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Metabolite Profiling and a Transcriptional Activation Assay Provide Direct Evidence of Androgen Receptor Antagonism by Bisphenol A in Fish

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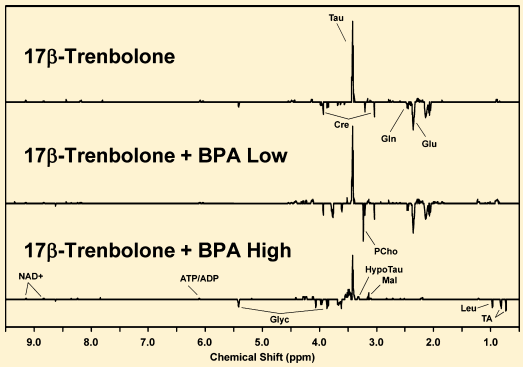
Supporting Information

ABSTRACT: Widespread environmental contamination by bisphenol A (BPA) has created the need to fully define its potential toxic mechanisms of action (MOA) to properly assess human health and ecological risks from exposure. Although long recognized as an estrogen receptor (ER) agonist, some data suggest that BPA may also behave as an androgen receptor (AR) antagonist. However, direct evidence of this activity is deficient. To address this knowledge gap, we employed a metabolomic approach using in vivo exposures of fathead minnows (FHM; *Pimephales promelas*) to BPA either alone or in a binary mixture with 17β-trenbolone (TB), a strong AR agonist. Changes in liver metabolite profiles in female FHM in response to these exposures were determined using high resolution ^{1}H NMR spectroscopy and multivariate and univariate statistics. Using this approach, we observed clear evidence of the ability of BPA to mitigate the impact of TB, consistent with an antiandrogenic MOA. In addition, a transcriptional activation assay with the FHM AR was used to confirm the AR antagonistic activity of BPA in vitro. The results of these in vivo and in vitro analyses provide strong and direct evidence for ascribing an antiandrogenic MOA to BPA in vertebrates.



INTRODUCTION

Bisphenol A (2,2-bis (4-hydroxyphenyl) propane; BPA) is a high-production volume chemical (>2 million metric tons/year [1]) used for several purposes, the most prevalent of which are the manufacture of polycarbonate plastics and epoxy resins.[2] Distribution of BPA in the environment is nearly ubiquitous, with detectable concentrations of the chemical present in virtually any biotic or abiotic compartment in which meaningful measurements can be made (for reviews see refs 2−4). This includes surface waters across the U.S., with concentrations varying dramatically from below detection limits to as high as 12 μg/L (mean-concentration range up to 1.78 μg/L).[5] This has created concern in regard to the risks posed to exposed organisms in such contaminated ecosystems. Consequently, there has been a significant amount of work examining the impact(s) of exposure in a variety of mammalian and nonmammalian species. Much of this work has been focused on the potential for BPA to disrupt function of the hypothalamic−pituitary−gonadal (HPG) axis of vertebrates.

Rat studies conducted more than 70 years ago showed that BPA can act as an effective estrogen receptor (ER) agonist.[6,7] These observations have served as a basis for a large amount of research concerning mechanisms of action (MOA) and toxicity of BPA in mammals (for reviews see refs 3,4,8,9). An extensive body of evidence indicates that BPA binds to mammalian ERs (albeit with affinities several thousand-fold lower than 17β-estradiol) and at least in vitro can activate the ER. However, in vivo activation of estrogen-responsive pathways by BPA in mammals varies substantially among studies. This may be due in part to BPA displaying various levels of agonism for multiple ERs, including ERα, ERβ, and ERRγ.[10−12] In addition, responses have been found to be highly dependent on magnitude of dose and route of exposure due to efficient first-pass hepatic metabolism of orally administered BPA. This has not been the case for aquatic species exposed via the water, where uptake across gills (i.e., inhalation) is the predominant route of exposure. Although gills exhibit some degree of metabolic capacity for environmental pollutants,[13,14] metabolism of BPA via this route would appear to be minimal. For example, in studies with a variety of fish species, waterborne BPA consistently produces strong estrogenic signals in vivo.

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Prominent among these is induction of the estrogen-responsive egg yolk protein precursor vitellogenin in males (for review see ref 15). Further, BPA has been shown to adversely affect reproduction (egg production) in fish (fathead minnows (FHM); *Pimephales promelas*).[16]

Some evidence exists suggesting that BPA could affect HPG function via mechanisms other than activation of the ER. For example, data from mammalian in vitro systems suggest that BPA may also act through androgen receptor (AR) antagonism.[17] In vivo mammalian studies that have directly evaluated this pathway[18,19] have not confirmed an antiandrogenic MOA for BPA. However, evidence of potential in vivo AR antagonism has been found recently for BPA in fish. Our research team conducted a series of experiments with the FHM in which fish were exposed to binary mixtures of the strong AR agonist 17β-trenbolone (TB) and chemicals known to impact the HPG axis via other mechanisms. (Note that exposures were also conducted with the various individual chemicals that comprised these binary mixtures). Bisphenol A was included as a putative weak ER agonist.[20] Consistent with studies on fish by other laboratories,[15] a 14 d water exposure to 100 μg/L BPA alone induced VTG synthesis, with the most pronounced effects occurring in males. Somewhat unexpectedly, BPA also appeared to antagonize effects of TB in the fish that were coexposed to the binary mixtures. Specifically, TB-induced morphological masculinization of female FHMs, as evidenced by production of malelike dorsal nuptial tubercles, was inhibited by the higher test concentration of BPA in the TB/BPA mixture. Inhibition of TB-induced tubercle formation in female FHMs is a response we have observed in similar coexposure experiments with the established AR antagonists flutamide, cyproterone acetate, and vinclozolin,[20−22] which led us to postulate that BPA may also be acting as an antiandrogen in the fish. This hypothesis was bolstered by data from Jolly et al.[23] who reported what appeared to be AR antagonism by BPA in an in vitro system composed of female kidney cells from the three-spined stickleback, a fish species commonly used in toxicological work in Europe.

In addition to measuring VTG and conducting morphological measurements for FHM in the BPA and TB/BPA coexposure experiments,[20] we collected urine from the male fish for use in metabolomic analyses. Specifically, NMR spectroscopy was used to determine if a BPA-induced antiandrogenic response was reflected in urinary endogenous metabolite profiles. This approach has proven valuable for assessing the MOA and toxicological impact of other antiandrogens in FHM.[24,25] As shown in Supporting Information Figure S1, urine collected from the male fish exposed to the low concentration of BPA (10 μg/L) alone did produce some metabolite changes consistent with the antiandrogens vinclozolin and cyproterone acetate, providing potential evidence for the antiandrogenicity of BPA in vivo in males. However, interpretation of this result was confounded by the presence of some of these same metabolite changes in the concurrent TB-only exposed males (although these changes were not observed in any of several previous TB-only exposures with male FHM (ref 24 and other unpublished results). This outcome with the low concentration of BPA, while not conclusive, provided the impetus to further pursue a metabolomic approach to investigate the possibility of an antiandrogenic MOA for BPA in fish.

The goal of the present study was to seek more conclusive evidence as to whether BPA indeed acts as an AR antagonist in fish. For this work, we assessed the hepatic metabolome (as measured by NMR spectroscopy) of female fathead minnows exposed to binary mixtures of TB and BPA to determine the degree of interaction between a known AR agonist and the putative antagonist. In addition to the metabolomic analyses, a transcriptional activation assay with the cloned fathead minnow AR was used to provide a complementary yet disparate line of evidence for the antiandrogenic nature of BPA.

■ MATERIALS AND METHODS

In Vivo Exposure: Experimental Design and Sample Collection. *Experimental Design.* The samples used for the current study were generated as part of an exposure that has been previously described.[20] In brief, sexually mature (ca. 5−6 month old) FHM from an on-site culture facility at the Duluth EPA lab were held in a mass culture prior to the exposures. To initiate the experiment, four males and four females were placed together in glass aquaria holding 10 L of Lake Superior water (4 μM filtered, UV sterilized) or test chemical(s) dissolved in Lake Superior water, which was continuously renewed at a rate of about 45 mL/min. Two males and two females were randomly selected from each of the quadruplicate tanks for each treatment condition, yielding an initial n = 8 for each sex and each treatment. The animals were held at 25 °C under a 16:8 L/D photoperiod and were fed brine shrimp to satiation twice per day.

Solvent-free solutions of TB (>95% pure; Sigma−Aldrich, St. Louis, MO, USA) and BPA (>99% pure, Sigma−Aldrich) were prepared as concentrated stocks in Lake Superior water. Exposures consisted of the following six treatments: controls (CON), TB (500 ng/L; nominal), BPA low (BPA-L, 10 μg/L; nominal), BPA high (BPA-H, 100 μg/L; nominal), TB+BPA-L (500 ng/L TB + 10 μg/L BPA), and TB+BPA-H (500 ng/L TB + 100 μg/L BPA). Exposures were conducted for 14 d. Concentrations of TB and BPA in the control and test tanks were monitored over the course of the exposure as previously described.[20] For both chemicals, measured concentrations in the water agreed well with target values (average deviation less than 10%).

Sample Collection. There were no treatment- or nontreatment-related mortalities. At the conclusion of the 14 d exposure, the fish were anaesthetized with buffered tricaine methane sulfonate (MS-222; 100 mg/L buffered with 200 mg/L $NaHCO_3$/L; Finquel, Argent, Redmond, WA, USA). Fish were weighed, and liver, gonads, and muscle tissue were removed, transferred to preweighed microcentrifuge tubes, flash-frozen in liquid nitrogen, and stored at -80 °C until extracted or analyzed. All laboratory procedures involving animals were reviewed and approved by the United States Environmental Protection Agency Mid-Continent Ecology Division Animal Care and Use Committee in accordance with Animal Welfare Act regulations and Interagency Research Animal Committee guidelines.

Sample Preparation and Analyses. Individual liver samples were extracted using a dual phase extraction procedure as described previously.[26] This procedure generates both a polar and a nonpolar metabolite phase. A total of 96 liver samples were processed batch wise, in a randomized fashion. Prior to NMR analysis, solvents were removed from the samples by drying using a vacuum concentrator (Thermo Scientific, Waltham, MA, USA). Each individual sample was then reconstituted in 145 μL of 0.1 M sodium phosphate buffered deuterium oxide (pH 7.4) containing 25 μM sodium

3-(trimethylsilyl) propionate-2,2,3,3-d_4 (TSP), vortexed briefly, and centrifuged at 10 600 relative centrifugal force for 15 min at 4 °C to remove any insoluble components. The supernatants were pipetted into a 96-well plate containing 500 μL glass inserts (Microliter Analytical Supplies, Inc., Suwanee, GA, USA) and run in automated fashion using a push through direct injection method.[27] Details relating to acquisition of NMR spectra and metabolite identification are described in the Supporting Information.

NMR Data Processing and Analysis. Spectra were processed using ACD/1D NMR Manager (Advanced Chemistry Development, Toronto, Canada). Specifically, spectra were zero-filled to 32 768 points, and an exponential line broadening of 0.3 Hz was applied prior to Fourier transformation. An automated routine was used to phase and baseline-correct each spectrum, which was then referenced to TSP (at 0.0 ppm). Subsequently, one spectrum (from a female control) that was characterized by poor shimming prior to acquisition (which produces overly broad peak shapes) was removed from the data set. Next, the spectra (0.50−10.00 ppm) were segmented into 0.005 ppm wide bins and imported into Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). Within Excel, bins in the region from 4.70−5.10 ppm were omitted in order to remove the residual water resonance. Bin values from 3.34 to 3.38 were omitted to remove a contaminating methanol resonance and bin values from 3.26 to 3.29 and from 3.90 to 3.93 were omitted to remove large betaine resonances that were highly variable, exhibited very strong leverage on the model, and were uninformative. The remaining 1794 bins were then normalized to achieve unit total intensity for each spectrum. This Excel spreadsheet of binned spectra was then imported into SIMCA-P+ (Umetrics Inc., Umea, Sweden) for multivariate data analysis (which was conducted on mean-centered and Pareto-scaled bins). Principal components analysis (PCA) was used to screen for outliers in the data set (none were found using the Hotelling's T^2 test at the 95% confidence interval for a scores plot of the first two components). Subsequently, partial least-squares discriminant analysis (PLS-DA) models were constructed to help establish and visualize the extent of impact for a given treatment, as well as the relative impacts of different treatments. We used the permutation testing routine[28] within SIMCA-P+ for PLS-DA models to assess the validity of assuming that the treatments had resulted in a discernible effect. To simplify interpretation, score values for classes in PLS-DA scores plots were averaged and are shown with the associated standard error. Student's *t* tests were conducted in Excel on component 1 score values to assess statistical significance of separation among classes.

The Excel spreadsheet of binned, edited, and normalized spectra was also used to construct "*t* test filtered difference spectra". In order to generate the difference spectra, an "average class spectrum" was first calculated by averaging the binned spectra across all class members. Next, the difference spectrum for each exposed class was generated by subtracting the averaged bins of the control class from those of the exposed class. Then, a *t* test was conducted on each bin to determine if the average for the exposed class differed significantly from that of the control class, using a p value <0.05. If not, the bin value for the difference spectrum was replaced with a zero. As previously described,[24] we use the known structure of NMR spectra to identify and zero out bins that are falsely discovered as positive. (On average, for the five difference spectra presented here, the percentage of bins rejected (of the total number tested) as falsely positive by this approach was 5.6 ± 0.7 (mean ± SD). Since 5% false positives are expected at $p < 0.05$, this result is consistent with an effective and statistically powerful method for rejecting false positives.) The resulting *t* test filtered difference spectrum for each exposed class displays peaks above the baseline that correspond to metabolites that were increased upon exposure, and peaks below the baseline that correspond to metabolites that decreased. More details on generating these difference spectra and on limiting their false positives rates have recently been published.[24]

In Vitro Transcriptional Activation Assay. *Reagents.* A plasmid pFAR-g expression cassette[29] was subcloned into pdeltaE1sp1A and used to generate Ad5FAR-g using standard techniques.[30,31] The Ad/mLuc7, which contains a luciferase gene regulated by the glucocorticoid-inducible hormone response element found in the mouse mammary tumor virus (MTV) LTR, and thus acts as an inducible reporter for AR activity, was a gift from Cary Weinberger (NIEHS; ref 32). Test reagents for the transcriptional activation assays were acquired as follows: 5 α-androstan-17β-ol-3-one (dihydro-testosterone (DHT); Sigma−Aldrich), BPA (Sigma−Aldrich), and 4-[2,2-dichloro-1-(4-hydroxyphenyl)vinyl]phenol) (hydroxy-flutamide; R.O. Neri Schering Corp, Bloomfield, NJ, USA).

Transcription Assay. CV-1 cells (ATCC CCL 70, Rockville, MD, USA) were maintained in a growth medium (RPMI-1640 (Sigma−Aldrich R8755) supplemented with 2.5 g of glucose/L, 10 nM HEPES, 1 mM sodium pyruvate, 1.5 g of $NaHCO_2$/L, and 10% dextran coated charcoal (DCC)-stripped fetal bovine serum (FBS; Hyclone, Logan, UT)) at 37 °C, 100% humidity, and 5% CO_2. The medium was devoid of phenol red and antibiotics. Cells were subcultured weekly 1:3 via standard trypsinization. Medium from a 7 d T25 flask of CV-1 cells was removed and replaced with 0.5 mL of growth medium containing 1×10^6 plaque forming units (pfu) of Ad5FAR-g and 1 x10^7 pfu of Ad/mLuc7. The flask was rocked every 15 min for 1 h and then incubated for 3 h, following which 3.5 mL of growth media was added and incubated for an additional 24 h. Cells were then trypsinized, suspended in 10 mL of growth media containing 5% DCC-stripped FBS, and 100 uL aliquots dispensed into a white-matted 96-well plate already containing 100 μL of test chemical solution at two-times the final target concentration in growth medium with 5% of the stripped FBS. The plate was incubated 24 h at 37 °C. Plates subsequently were observed for cytopathology, washed with phosphate-buffered saline (pH 7.4), decanted, lysed, and frozen at −80 °C, until determining luciferase activity as described by Hartig et al.[33]

Data Collection and Analysis. Before conducting experiments in which the combined effects of BPA and DHT were evaluated in the assay system, a complete DHT-only dose−response curve was generated. Based on this, 3.0 nM DHT was selected as an optimal concentration causing induction of luciferase in CV-1 cells transduced with the FHM AR and the luciferase reporter gene construct. The DHT/BPA interaction data presented herein were collected from four independent experiments, with four replicates/plates per treatment per experiment. A replicate here is defined as one 96-well plate, which included four independent observations of the media control (including ethanol, the dosing solution) and all other treatment groups. In each experiment, multiple concentrations of the putative AR antagonist (BPA), ranging from 1 nM to 100 μM, were tested in the presence of 3.0 nM DHT, and evaluations were made of relative reductions in luciferase

activity. Relative light units were converted to fold-induction above the media value, a background nonzero value that was set to unity. The highest BPA plus DHT-fold value was used to identify the top of the BPA response curve (percent of maximum). Curves were plotted using logistic regression, and IC50 values were calculated using Prism software version 5.03 (GraphPad, San Diego, CA, USA).

RESULTS

Metabolomic Analysis of Female FHM Exposed to BPA. We determined hepatic metabolome responses in female fish exposed to BPA at two different test concentrations: BPA-L (10 μg/L) or BPA-H (100 μg/L). A two-component PLS-DA scores plot generated with controls, BPA-L, and BPA-H is shown in Figure 1. Evident from this plot is a concentration-dependent response in the hepatic metabolite profiles from the two BPA exposures. Specifically, the larger impact elicited by BPA-H is apparent from the greater distance this class assumes from controls relative to the BPA-L class. For this comparison, it is instructive to consider the PLS-DA1 scores (plotted along the horizontal axis in Figure 1), since this (first) component captures the predominant differences between exposure classes. The first component scores for BPA-H are clearly more different from controls than are those for BPA-L (p values of 0.0068 versus 0.40, respectively). In addition, the first component scores of BPA-H and BPA-L are significantly different from one another ($p = 0.022$).

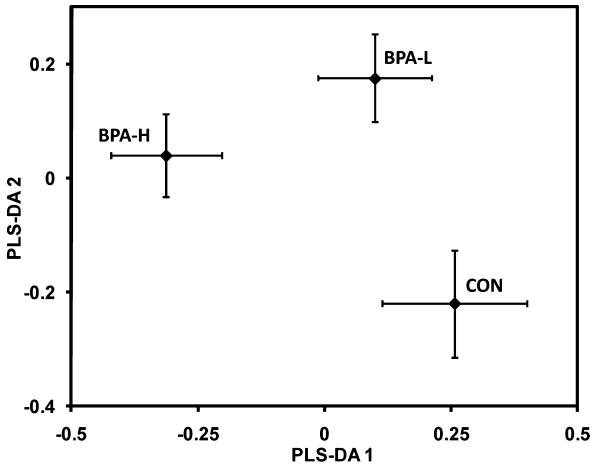

Figure 1. Two component scores plot from a validated partial least-squares discriminant analysis (PLS-DA) model of NMR spectra from polar extracts of livers collected from unexposed (CON, control) female fathead minnows, and those exposed to BPA either at the high concentration of 100 μg/L (BPA-H) or the low concentration of 10 μg/L (BPA-L). Each marker is the mean score value for a given treatment shown with its associated standard error.

In addition to modeling the data using PLS-DA, t test filtered difference spectra (Figure 2) were used to provide further evidence of a concentration-dependent response. This approach offers a quick and intuitive way to assess the overall impact of an exposure by observing the number and intensity of features present. In addition, these spectra enable rapid identification of specific metabolites that are changed upon exposure, along with the direction and extent of change. Metabolite changes observed for the BPA-H exposed fish included decreases in energetic metabolites such as ATP/ADP, glucose, and glycogen (Figure 2A). The largest metabolite change in the difference spectrum was a decrease in the amino acid glutamate. Decreases were also observed for creatine and choline. Conversely, the levels of the amino acids phenylalanine and lysine were increased upon exposure. In addition, we observed a relatively large increase in lactate.

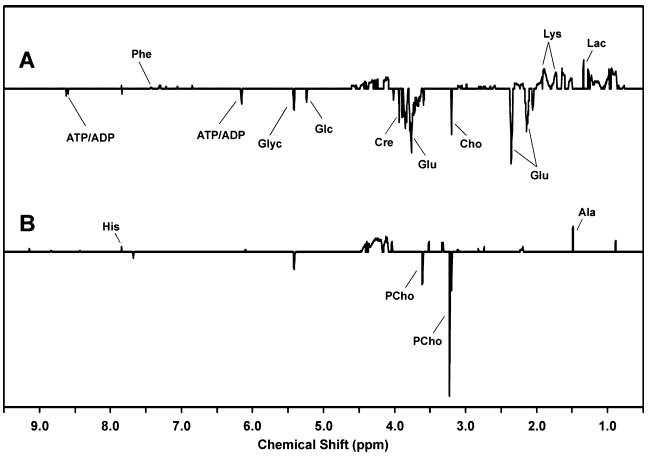

Figure 2. Average difference spectra (exposed minus control) generated both to assess the overall impact of exposure and to determine changes in liver metabolite profiles of female FHM exposed to either: (A) the high concentration BPA (BPA-H, 100 μg/L) or (B) the low concentration of BPA (BPA-L, 10 μg/L). Metabolite peak label abbreviations are as follows: Lac, lactate; Ala, alanine; Lys, lysine; Glu, glutamate; Cho, choline; PCho, phosphocholine; Cre, creatine; Glc, glucose; Glyc, glycogen; ATP/ADP, adenosine tri/di phosphate; Phe, phenylalanine; His, histidine. Note that all difference spectra are displayed using the same Y scale. Therefore, differences in the magnitude of metabolite changes across groups are meaningful.

Figure 2B shows the t test filtered difference spectrum for the BPA-L exposed fish. Consistent with a smaller impact on the metabolome than for BPA-H (Figure 1), considerably fewer peaks were observed. Among the metabolite changes were decreases in phosphocholine, choline, and glycogen. Increases were observed for the amino acids alanine and histidine. Taken collectively, results from both difference spectra and PLS-DA modeling support a concentration-dependent response in the hepatic metabolite changes when females are exposed to these two concentrations of BPA.

Metabolomic Analysis of Female FHM Coexposed to TB and BPA. The potential antiandrogenic activity of BPA was assessed using coexposures with the potent synthetic androgen TB. Again, a validated PLS-DA model of female liver metabolite profiles was used to determine the impact(s) of exposures (Figure 3). The greatest separation along the first PLS-DA component is between the controls and both the TB and TB +BPA-L classes ($p < 0.0001$). In contrast, there is significantly less separation between controls and the TB+BPA-H class along the first component ($p = 0.068$). This proximity indicates greater similarity of the first component scores for the TB +BPA-H class with those of the control class than with either of the other exposed classes. (The p value for the first component scores of the TB+BPA-H class compared to those of the TB and TB+BPA-L classes is 0.010 and 0.011, respectively.)

Metabolite changes produced by the TB, TB+BPA-L, and TB +BPA-H exposures were again identified by difference spectra analysis (Figure 4). A large number of relatively intense peaks were observed for the TB exposure, which is consistent with the large impact observed in the PLS-DA model (Figure 3). The majority of these peaks corresponded to decreases in

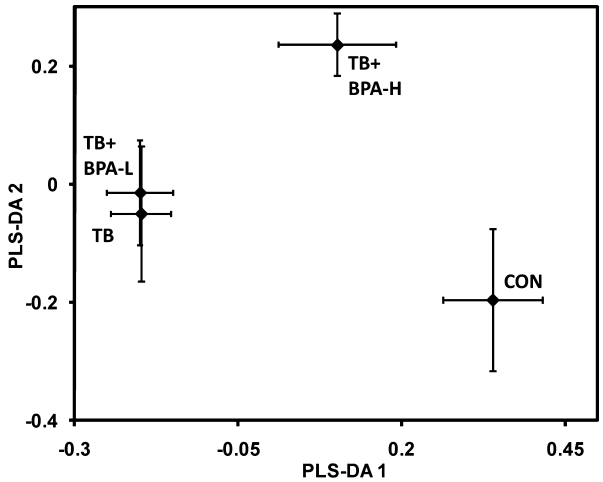

Figure 3. Two component scores plot from a validated partial least-squares discriminant analysis (PLS-DA) model of NMR spectra from polar extracts of livers collected from unexposed (CON, control) female fathead minnows, and those exposed to the following: 17β-trenbolone (TB, 500 ng/L), coexposure of TB with 100 μg/L BPA (TB+BPA-H), and coexposure of TB with 10 μg/L BPA (TB+BPA-L). Each marker is the mean score value for a given treatment, shown with its associated standard error.

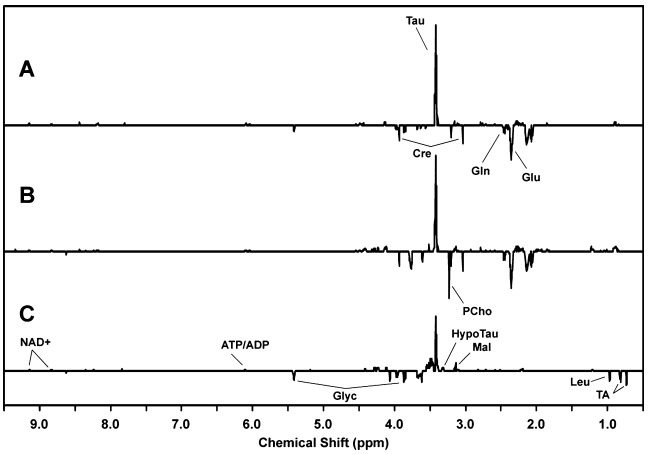

Figure 4. Average difference spectra (exposed minus control) generated both to assess the overall impact of exposure and to determine changes in liver metabolite profiles of female FHM exposed to either (A) 17β-trenbolone (TB, 500 ng/L), (B) TB with 10 μg/L BPA (TB+BPA-L), and (C) TB with 100 μg/L BPA (TB+BPA-H). Metabolite peak label abbreviations are as follows: TA, taurocholic acid; Leu, leucine; Glu, glutamate; Gln, glutamine; Cre, creatine; Mal, malonate; PCho, phosphocholine; HypoTau, hypotaurine; Glyc, glycogen; ATP/ADP, adenosine tri/di phosphate; NAD$^+$, nicotinamide adenine dinucleotide. Note that all difference spectra are displayed using the same Y scale. Therefore, differences in the magnitude of metabolite changes across groups are meaningful.

metabolite abundances upon exposure (Figure 4A). Among these was a decrease in the amino acid glutamate as well as a decrease in creatine. The largest increase was measured for the amino sulfonic acid taurine at 3.42 ppm.

The TB+BPA-L binary mixture produced metabolite changes (Figure 4B) that were very similar to those observed when fish were exposed to TB alone (which, again, is consistent with the PLS-DA results; Figure 3). This general similarity (compare Figure 4A and B) applies to both the number and types of metabolites and also to the direction and extent of change. Conversely, the difference spectrum for the TB+BPA-H exposure (Figure 4C) was not very similar to that observed for the TB exposure. Indeed, fewer and generally less intense peaks were observed, which is consistent with the decreased impact of the TB+BPA-H mixture relative to that of TB alone that was inferred from PLS-DA (Figure 3). The presence of BPA-H in the TB+BPA-H mixture seemingly had the effect of "canceling" many of the peaks that were observed when fish were exposed to TB alone. Also, there are a number of peaks observed in the TB+BPA-H difference spectrum that were not observed for TB or for TB+BPA-L. Metabolite changes observed for the TB+BPA-H exposure included increases in taurine, NAD+, ATP/ADP, malonate (tentative assignment), and hypotaurine. Decreases were measured for taurocholic acid, leucine, and glycogen.

Transcriptional Activation Assay. There was no evidence of cytopathology associated with chemical treatment of the CV-1 cells in any of the transcriptional activation assays. Each well of the 96-well plate was visually inspected and assessed on the standard 4 point scale (+1, +2, +3, +4) for chemically induced cytopathic effect. None was noted. When harvested for luciferase quantitation, all cultures were healthy and confluent and remained attached during the washing phase until disrupted with the lyses buffer. Transcriptional activation of the FHM AR was efficiently induced by DHT, with dose–response curves demonstrating maximal expression from 20 to 40 fold above the background level (data not shown). The ability of BPA to inhibit induction of luciferase was tested in the presence of 3 nM DHT (Figure 5). A concentration of 3 nM

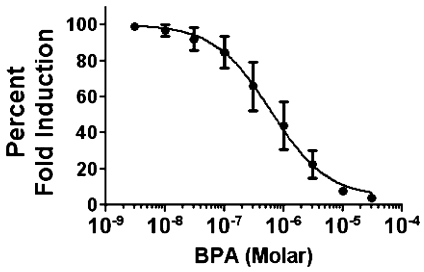

Figure 5. Effects of increasing concentrations of BPA on induction of luciferase activity by 3 nM dihydro-testosterone (DHT) in a fathead minnow androgen receptor–reporter transcriptional activation assay. Values are presented as a function of the maximal-fold induction based on curves constrained at 4.2 (media baseline) and 100%. Each data point represents the mean (±SD) derived from four separate experiments.

BPA had no effect on DHT induction of luciferase, while a concentration of 100 μM BPA effectively inhibited all DHT induction. The log IC50 of BPA, determined from the means of four independent experiments, was −6.2 (± log 0.087) with an r^2 =0.87. The shape of the dose–response curve and fit are entirely consistent with BPA behaving as an AR antagonist.

DISCUSSION

Suppression of TB-Induced Metabolite Changes by Coexposure with BPA-H. Previous studies have demonstrated that TB is a potent reproductive toxicant that binds with high affinity to the fathead minnow AR and causes a wide variety of in vivo responses in females indicative of masculinization, including enhanced growth, changes in coloration, and production of dorsal nuptial tubercles.[34]

Ekman et al.[35] also found that exposure of female fathead minnows to TB resulted in NMR-based hepatic metabolite profiles that more closely resembled those of males (as compared to unexposed females). These observations provided us with a logical basis by which to evaluate the hypothesis that BPA operates as an AR antagonist in vivo. Initial binary mixture studies by our group demonstrated that exposure to BPA inhibited the morphological masculinization of females by TB.[20] The current study extends this analysis through demonstration of the ability of BPA to mitigate the effects of TB on the hepatic metabolome in female fathead minnows, thus providing additional physiological evidence for AR antagonism. This was clearly seen in the two component scores plot generated from a PLS-DA model constructed for the control, TB, TB+BPA-L, and TB+BPA-H classes (Figure 3). While no reduction of the effects of TB was observed when coexposed with BPA-L, a substantial movement along the first component in the direction of the controls was observed after coexposure with BPA-H. This occurred to such an extent that statistical analyses (Student's *t* test) resulted in the classification of the first component scores of the TB+BPA-H and control classes as indistinguishable at the 95% confidence level.

Beyond PLS-DA scores plots, the use of *t* test filtered difference spectra provided a means to rapidly identify the TB-induced metabolite changes most impacted by coexposure with BPA-H. The most obvious of these was a decrease in the intensity of a TB-induced taurine resonance and a complete cancellation of the TB-induced reduction in glutamate (compare Figure 4A and C). In a previous study in which the effects of TB on female FHM after 8 d of exposure were examined, we also found significant changes in taurine and glutamate.[35] In that study, we found that the changes occurred in a concentration-dependent manner and hypothesized that taurine was acting in a hepato-protective capacity. Coexposure to BPA-H reduced that effect here, suggesting a reduced demand for this potentially protective measure (Figure 4C) as the toxic effects of TB exposure were mitigated. It is notable that BPA-H alone had no impact on hepatic taurine levels (Figure 2A), further suggesting its antagonist activity with the AR.

Inhibition of the TB-induced decrease in glutamate by BPA is somewhat less interpretable as BPA-H alone also produced a decrease in glutamate. A likely cause of this result is a general stress response to the individual exposures involving the activities of glutamate/oxaloacetate transaminase (GOT) and/or glutamate/pyruvate transaminase (GPT). Hepatic GOT (and to a lesser extent GPT) has been consistently shown to be activated by the stress hormone cortisol in teleosts.[36] This could result in the increased conversion of glutamate to α-ketoglutarate for energy production (via the TCA cycle). This mobilization of energetic substrates in response to stress (be it chemical, temperature, salinity, etc.) is well-established in vertebrates. Beyond glutamate, the responses differ between the TB and BPA-H exposures with regard to energetic metabolites. For example, BPA-H produced substantial decreases in glycogen, glucose, and ATP/ADP, while TB produced only a small change in glycogen after 14 d of exposure, suggesting that the fish exposed to BPA-H experienced a greater energetic cost. This is not entirely unexpected given the substantial resources required for VTG production which Ankley et al.[20] reported as being induced in these same fish.

In addition to what appears to be a complete or nearly complete mitigation of the TB-induced changes in taurine, glutamate, and several other metabolites by coexposure with BPA-H, metabolite changes characteristic of neither the TB-only or BPA-H-only exposures were also observed in females from the binary mixture experiment. Specifically, a decrease in taurocholic acid (a bile acid) and increases in NAD+, ATP/ADP, malonate (tentative assignment), and hypotaurine were observed. It is entirely plausible that the two chemicals could interact with one another relative to effects on biological pathways not involved with the AR. This is perhaps not unreasonable given the potency of TB and the numerous potential activities ascribed to BPA (e.g., ER agonist, thyroid receptor antagonist, estrogen-related receptor-γ ligand, glucocorticoid receptor agonist, and aryl hydrocarbon receptor ligand[37]). Regardless, these metabolite changes were of relatively low magnitude in comparison to the majority of metabolite changes observed for the individual exposures, again signifying that coexposure with TB and BPA-H had significantly less impact on the fish than either of the individual exposures. Indeed, this reduced impact in the difference spectrum, when combined with the results of the PLS-DA scores plot, clearly portrays mitigation of the TB exposure by BPA-H, indicating a potential agonist/antagonist interactive relationship between these compounds mediated through the AR.

The nonpolar liver extracts of these same fish were also analyzed. By using gas chromatography−mass spectrometry (GC-MS), the impact of the exposures on the various lipids (fatty acids, cholesterol, etc.) present in the liver was measured in females (data not shown). The overall impact of TB on this class of metabolites was found to be relatively minor in comparison to that seen in the polar fraction. Thus, it was not a useful approach for investigating the antiandrogenic effect that occurred in coexposure with BPA. It is unclear as to why the overall lipid responses to TB were less than that observed with the polar metabolites. However, earlier work has shown that different classes of metabolites often respond on a different time scale to an exposure.[38] Unfortunately for this study, there was no temporal component (all samples were taken after 14 days of exposure). There may in fact be more significant changes in lipid metabolites occurring at other times during the exposure that were not captured here.

Transcriptional Activation of the FHM AR Is Blocked by BPA. The ability of BPA to inhibit morphological masculinization of female fathead minnows exposed to TB and mitigate effects of the androgen on hepatic metabolite profiles together provide compelling evidence for AR antagonism. This evidence is strengthened further through the demonstrated inhibition by BPA of transcriptional activation of the cloned fathead minnow AR by DHT in vitro. The transcriptional activation assay enables a level of specificity with regard to direct AR interactions that cannot be easily achieved in vivo.

We have previously utilized adenovirus transduction to deliver the human AR gene for testing transcriptional activation.[33] Our primate AR assays based on this approach have shown a greater dynamic range than in vitro assay systems based on the stable integration of reporter genes into cell lines.[39] In addition, the constructs needed for viral delivery can be made more expeditiously than generation of a totally new cell line. As such, this approach represents a robust system capable of assessing the interactions of chemicals with a diversity of receptors, for example, from different species. Previous in vitro studies based both on mammalian and fish ARs have indicated that BPA could act as an antiandrogen (e.g.,

see refs 17,23,40,41); the present study confirms these observations through demonstration of a direct antagonistic interaction of BPA with the FHM AR in a well-defined system. Interestingly, in comparing our results with those of Sultan et al.,[41] it would appear that the in vitro potency of BPA as an AR antagonist in two reasonably analogous assays may be similar for human and FHM ARs. This is not unexpected based on cross-species conservation of the AR.[42]

To summarize and conclude, the high production volume, extensive environmental distribution, and established estrogenic potential of BPA have made it a priority contaminant of concern. It is reasonable to infer from the literature that species exposed primarily via an oral route (e.g., humans) may be at a relatively low risk of adverse effects from environmentally relevant BPA exposures, due to first-pass hepatic metabolism.[3,8] Aquatic species however can experience exposure to BPA via routes (e.g., inhalation) that may result in limited metabolism and hence increased potential toxicity. For example, while BPA administered orally in mammals is at most weakly estrogenic, BPA has repeatedly been shown to be estrogenic in fish exposed via the water, producing responses such as VTG induction in males.[15] Our work indicates that BPA has the potential to affect fish, not only through activation of the ER, but through antagonism of the AR, potentially adding further threat to aquatic species exposed to waterborne BPA. Consequently, this finding will likely weigh on decisions concerning acceptable levels of exposure to fish (and potentially other organisms) in contaminated surface waters.

Although our work concerning the antiandrogenic properties of BPA has focused on the FHM as a model, conservation of AR structure and function across vertebrates suggest that AR antagonism by BPA is likely to occur across a broad range of vertebrate species. Hence, it seems prudent to consider an antiandrogenic MOA when assessing risks posed by BPA, particularly at sensitive life stages. Highlighting this is the fact that, at least in the adult FHM, BPA simultaneously acts as an AR antagonist and an ER agonist at the same exposure concentration(s).[20] Further efforts to deduce the concentration and duration dependence, as well as tissue specificity with regard to this important MOA will be necessary as evaluation of the environmental risks posed by BPA move forward.[5] Moreover, in light of the near ubiquity of BPA in the aquatic environment, these evaluations should be given priority in future assessments.

■ ASSOCIATED CONTENT

Supporting Information

Description of the NMR spectral acquisition parameters used and the method employed for metabolic identification, a reporting of the average numbers of false positives identified across different spectra, and a figure of the stacked difference spectra of male FHM urine from vinclozolin, cyproterone acetate, and BPA exposures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ NOTE ADDED AFTER ASAP PUBLICATION

There were errors in the last paragraph of the Results section in the version of this paper published August 22, 2012. The correct version published August 24, 2012.