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# Differential Modulation by Aromatic Hydrocarbon Receptor Agonist of Circulating Estradiol-17 $\beta$ and Estrogen-Receptor DNA-binding Capability in Female Rainbow Trout (*Oncorhynchus mykiss*)

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**ABSTRACT**—We report alterations in serum estradiol-17 $\beta$  (E<sub>2</sub>) and hepatic estrogen receptor (ER) DNA-binding activity in female rainbow trout given a single ip injection of 0, 6.25, 12.5, 25, or 50 mg  $\beta$ -naphthoflavone (BNF)/kg body weight, followed by sacrifice at 24 or 48 hr. BNF affected E<sub>2</sub> in a dichotomous fashion at 24 hr, and reduced E<sub>2</sub> at 48 hr with increasing BNF. DNA binding by ER was decreased in an apparently dose-dependent manner after 24 hr and increased after 48 hr. These data suggest that the regulation of ER by AHR agonists may occur both at the ER promoter, and also secondarily via E<sub>2</sub>, and that the differential effects observed are both time and dose dependent.

## INTRODUCTION

Certain aromatic hydrocarbons (AH), the most toxic of which is the environmental pollutant 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD), can behave as potent endocrine disruptors in most vertebrate species studied to date (Cooper *et al.*, 1997). Rainbow trout appear to be especially sensitive to aromatic hydrocarbon receptor (AHR) agonists (Thomas, 1990; Anderson *et al.*, 1996). In general, the actions of AHR agonists are thought to be antiestrogenic (Safe, 1997) or at least to modulate the estrogen-signaling pathway (Chaffin *et al.*, 1996) in mammalian systems as measured by a reduction in both circulating E<sub>2</sub> and DNA-binding capability of hepatic estrogen receptor (ER). Potential mechanisms include direct regulation at the level of the ER gene, or via secondary endocrine changes (e.g., in serum E<sub>2</sub> concentrations). Herein we describe the effects of acute administration of a less-toxic AHR agonist,  $\beta$ -naphthoflavone (BNF), on circulating E<sub>2</sub> in rainbow trout and on the E<sub>2</sub>-signaling pathway as determined by ER DNA-binding activity using gel electrophoretic mobility-shift assay to estimate active ER present.

## MATERIALS AND METHODS

### Animals and experimental manipulations

This study was approved by the University of Wisconsin-Milwaukee Animal Care and Use Committee. Eight rainbow trout (*Oncorhynchus mykiss*) per group were food deprived for the duration of this study; and housed 8 fish per 50-gal tank, at 3–4°C, at a 12L:12D light cycle in environmental rooms. Water flow rate was 1–1 1/2 L/min. The study was performed twice.

Fish, (sexed post-mortem; 150–350 g; Ennis National Fish Hatchery, Ennis, MT, USA) were sedated with MS222, weighed, and given a single intraperitoneal (ip) injection (site: ventral aspect, anterior to the pelvic fins) of BNF (Sigma Chemical Co., St. Louis, MO, USA; 90–95% purity by manufacturer; micronized by sonication for 5–10 min) in olive oil (0, 6.25, 12.5, 25, or 50 mg/kg) or olive oil alone as vehicle control. One to two ml of blood was taken from the caudal vein posterior to the anal fin under MS222 anesthesia. Sera were frozen for subsequent radioimmunoassay for estradiol-17 $\beta$ , and validated by demonstrating parallelism between increasing aliquots of fish serum and authentic standards, as done for other species (Hutz *et al.*, 1990).

### Gel-mobility shift assay

Following sacrifice under MS222 overdose at 24 or 48 hr after BNF, the livers were removed and immediately frozen at –80°C for whole cell protein extraction according to the method described by Bettini *et al.* (Bettini *et al.*, 1992). For the gel-shift assay, 20 mg of whole-cell protein extract, as measured by the Bradford assay (Bradford, 1976) was incubated with 80 fmol of <sup>32</sup>P-labeled oligonucleotides containing the estrogen response element (ERE) (see below) and 0.7 mg poly dI-dC (to control for non-specific binding), with or without 200-fold molar excess of non-radiolabeled ERE as specific competitor for 30 min at room temperature. Presence of the unlabeled, excess ERE reduced signal to that of background. The gel-shift thereby reflected true ER/ERE complexes. Protein-DNA complexes were resolved on an 8% non-denaturing acrylamide gel fol-

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lowed by autoradiography. Shifted bands were reduced to background levels with the addition of the excess unlabeled ERE (see Fig. 3). Oligonucleotides were commercially synthesized by Biosynth, Inc. (Denton, TX, USA). The canonical, palindromic ERE was from the *Xenopus* vitellogenin promoter (5'-CAAAGTCAGGTCACAGTGACCTGATCAAA-3'). The cognate response element is in bold face. Single-stranded oligonucleotides were end-labeled with  $^{32}$ P-dATP using T4 polynucleotide kinase and annealed to form double-stranded response elements with the complementary strand. A small portion of the liver (300 mg) from each fish was also used for RNA extraction with the Trizol reagent (GIBCO/BRL Life Technologies, Gaithersburg, MD, USA) for other studies. The shifted bands generated from the autoradiography of the acrylamide gels were quantified on a densitometer, using an Optimas image analysis system (Bioscan, Inc., Edmonds, WA, USA) and expressed as relative optical density (O.D.) units (Chaffin *et al.*, 1996).

### Histology

Gonads were analyzed under a dissecting microscope at necropsy to determine gender; those gonads characterized as sexually indeterminate were fixed in 4% formalin, cleared in 70% ethanol, and processed in an automatic tissue processor through a graded series of ethanol, and then infiltrated with xylenes and paraffin. Sections were cut at 10  $\mu$ m, deparaffinized, and re-hydrated, stained with hematoxylin and eosin, cover slipped and analyzed microscopically for evidence of ovarian or testicular tissue before assignment of fish

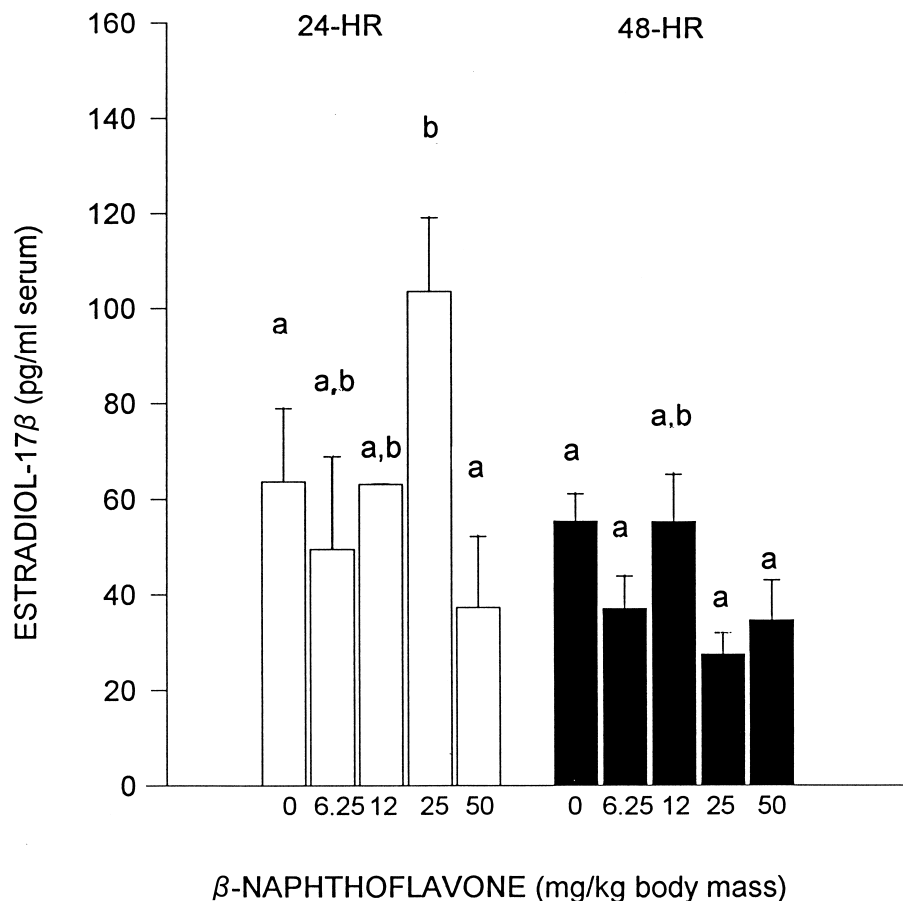
as to gender. Only data from female fish were used. Ovaries were exclusively immature, with oocyte < 0.5 mm in diameter, and at the pre- and vitellogenic stages (Weil *et al.*, 1995).

### Statistical analysis

Completely independent two-way ANOVA was performed with dose and time as the two factors; and Student-Newman-Keul's multiple-range test was used to make multiple comparisons among all groups (after log [X + 1] transformation when heteroscedasticity was present) (SPSS-X; SPSS, Inc., Chicago, IL, USA). Student's T-test after transformation was also used for the comparison of two independent groups (Zar, 1974). Sigmaplot (Jandel Scientific, San Rafael, CA, USA) was used for graphics presentation.  $P < 0.05$  was considered to be significant. Any males were deleted from the analysis.

## RESULTS

Using ANOVA, we did not observe a classical dose-dependent effect of BNF on circulating concentrations of estradiol-17 $\beta$  in female *O. mykiss* at 24 or 48 hr (Fig. 1), rather a dichotomous effect. There was a significant increase in  $E_2$  between 0 and 25 mg doses of BNF, and then a reduction between 25 and 50 mg doses of BNF (Fig. 1). Importantly, there was a significant difference between control and the 25-



**Fig. 1.** Circulating estradiol-17 $\beta$  concentrations in adult female *O. mykiss* (mean  $\pm$  S.E.M.) administered a single graded dose of  $\beta$ -naphthoflavone [(BNF); mg/kg] and sacrificed 24 or 48 hr later. <sup>a,b,c,d</sup> Superscripts without a common letter denote significance ( $p < 0.05$  by two-way ANOVA).

mg dose, and between the 12 mg and 25 mg BNF/kg body mass doses at 48 hr (Fig. 1;  $p < 0.05$  by T-test only). The circulating estrogen concentration at 24 hr in the 25-mg dose group was also significantly higher than four of the five groups at 48 hr using ANOVA (Fig. 1). There was a significant effect of time ( $p < .003$ ) with a reduction at 48 hr vs 24 hr ( $p < 0.05$ ). There was no interaction by ANOVA between the main effects of dose and time.

There was a 50% decrease in mean circulating  $E_2$  when comparing all female trout at 24 hr regardless of dose {67.7  $\pm$  9.1 [15] at 24 hr vs. 42.4  $\pm$  4.1 pg/ml [18]} at 48 hr (T-test,  $p < 0.05$ ).

As control groups showed the same concentrations of  $E_2$  at 24 hr {63.57  $\pm$  15.37 [4] vs. 55.42  $\pm$  5.67 pg/ml [4] at 48 hr}, we also evaluated controls relative to the rest of the treatments at each time. At 24 hr there was no effect of BNF on circulating  $E_2$  {63.6  $\pm$  15.4 [4] vs. 69.2  $\pm$  11.5 [11]}. But at 48 hr, BNF significantly reduced circulating  $E_2$  {55.42  $\pm$  5.67 pg/ml controls [4] vs. 37.47  $\pm$  4.46 treated [15],  $p < 0.05$ ).

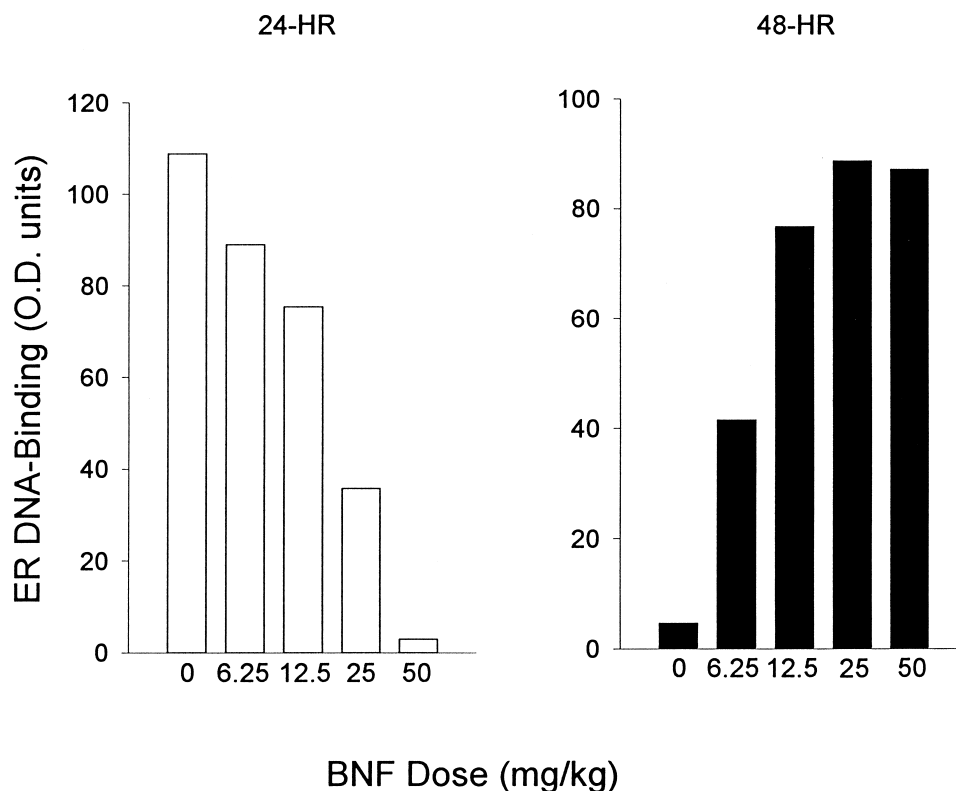
Twenty-four hours after injection with BNF, hepatic ER DNA-binding activity was reduced in a dose-dependent manner (Fig. 2), while after 48 hr, ER DNA-binding was increased (Fig. 2 and 3).

## DISCUSSION

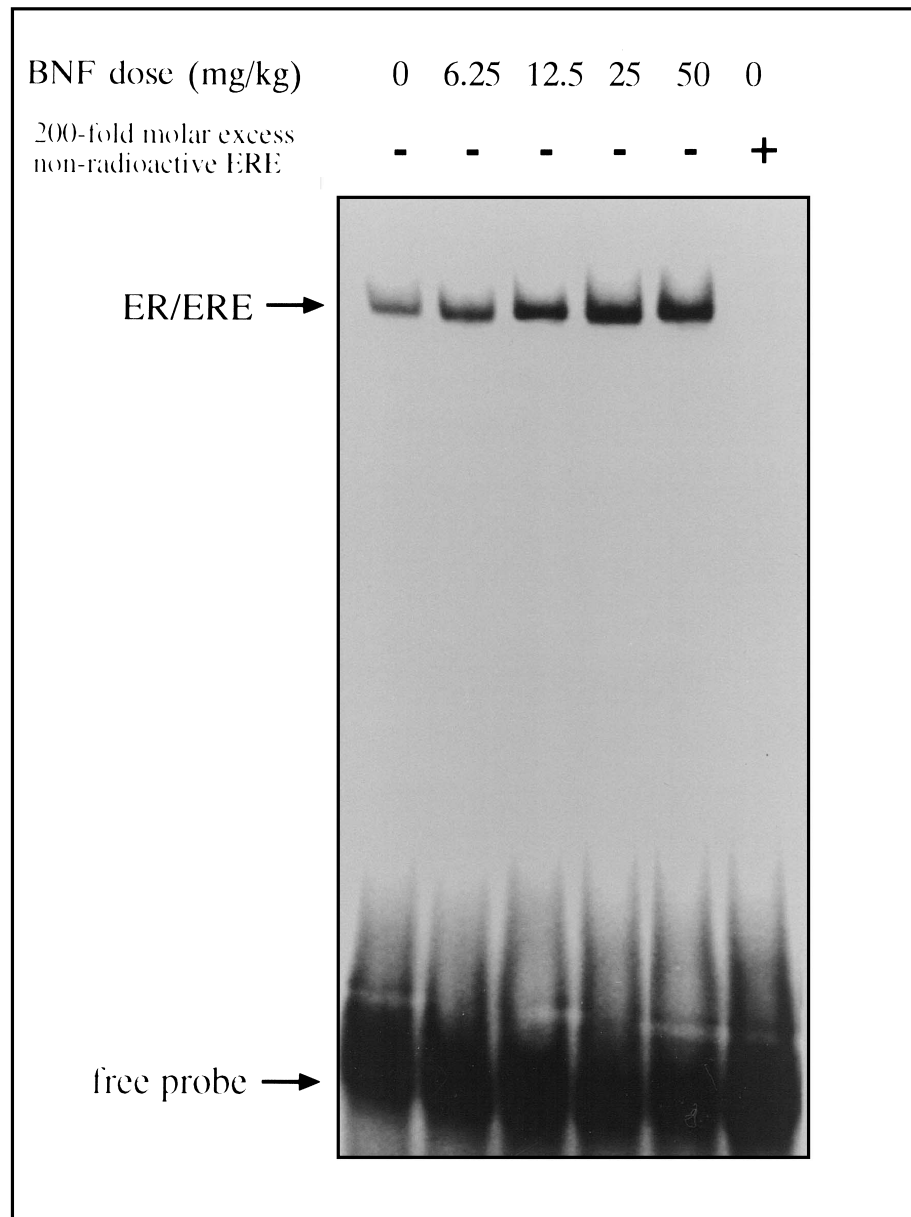
Our present model for evaluating TCDD activation of its aromatic hydrocarbon receptor (AHR) is to use a less-toxic AHR-receptor agonist,  $\beta$ -naphthoflavone (BNF), for our *in vivo* aquatic studies. The reason for our interest in AHR agonists is the apparent receptor cross-talk observed between the AHR- and ER-signaling pathways. This interaction may be responsible for the changes we have seen in reproductive parameters such as ER mRNA, circulating  $E_2$ , etc., in other paradigms (Chaffin *et al.*, 1996).

Circulating  $E_2$  concentrations in the female *O. mykiss* were reduced in general at the later time relative to the 24-hr time period, at certain doses, which suggests a threshold effect. The effect of dose at 24 hr, at 25 mg and 50 mg/kg was replicated with identical results. We believe therefore that the results are real and physiologic with respect to effect, and that the presence of BNF has the effect of reducing  $E_2$  with time relative to vehicle-treated controls. Certainly, AHR agonists have been shown to generally modulate expression of estrogen-sensitive biomarkers.

Since fish liver and other organ systems synthesize the estrogen-inducible egg yolk precursor protein vitellogenin, which is necessary for oocyte maturation and ovarian development, this is one such important estrogenic endpoint (Lee



**Fig. 2.** Estrogen receptor DNA-binding activity in *O. mykiss* administered a single graded dose of  $\beta$ -naphthoflavone [(BNF) mg/kg] and sacrificed 24 or 48 hr later. Gel mobility assays were performed using an estrogen response element from the *Xenopus* vitellogenin A2 gene promotor and 20 mg of whole cell liver protein extracts. The bands generated from the autoradiography of the acrylamide gels were quantified on a densitometer, using an Optimas image analysis system and expressed as relative optical density (O.D.) units.



**Fig. 3.** Photograph of a gel-shift autoradiogram. DNA response element (ERE)/estrogen receptor binding was observed in liver protein extracts from *O. mykiss* given a single graded dose of  $\beta$ -naphthoflavone (BNF) and sacrificed 48 hr later. Note the increase in intensity of bands with increasing dose of BNF, and complete band absence with competing, unlabeled ERE.

*et al.*, 1996). Dioxins and BNF reduced vitellogenin synthesis in fish primary hepatocyte cultures (Anderson *et al.*, 1996). Haasch *et al.* (Haasch *et al.*, 1989) demonstrated a negative correlation between *cyp1A1*-inducing capability and antiestrogenicity; this fits with our results in which presumed greater CYP1A1 activity in response to AHR agonist could be expected with increasing BNF dosage and time. The inhibition of circulating  $E_2$  at 48 hr [also observed by Anderson *et al.*, (1996) may be due to glucuronid conjugation (phase II) of  $E_2$  as has been shown in *O. mykiss* *in vivo* after exposure to BNF (Forlin and Haax, 1985)]. Chronic dietary exposure to the CYP1A1-inducing compound Arachlor 1254 reduced the

plasma vitellogenin response by *O. mykiss* to  $E_2$  (Chen *et al.*, 1986). Atlantic croakers showed reduced circulating  $E_2$  and impaired gonadal function in general due to AHR agonists (Thomas, 1990). BNF, over the dose range 0.5-5  $\mu$ M, was also recently shown to reduce significantly estradiol production (without affecting testosterone production) by ovarian follicles taken from coho salmon and cultured *in vitro* for 18 h (Afonso *et al.*, 1997). Our dose range encompasses these doses, and extends them ten-fold higher. Collectively, the observed effects may have been due to direct steroidogenic impairment at the ovary (*e.g.*, inhibition of aromatase) or disruption of the hypothalamic-pituitary-gonadal axis.

Regardless of the locus of BNF effect, the decrease observed in circulating  $E_2$  with time may be due to actual diminished synthesis/secretion at the ovary (Hutz and Gold, 1987), *i.e.*, a possible direct effect of BNF on aromatase; or preferential metabolism to some other form that does not cross-react in our highly specific radioimmunoassay—such as 2- or 4-OH-estrone (*i.e.*, catecholestrogens), a known consequence of AHR agonist effect in some systems (Safe, 1997). This change in peripheral  $E_2$  typically modulates ER site number (Bethea *et al.*, 1996). The dichotomous change we observed in circulating  $E_2$  with two highest doses is typical of many hormonally regulated systems (Bethea *et al.*, 1996; Hutz and Gold, 1987).

Twenty four hr after exposure to BNF, we observed a diminution in ER DNA-binding activity (a presumed down-regulation in receptor) with increasing dosage of BNF, whereas with longer exposure, there was an apparent increase (Fig. 2). This apparent time-dependent regulation leads us to hypothesize that the initial decrease in ER DNA binding may be due to a direct effect of AHR agonists on the upstream regions of the ER gene (Piva *et al.*, 1992), due to the presumed rapidity of the ER gel-shift change. Increases seen in *cyp1A1* (Haasch *et al.*, 1989) and decreased ER appear to be direct. Long-term BNF treatment in contrast diminished *cyp1A1* activity possibly causing ER to upregulate (Haasch *et al.*, 1989).

The increased ER DNA binding observed in the present study 48 hr after exposure is suspected to be due to the BNF-induced reduction in serum estradiol-17 $\beta$  concentrations seen at 48 hr, and then possibly causing an upregulation of ER expression [seen with low concentrations of hormones over time (Bethea *et al.*, 1996)]. There may be a direct effect of BNF on ovarian steroid secretion, thereby causing the characteristic increase in DNA-binding capability; this might allow functional receptor and presumed downstream bioactivity to compensate for diminished sensitivity. Therefore, with time, circulating  $E_2$  appears to be affected by this AHR agonist. [Interestingly, there was no dose-dependent alteration in AHR DNA binding with BNF in this paradigm (Chaffin and Hutz, unpublished observations)]. Collectively, these data suggest that AHR regulation of ER, at least in this model, may be dose dependent, although this does not hold true classically for circulating  $E_2$ . This demonstrates the importance of exposure dose, duration, specific endpoint and presumably other parameters in study outcomes; and may be relevant in terms of the marked discrepancies observed among laboratories pursuing the issue of ER regulation by AHR agonists.

In conclusion, (1) serum estrogen concentrations appeared to be affected by BNF in a time-dependent manner, but  $E_2$  changes were not shown to be dose dependent in a classical sense, but rather showed dichotomous effects dependent upon dose; and (2) 24 hr after exposure to BNF, hepatic ER DNA-binding activity was reduced in a dose-dependent manner, while after 48 hr, ER DNA binding was increased, showing another dichotomous effect. These results suggest that multiple mechanisms may be responsible for alterations in hepatic ER. We hypothesize that regulation of ER by BNF

may be directly at the ER gene promoter via the estrogen-response element, or that ER is regulated secondarily by changes in serum estrogen. Importantly, it must be recognized that AHR agonists such as TCDD and BNF may not be purely antiestrogenic, but rather estrogen modulatory and dependent upon dose, time, species, tissue, endpoint, *etc.*

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