In ovo inhibition of steroid metabolism by bisphenol-A as a potential mechanism of endocrine disruption

Sandrine G. Clairardin, Ryan T. Paitz and Rachel M. Bowden

School of Biological Sciences, Illinois State University, Campus Box 4120, Normal, IL 61790-4120, USA

During embryonic development, endogenous signals, for example steroid hormones, and exogenous signals, for example endocrine disrupting chemicals (EDCs), have the capacity to produce phenotypic effects that persist into adulthood. As the actions of steroids are mediated through the binding of steroid receptors, most studies of EDCs have assumed that they too elicit their effects by binding steroid receptors. We tested an alternative hypothesis, namely that EDCs elicit their effects during embryonic development by disrupting the metabolism of maternally derived steroids, thereby allowing maternally derived steroids to bind steroid receptors and elicit effects. Specifically, we examined the ability of the EDC, bisphenol-A (BPA) to inhibit the normal metabolism of oestradiol during the first nine days of embryonic development in the red-eared slider turtle (Trachemys scripta). We found that, when BPA was present, oestrogen metabolism was inhibited when compared to control eggs. In particular, the formation of oestrone sulfate was blocked in BPA-treated eggs. We postulate that the oestrogenic effects of EDCs may be driven, at least in part, by inappropriate oestrogen signalling. The retention of oestrogens at points of development when they would normally be metabolized to inactive forms might also help explain low-dose effects frequently reported for EDCs.

1. Introduction

It has long been recognized that the endocrine environment experienced by developing vertebrate embryos can produce long-lasting phenotypic effects (reviewed in [1]). Many of these effects are unique to the embryonic period such that similar endocrine signals later in life are not capable of producing the same effect. The heightened sensitivity of embryos to the endocrine environment has led to a great deal of research focused on understanding how early endocrine signals influence development. During embryonic development, vertebrate embryos are exposed to steroids of embryonic and maternal origin. Because one of the main functions of oestradiol is to regulate reproduction in females [2], there is an inherent link between maternal oestradiol and offspring development. For many vertebrates, maternal oestradiol levels increase during reproduction [2]. In placental vertebrates, this results in embryos developing within a maternal environment that contains elevated oestradiol concentrations [3], whereas in oviparous vertebrates, this results in the presence of oestradiol, and other lipophilic steroids, in the yolk at oviposition [4].

Research on the early endocrine environment has taken on additional importance in light of the fact that a large number of environmental and man-made chemicals often interfere with normal endocrine signalling, and produce long-lasting phenotypic effects, including effects similar to those observed following oestrogen exposure [5]. These endocrine disrupting chemicals (EDCs) are comprised of a structurally diverse group of compounds that are also known as ‘xenoestrogens’ [6], ‘endocrine disrupting chemicals with oestrogenic activity (EEDCs)’ [7], ‘oestrogen mimics’ [8] and ‘environmental oestrogens’ [9]. A major focus of ongoing research is to determine how such a structurally diverse group of chemicals could produce effects similar to such a tightly regulated ligand, for example oestradiol, with an emphasis on the ability of EDCs to
bind to steroid receptors as a potential mechanism for producing steroid-like effects [5].

Previous research has found that a variety of EDCs can bind to oestrogen receptors (ERs) [10,11], suggesting that these EDCs may elicit oestrogenic effects by direct ER activation, thereby increasing ER-dependent gene transcription [11,12]. This model of EDC action via ER binding assumes a promiscuous ER-binding site owing to the extreme variation in EDC structure [13]. While it is clear that EDCs can bind ERs, when binding does occur, only weak, partial agonistic actions are noted [14], such that the activity of an EDC is often significantly weaker than that of the natural ligand [15,16]. Together, the evidence suggests that while ER activation by EDCs may play a role in producing oestrogenic endpoints, it is not likely the sole mechanism for oestrogenic effects in all tissues. Other possible mechanisms for the oestrogenic effects of EDCs include the induction of oestriadiol production [17] and the inhibition of oestriadiol metabolism [18,19]. Both of these mechanisms would result in elevated oestriadiol levels and do not require a direct interaction between the EDC and an ER.

Understanding how EDCs influence steroid metabolism in developing embryos could be especially important because of the high sensitivity of the embryo to steroid signals paired with the elevated levels of maternal oestrogens. A key metabolic pathway in the metabolism of maternal steroids, including oestriadiol, is sulfonation [20] because the primary conjugative pathway found in adults (glucuronidation) does not fully develop until after birth [21]. The sulfonation of steroids is carried out by a family of sulfotransferase enzymes that transfer a sulfonate group to the acceptor steroid and use a universal sulfonate donor (3′-phosphoadenosine 5′-phosphosulfate or PAPS) to carry out this reaction [22]. The sulfonation of steroids typically reduces or eliminates the ability of the steroid to bind its respective steroid receptor [23], while also decreasing the hydrophobicity of the steroid to facilitate clearance [21]. Recent work in reptiles [24–27] and birds [28–30] has demonstrated that in ovo metabolism of maternal steroids occurs. Characterization of this metabolism in the red-eared slider turtle (Trachemys scripta) demonstrates that oestriadiol is converted to a variety of oestrogen sulfates (primarily oestrone sulfate) [24,26,27,31] through sulfonation. Interestingly, sulfotransferase enzymes not only can be subjected to inhibition by EDCs [32,33], but are also vital to the metabolism and clearance of exogenous chemicals such as those that act as EDCs [34], creating a situation where there is a shared metabolic fate for both maternal steroids and EDCs. Given that both endogenous steroids and EDCs use sulfonation for clearance, it is possible that the presence of an EDC might affect the normal clearance of oestriadiol.

Here, we investigate the effect of the EDC, bisphenol-A (BPA), on the metabolism of maternal oestriadiol in T. scripta. BPA is a commonly used plasticizer that is one of the most mass-produced chemicals in the world with an essentially ubiquitous presence in the environment ([35], reviewed in [36]). It has been found around the world in human [36,37] and non-human animals alike, and is known to have oestrogenic effects on developing organisms across vertebrate and invertebrate taxa (reviewed in [38–41]). Although BPA has been shown to bind to both ERα [15,42] and ERβ [42], the binding affinity for BPA is up to 12,500-fold lower than for oestriadiol [43]. Importantly, it has been shown that BPA is subject to sulfonation [44,45] and can also inhibit the sulfonation of oestriadiol in vitro [18]. These characteristics make BPA an ideal compound for investigating the potential for EDCs to produce oestrogenic effects by inhibiting the natural metabolism of maternal oestriadiol.

2. Material and methods

(a) Egg collection

In this species, there is consistent variation in levels of maternal oestriadiol with concentrations in the eggs from a female’s second clutch of the nesting season being approximately 10-fold higher than those from her first clutch of the season [25]. In both first and second clutches, these concentrations decline to undetectable levels early in development [25]. For this study, eggs were collected at Banner Marsh State Fish and Wildlife Area in Fulton Co., IL, USA [24,25] either from freshly laid nests in the field or from gravid females that were caught in baited hoop traps and transported to Illinois State University where egg laying was induced by injection of oxytocin [46,47]. All adult trapping, handling and egg collection methods were approved by the Illinois Department of Natural Resources Illinois (IDNR permit NH11-2084) and the Illinois State University Institutional Animal Care and Use Committee (04-2010).

(b) Effect of bisphenol-A in ovo oestriadiol metabolism

To determine the effect of BPA on the early in ovo decline of oestriadiol in T. scripta, seven clutches were collected and eggs from each clutch were randomly allocated to one of two treatments: control eggs ¼ 0.1 μg oestriadiol in 5 μl vehicle (70% ethanol), or BPA-treated eggs ¼ 0.1 μg oestriadiol plus 40 μg BPA in 5 μl vehicle. For oestriadiol, the dose was selected to elevate levels of oestriadiol in the early season clutches to levels found in late season clutches [25]. For BPA, we performed a pilot study (data not shown), basing our initial doses on those used in Stoker et al. [48]; we determined that 40 μg of BPA, given on day 0 of incubation, did not result in significant egg mortality in T. scripta. Treatment occurred within 24 h of the start of chalking, a sign that the egg contains a viable embryo that has begun to develop. For this study, only clutches (n ¼ 5) in which all eggs began chalking within the first 24 h after laying were used to control timing of treatment. Within each treatment, eggs from each clutch were randomly assigned to a sampling day either 1, 3, 6 or 9 days post-treatment. Eggs (n ¼ 4–5 per sampling day and treatment) were incubated at 27°C in moist vermiculite (approx. −150 kPa) until sampling, at which point the eggs were frozen at −20°C until analysis. For analysis, each egg was dissected and components (yolk and albumen/fluid) were separated and weighed to the nearest 0.01 g.

Levels of oestrogens in the yolk were measured using radioimmunoassay (RIA) (modified from [49,50]). Oestriadiol and oestrone were measured in independent assays with slightly modified extraction techniques. For oestriadiol, 50 mg of yolk was diluted and homogenized in 1 ml distilled water. Two thousand cpm of tritiated oestriadiol (Perkin Elmer, Waltham, MA, USA) was added to each sample to allow for the calculation of percent recovery. Steroids were extracted with ether (30:70, petroleum ether:d.i.e. ether) producing two phases: an aqueous phase and an organic phase, which contain the water-soluble and lipid-soluble molecules, respectively. The organic phase, containing any free steroids present in the sample, was then dried down under nitrogen gas, resuspended in 1 ml of 90% ethanol, vortexed and held at −20°C overnight. The samples were then centrifuged at 2000 r.p.m. for 5 min at 0°C to remove neutral lipids and proteins. The supernatant was
poured off, dried down under nitrogen gas and resuspended in 10% ethyl acetate in isooctane and loaded onto celite-packed columns. For oestrone, 100 mg of yolk was diluted and homogenized in 200 μl of distilled water and 2000 μml of tritiated oestriol (Perkin Elmer) was added to calculate recoveries. To precipitate proteins and neutral lipids, 1 ml of methanol was added and samples were held at −20°C overnight. The samples were centrifuged at 2000 r.p.m. for 15 min and the supernatant was decanted. An additional 1 ml of methanol was added, and the samples were centrifuged again, and the supernatants were combined and dried under nitrogen. The dried sample was resuspended in 1 ml of distilled water and 2 ml of diethyl ether was added, producing an aqueous phase and an organic phase, respectively. The extraction was repeated, the organic phases combined and dried under nitrogen gas, and resuspended in 10% ethyl acetate in isooctane loaded onto celite-packed columns.

Both steroids were eluted with solutions containing progressively higher concentrations of ethyl acetate in isooctane (0, 10, 20 and 40%). Oestrone was isolated with the 20% fraction, whereas oestradiol was isolated with the 40% fraction. Duplicate aliquots of each steroid were subjected to RIA using specific antibodies (oestrone antibody E3135, Sigma, USA; oestradiol antibody 7010, Biogenesis, UK). To quantify endogenous oestrogens, a standard curve (range: 1.95–500 pg) was used for comparison. Oestrone was analysed in a single assay, with an intra-assay coefficient of variation (CV) of 4%. Oestradiol was analysed across two assays, with intra-assay CVs of 13 and 20%, and an inter-assay CV of 23%.

Oestrone sulfate, oestradiol sulfate and oestradiol sulfate were quantified using liquid chromatography–mass spectrometry (LC/MS/MS). Samples were prepared by vortexing 1 ml of albumen with 3 ml of methanol (HPLC grade), frozen overnight and centrifuged (2000 r.p.m.) at 0°C for 20 min. The supernatant was poured off and kept at −20°C. A second methanol extraction was performed on the pellet with 2 ml of methanol following the same protocol. Supernatants from both rounds were consolidated, dried down under nitrogen gas and resuspended in 5 ml nano-pure water. Water-soluble metabolites were extracted using Sep-pak cartridges (C18 plus short cartridge, 55–105 μm particle size; Waters, Milford, MA, USA). For each sample, two Sep-pak cartridges were stacked and charged with 5 ml methanol followed by 5 ml nano-pure water. The samples were then loaded onto the cartridges. Lipid-soluble molecules were eluted with 5 ml ether followed by elution of the water-soluble metabolites with 5 ml methanol. The methanol eluate was then dried down under nitrogen gas and metabolites characterized using LC/MS/MS.

The LC/MS/MS analysis was performed in the Metabolomics Center at University of Illinois at Urbana–Champaign with a 5500 QTRAP mass spectrometer (AB Sciex, Foster City, CA, USA), that is equipped with a 1200 Agilent HPLC. ANALYST (v. 1.5.1, Applied Biosystems) was used for data acquisition and processing. The HPLC flow rate was set at 0.35 ml min⁻¹. HPLC mobile phases consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The autosampler was kept at 5°C. The injection volume was 5 μl. An Agilent Zorbax SB-Aq column (3.5 μm, 50 × 4.6 mm) was used for the separation with the following gradient: 0–2 min, 80% A; 2–10 min, 25% A; 11–16.5 min, 80% A. Mass spectrometer was operated under negative mode electrospray ionization. The electrospray voltage was set to −4500 V, the heater was set at 600°C, the curtain gas was 35, and GS1 and GS2 were 50, 65, respectively. Quantitative analysis was performed via MRM where m/z 349.1 → m/z 269.1 (3-OL-17-one; oestrone sulfate); m/z 351.1 → m/z 271.1 (17-β-oestradiol; oestradiol sulfate) and m/z 367.1 → m/z 287.1 (16-a-17-β-oestriol; oestrone sulfate) were monitored.

(c) Statistical analyses

Levels of oestrone, oestradiol and oestrone sulfate were analysed by ANOVA in PROC GLM (SAS 9.3 SAS Institute, Cary, NC, USA). For the ANOVAs, treatment (control or BPA treated), sampling day (1, 3, 6 or 9) and their interaction were included as fixed factors in the model. Clutch of origin was included as a random factor. We ran contrasts to probe any significant interactions. Levels of oestradiol sulfate were low to undetectable, while oestrone sulfate levels were below detection limits in all samples; neither was included in the final analyses. To meet normality assumptions of ANOVA, all steroids were log(10) transformed prior to analysis.

3. Results

Mean levels of oestradiol, oestrone and oestrone sulfate in eggs (n = 1 per clutch) sampled prior to any treatment were 2.7, 0.10 and 5.6 ng g⁻¹, respectively. Levels of oestradiol in the yolk were significantly affected by treatment, with eggs from the BPA treatment group having elevated levels of yolk oestradiol relative to those from the control treatment (F1,1 = 11.10, p = 0.003; figure 1a). There were no significant effects of either day (F1,3 = 2.50, p = 0.085), or the interaction of treatment × day (F1,3 = 0.19, p = 0.90) on yolk oestradiol.
control

\[ E_2 \rightarrow E_1 \rightarrow E_1S \]

BPA treated

\[ E_2 \rightarrow E_1 \times \rightarrow E_1S \]

**Figure 2.** Schematic of how steroid metabolism is altered during the first nine days of embryonic development in *T. scripta* when eggs are exposed to the endocrine disruptor bisphenol-A. In the control eggs, oestradiol (E2) is converted to oestrone (E1), and then to oestrone sulfate (E1S) which is present at high levels in the egg. Eggs treated with BPA maintained higher levels of both E2 and E1, with little to no E1S being formed. Our data indicate that BPA interferes with the metabolic conversion of E1 to E1S in ovo.

However, we did detect a significant cluch effect on yolk oestradiol levels ($F_{1,4} = 18.16, p < 0.0001$). Levels of oestrone in the yolk were also significantly affected by treatment, with eggs from the BPA treatment group having elevated levels of yolk oestrone relative to those from the control treatment ($F_{1,1} = 6.86, p = 0.015$; figure 1b). Yolk oestrone levels varied by sampling day ($F_{1,3} = 12.06, p < 0.0001$), but there was no significant interaction detected ($F_{1,3} = 0.09, p = 0.97$). Levels of oestrone sulfate in the albumen varied significantly by treatment group ($F_{1,3} = 19.30, p = 0.0002$) and by day ($F_{1,3} = 54.55, p < 0.0001$). There was a significant treatment × day interaction ($F_{1,3} = 4.94, p = 0.0866$; figure 1c). Probing this interaction, we found that the treatment groups varied on day 1 ($p < 0.0001$) and day 6 ($p = 0.006$), but not on days 3 and 9 ($p > 0.05$). There were no clutch effects detected for either oestrone or oestrone sulfate. Data used in these analyses are available as the electronic supplementary material.

### 4. Discussion

Here, we demonstrate that BPA alters the metabolism of oestrogens in ovo. We found that levels of both oestradiol and oestrone were higher, whereas levels of oestrone sulfate were lower, in eggs treated with BPA when compared with the control eggs. The low levels of oestrone sulfate paired with the elevated levels of free oestrogens indicate that oestrogen sulfotransferase activity is altered in the presence of BPA in *T. scripta* (figure 2). Because, however, there are several metabolic conversions required to produce oestrone sulfate from oestradiol, it is also possible that BPA could be affecting other steps in this process. For example, BPA could alter the conversion of oestradiol to oestrone instead of affecting the sulfonation of oestrone itself, thus producing the lower oestrone sulfate levels observed in the BPA-treated group. If the conversion from oestradiol to oestrone was the main pathway inhibited, we would have expected to see decreased levels of oestrone in the BPA-treated eggs. In fact, we found just the opposite pattern, indicating that BPA is inhibiting the conversion of oestrone to oestrone sulfate. Our data support the hypothesis that EDCs, for instance BPA, can produce their effects by increasing the bioavailability of active steroids during critical periods of development by effectively interfering with the metabolism of maternal steroids.

The ability of EDCs to inhibit the sulfonation of oestrogens has been demonstrated in a number of *in vitro* systems ([18,19], reviewed in [51]), but to our knowledge, this is the first time it has been shown that the sulfonation of maternal oestrogens is subject to inhibition by an EDC *in ovo*. In both placental and oviparous vertebrates, the sulfonation of steroids is thought to serve as a buffer that protects the embryo from being exposed to the high levels of maternal oestrogens [20,24]. The presence of increased levels of free oestradiol and oestrone in the yolks of BPA-treated eggs suggests that inhibiting sulfotransferase activity may lead to increased levels of active steroid that is potentially available for signalling within the egg.

Demonstrating that the sulfonation of maternal oestrogens is subject to inhibition by BPA has important implications for developing vertebrate embryos. Previous work has demonstrated that EDCs can be metabolized through the same enzymatic pathways as steroids, including sulfonation [34], and our data indicate that the presence of BPA results in the inhibition of oestrogen metabolism. At present, it is unclear if the observed inhibition is the result of competitive inhibition of BPA for access to the sulfotransferase enzyme, PAPS depletion, or if non-competitive or allosteric inhibition may be taking place. Regardless, if embryos are simultaneously exposed to both maternal oestrogens and exogenous compounds that can be metabolized through the sulfotransferase pathway, it appears that they may not be able to adequately remove all active compounds in a timely manner, ultimately producing effects on the offspring’s phenotype.

#### (a) EDC effects in vertebrates

EDCs have been shown to elicit a wide variety of effects from the ‘classic’ feminizing effects of DDT on gull embryos [52] to more recent work demonstrating transgenerational epigenetic effects (reviewed in [53]). Interestingly, many effects are observed when embryos are exposed to low doses of the compounds, and the effects of the EDC can be influenced by endogenous steroid levels. For example, fetal mice with higher levels of endogenous oestradiol responded to exposure to a low dose of BPA *in utero*, whereas those with low endogenous oestradiol levels did not [54]. It is possible, then, that even EDC exposures considered to be ‘low doses’, may be high enough relative to the available enzyme to produce altered steroid metabolism, resulting in the inhibition of sulfotransferase activity toward endogenous and exogenous compounds alike, which may account for the low-dose effects often observed (see [41] for discussion of dose–response curves for BPA).

This is the first study, to our knowledge, that demonstrates alteration of steroid metabolism by an EDC *in ovo*. While *in vitro* data provides evidence for the potential of an EDC to inhibit steroid metabolism, the *in vitro* approach may lack the feedback and compensatory mechanisms that may otherwise prevent alteration *in vivo*. By demonstrating alteration in a closed and complete developmental environment, we provide support for altered steroid metabolism as a mechanism of endocrine disruption in the whole organism. This altered steroid metabolism could produce inappropriate signalling by endogenous compounds, potentially producing or contributing to the known effects of EDCs on tissue differentiation. Although we have only discussed oestrogens here, the implications of this research may extend to any endogenous signalling molecules that use similarly promiscuous conjugation pathways. The relative promiscuity of conjugative enzymes as compared to steroid receptors suggests that altered steroid metabolism has the potential to be a more universal mechanism across...
structurally varied EDCs and to better account for low-dose effects than the traditional receptor-mediated mechanism.

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