partially attenuated in males by flutamide co-treatment, but not females, suggesting a partial androgenic mechanism to the P450 decreases as well as a fundamental sex difference responding to androgen receptor binding. Moreover, flutamide alone decreased P450 content by up to 30% in males and 40% in females. These effects may be mediated through direct androgen receptor binding irrespective of whether the binding is agonistic or antagonistic. This study indicates that androgen receptor agonists/antagonists can elicit significant antiestrogenic effects that may not necessarily be mediated through classic receptor binding mechanisms and signal transduction pathways.

Keywords: Dihydrotestosterone; Antiestrogenic responses; Rainbow trout

1. Introduction

Estrogenic compounds and their modulating effects on carcinogenesis, enzyme induction/inhibition and sex determination has become an increasingly important area of research. The influence of androgens in this field has been studied to a much lesser extent. Dehydroepiandrosterone (DHEA), a weak androgen, has been established as a potent liver tumor promoter in rainbow trout in the absence of peroxisome proliferation [1,2]. DHEA is also a precursor to estrogens such as 17β-estradiol (E_2), a potent promoter of carcinogenesis in many organs such as the liver and breast [3–8]. Androstenedione can be directly converted to testosterone via 17β-hydroxysteroid dehydrogenase or estrone via aromatase. E_2 is synthesized via aromatase from testosterone or estrone by the dehydrogenase enzyme [4]. Several mechanisms have been postulated for tumor promotion by estrogens such as production of catechol metabolites that can induce redox cycling and subse-

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quent DNA damage and covalent binding, to increases in cell growth and division [3–8]. Since DHEA is converted to androstenedione, a precursor to potent androgens such as testosterone, dihydrotestosterone (DHT) and in teleosts, 11-ketotestosterone (11kT), the ability to decipher between estrogenic and androgenic responses is critical.

Vitellogenin (Vg) is an egg-yolk protein precursor synthesized in the liver of oviparous animals such as fish, amphibians, reptiles and birds in response to estrogen receptor binding [9]. It is secreted into the blood where it is taken up by the ovaries to be incorporated into the developing eggs [10]. Vg synthesis is a well characterized, sensitive biomarker for estrogen receptor binding in the rainbow trout liver [11]. Many studies have utilized Vg production to screen for and determine the efficacy and potency of environmental contaminants such as alkylphenols, DDEs, and PCBs [12–18]. Phytochemicals and natural products that modulate Vg production have been postulated as possible promoting or chemopreventive agents in estrogen dependent cancers [19,20].

The contribution of natural and synthetic androgens to endocrine disruption has not been studied to nearly the degree of estrogens. In some cases androgens are investigated for estrogenic effects instead of pure androgenic mechanisms. In previous studies, the potent androgens, DHT and 17α-methyltestosterone were shown to induce Vg in fish [21,22]. These studies, however, were carried out at extremely high doses (1000 mg/kg) and were not believed to be physiologically relevant. In contrast, another study reported that 17α-methyltestosterone inhibited Vg gene expression and serum E_2 levels in tilapia [23]. One reason for the contrasting results in the few studies that have been performed, may be the lack of a well characterized biomarker like Vg. In mammals, screening for prostate specific antigen levels in blood serum is an accepted biomarker for androgen status to indicate high risk subjects for prostate cancer. In mice, the use of 6α/15α-OH ratios has been proposed as another biomarker, which is superior to measuring circulating hormone levels, which are highly variable and make detection of alterations by xenobiotics difficult [24]. In rats, testosterone and DHT were found to lower luteinizing hormone secretion in the presence and absence of E_2 [25].

Alterations in steroid metabolizing enzymes, either through modulation of synthesis or degradation could have profound effects on estrogenic pathways. Numerous hepatic Phase I and II enzymes metabolize estrogens and androgens [26–28]. Marked shifts in the levels and activities of xenobiotic metabolizing enzymes, as previously demonstrated [29–31], would presumably affect the type and degree of estrogenic and androgenic responses. The importance of this and other indirect mechanisms of action by androgens may affect responses that are classically regarded as estrogen mediated [32]. In one study, You et al. [33] found that male rats exposed to *p,p′* DDE had elevated cytochrome P4502B1 and 3A1 protein levels which resulted in an increase in the respective hydroxylated testosterone products. Administration of 17β-estradiol to rainbow trout lowered mean hepatic 6β-hydroxylase activity and total cytochrome P450 content, which indicates that steroids can modulate metabolic enzyme protein levels and activity [34].

In this paper, we compared the ability of DHEA, androstenedione, and DHT, a non-aromatizable androgen, to elicit estrogenic responses, by measuring Vg induction in rainbow trout. We also quantified blood plasma E_2 in order to measure conversion rates of each androgen. In an attempt to shed light on some possible metabolic effects of androgens, we quantified total liver microsomal cytochrome P450 content for each group. Additionally, we investigated the possible mediation of Vg, E_2 and total cytochrome P450 content through the androgen receptor utilizing the antiandrogen, flutamide. The aim of these studies was to determine sensitive endpoints and indicators of androgen exposure and to decipher them from estrogenic responses that would serve as a basis for future studies.

2. Methods and materials

Juvenile Mt. Shasta rainbow trout, *Oncorhynchus mykiss*, 12–18 months old, were kept in 375 l tanks at 14°C under a 12:12 light:dark cycle and fed a maintenance ration (2.8% w/w) of Oregon test diet (OTD). To determine the estrogenic contribution of the three androgens in this experiment, treatment groups of 12 rainbow trout were fed OTD containing DMSO ($< 0.2\%$ by volume) with or without dissolved DHEA, androstenedione or DHT (0.05–50 mg/kg per day) for 14 days. In a second study, we focused on the relationship of the effects observed in the first study with the androgen receptor. Rainbow trout, 16 per treatment group, were administered diets of OTD containing DHT (0, 50 or 100 mg/kg) and flutamide (0, 100, 250, 750 or 1250 mg/kg) dissolved in DMSO for 14 days.

On day 15 of both studies, fish were euthanized with an overdose of tricane methanesulfonate (MS222) following protocols approved by the Oregon State University Institutional Animal Care and Use Committee. Blood was extracted from the caudal vein into heparinized vials containing 1 mM EDTA and 50 KIU/ml aprotinin to slow Vg degradation. Plasma was collected by centrifugation of blood at $2000 \times g$ for 10 min at 4°C and stored at −80°C. Livers were weighed, snap frozen in liquid N_2 and stored at −80°C.

Blood plasma Vg was quantified using an ELISA described by Donohoe and Curtis [13], and Shilling and

Williams [35] with antibody raised in rabbits against chum salmon Vg (1:1500 dilution), which was graciously obtained by way of Donald Buhler, Oregon State University from A. Hara at Hokkaido University. Colormetric readings for the ELISA were performed on a microtiter plate reader (Biotek EL 340, Winooski, VT) and analyzed with plate reader software (Deltasoft 3, Princeton, NJ). The linear range of a given assay was determined to be between 6.25 and 3200 ng Vg/ml sample. Samples were diluted accordingly in phosphate buffered solution, pH 7.2, containing 1% bovine serum albumin, 0.1% Triton X-100 and 0.1% Tween-20. Although rabbit anti-trout Vg antibody was available, we found that the anti-chum salmon antibody gave relatively similar values throughout the linear concentration range with about a third of the background, allowing for greater sensitivity (data not shown). A 100-μl aliquot of each blood plasma sample was taken for steroid analysis. These samples were extracted twice with 2 ml diethyl ether and vortex shaken for 20 s. The tubes were placed in liquid N_2 for 8 s and then the ether layer was poured off and collected. The extract was concentrated to dryness using a SpeedVac concentrator system (Savant, Holbrook, NY), resuspended in 1 ml of EIA assay buffer and stored at −20°C. 17β-Estradiol (E_2) and testosterone (T) levels were measured by EIA with reagents from Cayman Chemical (Ann Arbor, MI). Statistical analyses were performed intrasex only using *t*-tests for treatment effect and one-way ANOVA and *F*-tests comparing differences between treatment groups.

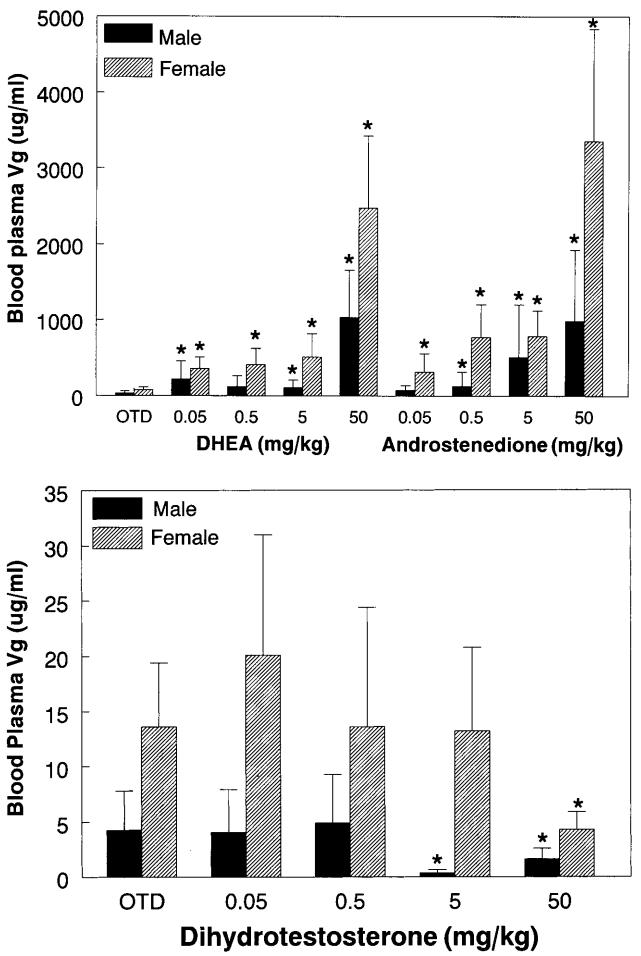

Fig. 1. Blood plasma Vg for male and female trout fed 0.05–50 mg/kg per day (A) DHEA and androstenedione and (B) DHT for 2 weeks. Significant sex differences were observed in most groups. (*) denotes significant Vg modulation compared to controls ($P < 0.05$, *t*-test assuming unequal variances). Error bars represent ± S.E. ($n = 4$–8, total $n = 12$).

Microsomes were prepared from livers by the method of Guengrich [36]. Briefly, tissue homogenized in cold 0.1 M phosphate buffer containing 0.15 M KCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) was spun for 25 min at 10 000 × *g* at 4°C. The microsomal fraction was obtained by spinning the supernatant at 100 000 × *g* for 95 min at 4°C. The pellet was washed by resuspension in 0.1 M potassium pyrophosphate containing 1 mM EDTA, 0.1 mM BHT and 0.1 mM PMSF followed by a second 95 min spin at 100 000 × *g*. The final microsomal pellet was resuspended in phosphate buffer containing 30% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF and stored at −80°C. Total liver microsomal P450 content was quantified by the CO versus CO-reduced difference spectra [37] on a Cary 300 UV-Vis spectrophotometer (Varian, Walnut Creek, CA). Values are reported as nmol P450 and normalized to protein content determined by the Lowry method using bovine serum albumin as the standard [38].

3. Results

After 2 weeks, untreated female trout were found to have significantly higher levels of Vg than males, representing a fundamental sex difference in basal Vg levels, even in immature trout ($P < 0.01$, *t*-test). Comparisons were performed intrasex only, due to significant sex differences in Vg levels ($P < 0.05$, 1 way ANOVA and ANOVA *F*-test) in most of the treatment groups as well. A dose responsive increase in blood plasma Vg was observed in trout fed DHEA and androstenedione (Fig. 1(A)). In trout fed DHEA, Vg levels increased in males and females with about 30-fold induction observed at 50 mg/kg per day ($P < 0.0001$) compared to corresponding control (OTD). The Vg response to androstenedione administration was similar to that of DHEA with female levels generally somewhat higher than male levels. Maximum induction at 50 mg/kg per day androstenedione was 45-fold for females and 30-fold for males. In contrast, induction of Vg was not observed in DHT fed groups (Fig. 1(B)). In fact, Vg significantly decreased by up to 80% in male and female

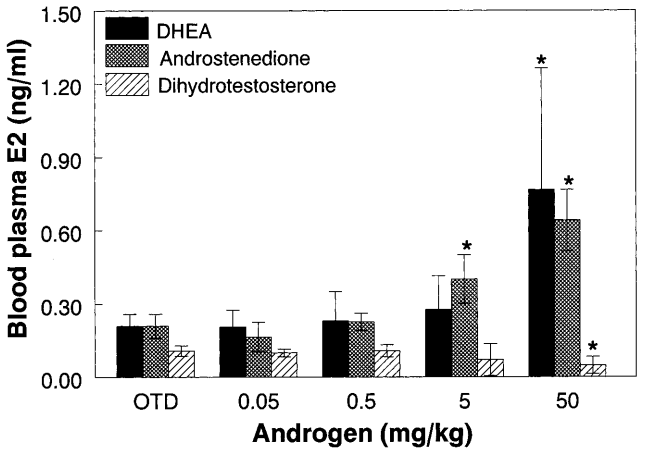

Fig. 2. Blood plasma E_2 levels in male trout fed 0.05–50 mg/kg per day DHEA, androstenedione and DHT for 2 weeks. E_2 was not reported for female trout due to loss of samples. Significant differences in E_2 from controls are denoted with (*) ($P < 0.05$, t-test assuming unequal variances). Error bars represent ± S.E. ($n = 4$–8).

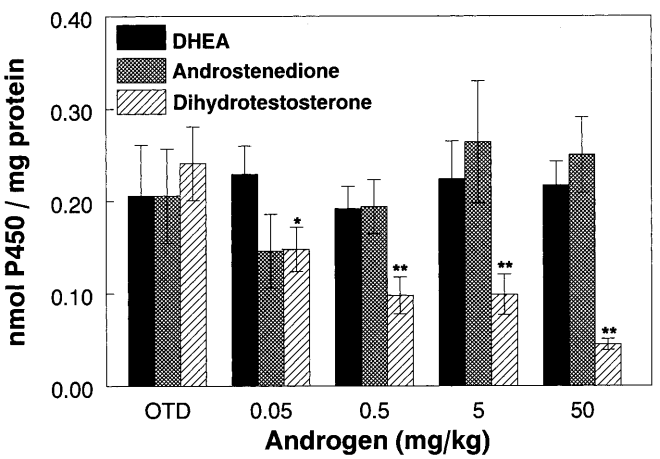

Fig. 3. Total P450 content in trout fed 0.05–50 mg/kg per day DHEA, androstenedione or DHT for 2 weeks. Sex data are pooled since no differences were observed in any group ($P > 0.05$). Error bars represent ± S.E. ($n = 12$ or 15, respectively). (*) and (**) denote values significantly lower than control ($P < 0.03$ and 0.0005, respectively, t-test assuming unequal variances).

trout fed DHT compared to trout fed vehicle only ($P < 0.05$, F-test).

As was the case with Vg, basal E_2 levels were twice as high in untreated females compared to untreated males ($P < 0.01$, t-test, Fig. 2). Unfortunately, we were only able to report values for males due to loss of some female samples. Blood plasma E_2 levels were significantly higher in males fed 50 mg/kg DHEA or 5 and 50 mg/kg androstenedione ($P < 0.05$, t-test). A three-fold induction compared to OTD was observed at 50 mg/kg for DHEA and androstenedione. These increases support the hypothesis that these androgens are indeed being converted to estrogens in vivo. If the previously observed estrogenic effects are through direct conversion to estrogens, we would not expect increased E_2 in trout fed an androgen that cannot be aromatized, such as DHT. At 50 mg/kg, we actually noticed a significant 55% reduction in male plasma E_2 (Fig. 2, $P < 0.005$, t-test). The E_2 decrease may be the basis of the Vg decrease since the values of the two parameters corresponded closely.

To investigate possible indirect effects on Vg and E_2 levels by androgens via alterations in metabolism, total microsomal cytochrome P-450 content was quantified. In trout fed DHEA or androstenedione, total cytochrome P-450 content was not affected (Fig. 3). DHT, however markedly decreased P450 content in a dose responsive fashion at all doses compared to OTD (Fig. 3, $P < 0.03$ at 0.05 mg/kg and $P < 0.0005$ at doses above 0.05 mg/kg, t-test). At the highest dose of DHT, an 81% decrease was seen.

In the first study, the potent non-aromatizable androgen, DHT, exhibited antiestrogen-like characteristics in the rainbow trout. We hypothesized that some of these effects may be mediated through androgen receptor binding and subsequent down-regulation of aromatizable androgen and/or estrogen synthesis. Either mechanism would explain the observed Vg and E_2 decreases. To test this hypothesis, DHT was fed at two high doses (50 and 100 mg/kg per day) to trout with flutamide, an androgen receptor antagonist. After 2 weeks, the antiestrogenic responses were observed in both male and female rainbow trout in the absence of flutamide. At 50 mg/kg per day, Vg levels were 80% lower than trout fed vehicle (Fig. 4). At 100 mg/kg per day, this response was not as marked, possibly due to some estrogen receptor agonism, especially in males where Vg levels

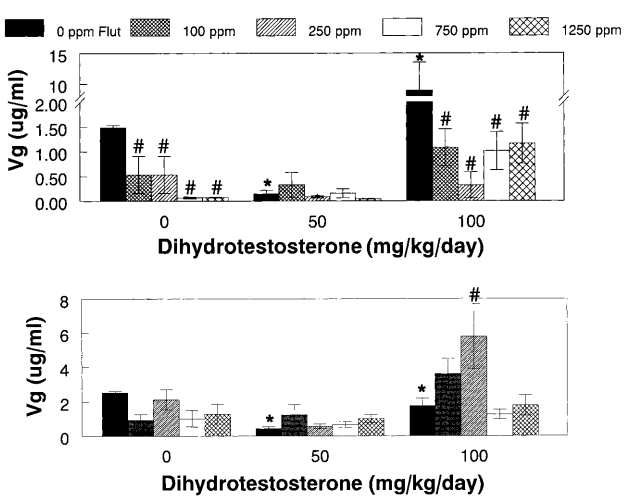

Fig. 4. Blood plasma Vg for male (upper graph) and female (lower graph) trout fed DHT and flutamide. Error bars represent ± S.E. ($n = 4$–12, total $n = 16$). (*) denotes values significantly different from trout fed vehicle ($P < 0.05$, t-test assuming unequal variances. (#) denotes significant difference from corresponding group fed DHT only ($P < 0.05$, ANOVA F-test).

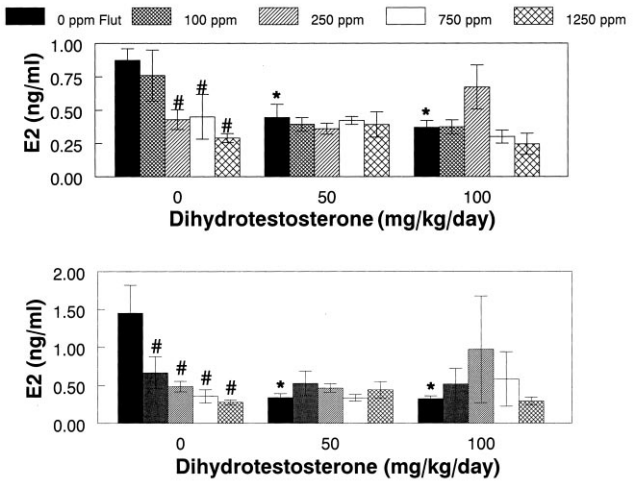

Fig. 5. Blood plasma E_2 for male (upper graph) and female (lower graph) trout fed DHT and flutamide. Error bars represent ± S.E. ($n = 4$–12, total $n = 16$). (*) denotes values significantly different from trout fed vehicle ($P < 0.05$, t-test assuming unequal variances. (#) denotes significant difference from corresponding group fed DHT only ($P < 0.05$, ANOVA F-test).

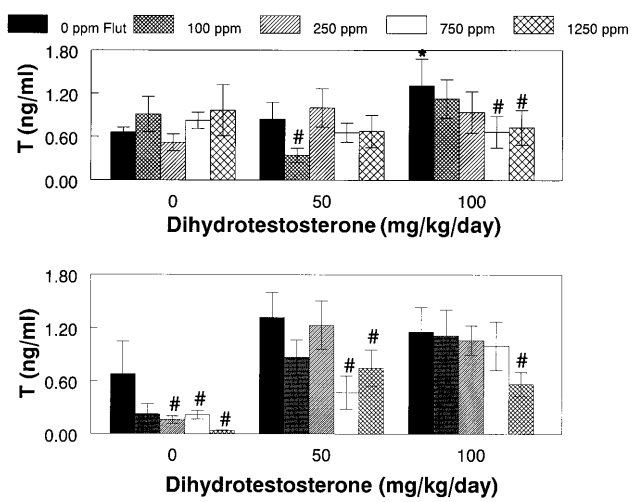

Fig. 6. Blood plasma T for male (upper graph) and female (lower graph) trout fed DHT and flutamide. Error bars represent ± S.E. ($n = 4$–12, total $n = 16$). (*) denotes values significantly different from trout fed vehicle ($P < 0.05$, t-test assuming unequal variances. (#) denotes significant difference from corresponding group fed DHT only ($P < 0.05$, ANOVA F-test).

were higher than controls. The decrease, however, appeared to be in response to a decline in E_2 levels which was also observed in males and females fed DHT (Fig. 5). Flutamide did not reverse the DHT effect, suggesting that the Vg and E_2 decreases were not due to pathways directly related to androgen receptor binding. It does seem that the androgen receptor plays some role because this receptor antagonist was as effective as DHT in decreasing Vg and E_2 levels, particularly in males. DHT increased T levels two-fold at 100 mg/kg, an effect that was partially blocked by increasing flutamide dose (Fig. 6). Flutamide had no effect on trout fed 50 mg/kg DHT and there was high variability, making it difficult to generate relationships between T and the Vg and E_2 decreases. Flutamide alone, however, had a profound effect on female T levels, decreasing the steroid by 90% at 1250 mg/kg ($P < 0.0005$, F-test). This decrease was not observed in males, suggesting the presence of a sex-specific antiandrogenic feedback mechanism.

We investigated a possible metabolic effect of DHT through androgen receptor binding by measuring total P450 content in liver microsomes from trout fed DHT and flutamide. Although there were no significant differences in liver somatic index between groups, DHT lowered P450 content significantly in males and females (Fig. 7). Flutamide had a substantial impact on this effect in males, blocking P450 decreases induced by DHT in a dose dependent manner. In females, total P450 content was similar with or without flutamide. Flutamide alone also lowered total P450 content by 30% in males and 40% in females. These results suggest a sex specific role for the androgen receptor pathway that may not be related to classic agonistic/antagonistic mechanisms.

4. Discussion

We observed striking differences in responses of the trout fed the aromatizable androgens, DHEA and androstenedione, and the structurally similar non-aromatizable androgen, DHT. DHEA and androstenedione significantly induced blood plasma Vg and E_2 levels in

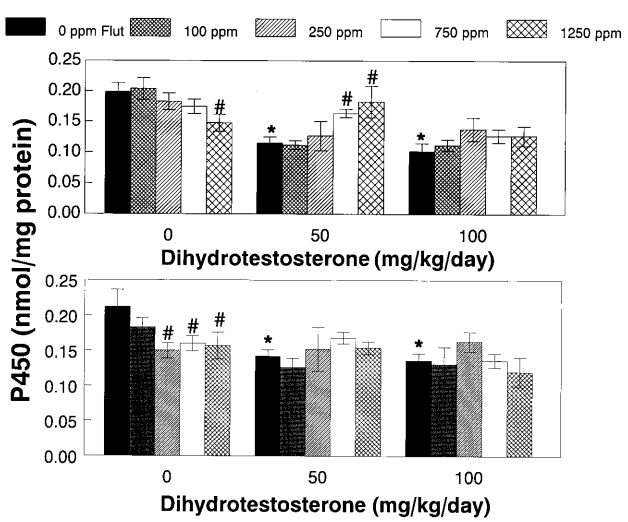

Fig. 7. Total P450 content for male (upper graph) and female (lower graph) trout liver microsomes. Error bars represent ± S.E. ($n = 4$–12, total $n = 16$). (*) denotes values significantly different from trout fed vehicle ($P < 0.05$, t-test assuming unequal variances. (#) denotes significant difference from corresponding group fed DHT only ($P < 0.05$, ANOVA F-test).

juvenile rainbow trout demonstrating the ability for these androgens to elicit an estrogenic response. DHT did not induce Vg or E_2 levels, suggesting induction by DHEA and androstenedione was not by direct estrogen receptor binding, but by conversion to estrogens. Support for this conclusion was seen in the DHEA and androstenedione treated fish, where E_2 levels increased in concert with the Vg increases, albeit with less sensitivity. In fact, DHT markedly lowered Vg and E_2 levels in the absence of toxicity, which was assessed grossly, by body weight, and liver somatic index. Decreases in Vg and E_2 levels in trout fed DHT suggest that this androgen does not bind the trout estrogen receptor or it is an estrogen receptor antagonist. Receptor binding assays are underway to answer this question. There is also the possibility of estrogen down regulation by this androgen. Perhaps it is inhibiting aromatase, the enzyme that converts androgens to estrogens. Our lab has been unsuccessful in uncovering an effective aromatase inhibitor for trout in vivo to test this hypothesis. The conversion could be blocked in another way such as inhibition of luteinizing hormone (LH) secretion that would be a signal to synthesize estrogens from androgens. Turgeon and Waring [25] found that DHT blocked gonadotropin-releasing hormone induced LH secretion in female rat gonadotropes in vitro.

In the second study, DHT elicited antiestrogenic-like responses similar to the first study, reducing Vg levels to 20% of control levels at 50 mg/kg in both males and females. Flutamide alone, however, also decreased Vg to levels comparable to DHT. DHT and flutamide normally act as opposite entities in regard to androgenic responses, but both of these evoked similar responses concerning Vg suppression. There may be some signal transduction pathways initiated by androgen receptor binding separate from classic androgen/antiandrogen pathways that ultimately reduce E_2. Females may be less sensitive to E_2 decreases due to higher estrogen receptor levels and/or affinity for DHT and/or flutamide as a substrate, which could offset decreases in E_2 levels. Carlson and Williams [39] found that even in immature rainbow trout there is a sex difference in Vg production in response to E_2. This difference may be one of several between males and females regarding steroid production and metabolism.

We also examined the potential for DHT to block the production of endogenous T, thus depleting the available pool of androgens for conversion to estrogens. DHT increased T levels, an effect that may be an artifact of crossreactivity in the EIA, which has been estimated by the vendor to be 20%. Although flutamide significantly decreased E_2 in both sexes, T levels were only lowered in females. It is possible that decreases in E_2 could be a consequence of a depleted androgen pool, induced by androgen receptor binding. Perhaps separate mechanisms exist that control E_2 and T levels in response to androgen receptor binding. Moreover, these differences may be sex-specific.

Increases in estrogen metabolism by specific cytochrome P450s provide an explanation for the E_2 decreases that ultimately would lead to suppressed Vg levels. The highly significant, dose responsive decrease in total liver P450 content by DHT does not support this hypothesis. Feedback mechanisms by DHT that decrease syntheses of estrogens or estrogen precursors, however, would decrease E_2 and Vg, a hypothesis that is supported by the DHT data. A previous study in rainbow trout, implanted with 5 mg of different androgens and E_2, found that DHT, T, and 17α-methyltestosterone had no effect on total P450 content, however, although 11kT acted as an inducer [40]. Decreases in hepatic P450 levels may mirror repression of extrahepatic P450s or other sex-steroid-regulated enzymes important for E_2 and/or Vg synthesis. Perhaps the levels of aromatase in Sertoli cells, a cytochrome P450 ($P450_{arom}$) responsible for the conversion of androgens to estrogens, is also being lowered in response to DHT, resulting in an accumulation of T. Our laboratory is currently investigating the androgenic effects on specific P450 isozymes, such as aromatase. Although DHT significantly lowered P450 content in males and females, the antiandrogen flutamide attenuated this effect in males only. It is possible that total P450 levels may be under androgen control in males in a fashion that is quantitatively or qualitatively different from females.

We hope to compare the responses by trout to DHT, the major circulating androgen in mammals to 11kT, the major androgen in trout, which is also non-aromatizable. This will allow us to study a physiologically relevant androgen in trout to determine if the DHT responses are predictive of those of the major teleost androgen. This is critical since significant differences in Phase I and Phase II activities have been reported between trout and other species [41,42].

The reported and current studies will hopefully provide a basis for investigating mechanisms and sensitive endpoints of androgen exposure. Endogenous and exogenous androgens may play an important role in modulating endocrine and metabolic pathways in trout as well as higher vertebrates.

In summary, trout fed DHT have lower Vg, E_2, and total P450 content without the standard indications of toxicity. The antiandrogen, flutamide also had some antiestrogenic-like effects in the trout supporting the role of the androgen receptor in these responses. The mechanisms seem less clear due to the similar responses elicited by these different compounds. There may be some metabolic modulation associated with DHT treatment that may or may not be related to the antiestrogenic effects. The data presented suggest that antiestrogenic responses can result from androgenic and antiandrogenic compounds acting in a direct or indirect manner.

Acknowledgements

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