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An Aryl Hydrocarbon Receptor Agonist Amplifies the Mitogenic Actions of Estradiol in Granulosa Cells: Evidence of Involvement of the Cognate Receptors[1]

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ABSTRACT

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that, besides mediating toxic responses, may have a central role in ovarian physiology. Studying the actions of AHR ligands on granulosa cells function, we have found that beta-naphthoflavone amplifies the comitogenic actions of FSH and 17beta-estradiol in a dose-dependent manner. This amplification was even greater in cells that overexpress the AHR and was reversed by cotreatment with the AHR antagonist alpha-naphthoflavone, suggesting that this effect is mediated by the AHR. The estrogen receptor is likewise implicated in this phenomenon, because a pure antiestrogen abolished the described synergism. However, the more traditional inhibitory AHR-estrogen receptor interaction was observed on the estrogen response element-driven transcriptional activity. On the other hand, alpha-naphthoflavone inhibited dose-dependently the mitogenic actions of FSH and 17beta-estradiol. Beta-naphthoflavone induced the expression of *Cyp1a1* and *Cyp1b1* transcripts, two well-characterized AHR-inducible genes that code for hydroxylases that metabolize estradiol to catecholestrogens. Nevertheless, the positive effect of beta-naphthoflavone on proliferation was not caused by increased metabolism of estradiol to catecholestrogens, because these compounds inhibited the hormonally stimulated DNA synthesis. This latter inhibition exerted by catecholestrogens suggests that these hydroxylases would play a regulatory point in granulosa cell proliferation. Our study indicates that AHR ligands modulate the proliferation of rat granulosa cells, and demonstrates for the first time that an agonist of this receptor is able to amplify the comitogenic action of classical hormones through a mechanism that might implicate a positive cross-talk between the AHR and the estrogen receptor pathways.

estradiol, estradiol receptor, granulosa cells, ovary, toxicology

INTRODUCTION

The aryl hydrocarbon receptor (AHR) is a ligand-activated basic-helix-loop-helix-PAS (bHLH-PAS) transcription factor that mediates a variety of toxic and biological effects of different classes of aromatic compounds: halogenated aromatics, e.g., 2,3,7,8-tetrachlorodibenzeno-*p*-dioxin (TCDD); polycylic aromatic hydrocarbons (PAHs), e.g., 3-methylcholanthrene (3-MC); and heteropolynuclear aromatic hydrocarbons, e.g., β-naphthoflavone (5,6-benzophlavone) and α-naphthoflavone (7,8-benzophlavone). This receptor and homologues are widely and ubiquitously expressed in diverse tissues throughout different groups of vertebrates and invertebrates (reviewed in [1, 2]).

Upon binding agonists, the AHR translocates from the cytoplasm to the nucleus, where it forms a heterodimer with another bHLH-PAS protein known as the aryl hydrocarbon nuclear translocator (ARNT). This heterodimeric complex binds to cognate DNA sequences, known as xenobiotic response elements, and upregulates the transcription of a battery of xenobiotic metabolizing enzymes (XMEs). These regulated XMEs include the cytochrome P450s CYP1A1, CYP1B1, and CYP1A2 and the phase II enzymes GSTA5 and UGT1A1 (reviewed in [3–6]).

The environmental contaminant TCDD is the most potent congener among these aromatic compounds and the prototype of these receptor agonists. Its effect and the involvement of the AHR in acute toxicity and teratogenesis have been well studied (reviewed in [7]). Besides, AHR agonists have been extensively investigated as antiestrogens in the rodent mammary gland and uterus and in human breast cancer cell lines [8], this effect being complex and related to the inhibitory cross-talk between the AHR and the estrogen receptor [4, 9–11]. The role of the AHR in carcinogenesis has also been studied and discussed (reviewed in [11]).

Several reports have shown constitutive activation of the AHR in the absence of an exogenous ligand, suggesting that the AHR may play important roles not only in the regulation of xenobiotic metabolism but also in the maintenance of homeostatic function [12–15]. It has been demonstrated that the absence of this receptor produces, among other effects, impairment in liver development (reviewed in [7]), reduced incidence of blastocyst formation, and smaller mean cell number in cultured embryos [16]. In addition, it has been demonstrated that the AHR plays a role in cell cycle regulation in some systems (reviewed in [17]).

The presence of the AHR has been described in oocytes and granulosa cells in the rat ovarian follicles [18]. Deletion of this receptor produces changes in follicular selection processes, apparently caused by alterations in growth of preantral and antral follicles, and alterations in embryonic implantation in animals lacking this receptor have also been described [19–21].

With the aim of studying the possible modulation that the AHR may exert on granulosa cells function, we investigated in the present study the role of AHR ligands on the growth of these cells. The effects of two aromatic hydrocarbons (β-naphthoflavone and α-naphthoflavone) that have been described as agonist and antagonist of this receptor in a variety of systems [22–30] were tested in a defined culture system of rat granulosa cells that has been extensively characterized in terms of hormonally regulated cell proliferation [31].

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MATERIALS AND METHODS

Hormones and Chemicals

Ovine FSH (NIDDK-oFSH-20) was obtained from the National Hormone and Pituitary Program. Two-hydroxyestradiol and 2-methoxyestradiol were purchased from Steraloids, Inc., ICI 182,780 was purchased from Tocris, and [*methyl*-^{3}H]-thymidine from Dupont NEN Research Products. Tissue culture reagents, 17β-estradiol (estradiol), and all other chemicals were obtained from Sigma Chemical Co., unless otherwise indicated. Collagen was prepared from rat tails as previously described [32].

Granulosa Cell Preparation and Culture

Ovaries were obtained from 24- to 26-day-old female Sprague-Dawley rats, from the institute colony, after 4 days of diethylstilbestrol (DES) treatment (subcutaneous Silastic implants containing 5 mg DES). The animal procedures were reviewed and approved by the Animal Research Committee of our Institution, which follows the guidelines of the National Institute of Health. Granulosa cells were prepared and cultured as previously described [33]. Briefly, the ovaries were punctured with a 30-gauge needle and incubated in Dulbecco modified Eagle medium (DMEM, 4.5 g glucose/liter)-Ham F12 (1:1; Gibco), EGTA (6.8 mM), and Hepes (10 mM; 15 min at 37°C), and then washed and incubated in DMEM-F12 (1:1), sucrose (0.5 M), and Hepes (10 mM; 5 min at 37°C). After incubation, the medium was diluted with 2 volumes of DMEM-F12 and Hepes (10 mM), and ovaries were allowed to sediment. Granulosa cells were obtained by pressing ovaries within two pieces of nylon mesh (Nytex 50). To eliminate contaminating theca/interstitial cells, the crude granulosa cell suspension was layered over a 40% Percoll solution in saline, and centrifuged at 400 × *g* for 20 min. The purified granulosa cell layer was aspirated from the top of the Percoll solution and resuspended in DMEM-F12 (1:1) containing bicarbonate (2.2 g/L; pH 7.4), unless otherwise indicated. Cells were seeded on plastic plates (Nunc) precoated with collagen. Cells were maintained at 37°C with 5% CO_2. After 2 h, media were changed to remove nonattached cells and were replaced by fresh media containing the different factors to be tested.

DNA Synthesis Assay

DNA synthesis was determined by [^{3}H]-thymidine incorporation according to a method previously validated in these culture conditions [31–34]. Briefly, granulosa cells were cultured in 96-well plates at an initial plating density of 3×10^5 viable cells/cm^2, in the presence of different hormones and compounds. Tritiated thymidine (4 μCi/ml) was added to the cultures 24 h after plating. Cells were harvested 24 h later in glass hollow fibers with a multiwell cell harvester (Nunc) and radioactivity was measured in a scintillation counter.

Western Blot Analysis

Granulosa cells were cultured in P6 multiwell plates at a density of 2.5×10^6 viable cells/well. After 36 h of incubation with the different stimuli, the cells were taken up in lysis buffer and processed for protein extraction. Twenty-five micrograms of total protein were electrophoresed under reducing conditions in 12% polyacrylamide gels and electrotransferred to nitrocellulose membranes. To corroborate equal protein load in each lane, membranes were stained after transfer with Ponceau S. Proliferating cell nuclear antigen (PCNA) and G1/S-specific cyclin D2 (CCND2) expression was assessed with standard Western Blot techniques using an antibody against PCNA raised in mouse (PC10; Santa Cruz Biotechnology, Inc.; diluted 1:5000) or an antibody against CCND2 raised in mouse (M10; Santa Cruz Biotechnology, Inc.; diluted 1:100), respectively, and the appropriate peroxidase-conjugated second antibody (Amersham Biosciences). Detection was performed with a chemioluminiscence kit (DuPont NEN). The assay was performed for three independent experiments, obtaining the same pattern of response in each separate experiment.

Overexpression of AHR in Granulosa Cells and Immunoflorescence for CCND2

Granulosa cells were cultured in a LAB-TEK Chamber Slide (Nalge Nunc International Corp.) at a density of 3×10^5 viable cells/well. After 18 h, cells were transiently transfected with an expression vector encoding the rat AHR (kindly provided by Dr. M.S. Denison, University of California, Davis, CA). The transfection was made using Lipofectamine 2000 (Invitrogen, Molecular Research Center, Inc.) at 0.75 μl liposomes/cm^2 and 500 ng DNA/cm^2. Twenty-four h posttransfection, media were changed and the different stimuli were added. After 36 h of stimuli, cells were fixed in P-formaldehyde 4% for 10 min. After washing cells with PBS, cells were permeabilized for 30 min in Triton ×100 0.25%, washed afterwards with PBS and blocked for 30 min with 10% goat normal serum-1% BSA in PBS-Tween 20 (0.05%). Incubation with first antibodies against CCND2 (Santa Cruz Biotechnology, Inc.; diluted 1:500) and AHR (Affinity BioReagents; diluted 1:500) was done ON at 4°C. Thereafter, cells were washed with PBS and incubated with Cy3-conjugated anti-rabbit IgG and Cy2-conjugated anti-mouse IgG (Jackson ImmunoResearch; BA, diluted 1:100) for 1 h at room temperature. After washing with PBS, samples were allowed to dry and then mounted with Mowiol mounting media. Images were then analyzed by confocal microscopy.

Semiquantitative RT-PCR

Levels of *Cyp1a1* and *Cyp1b1* mRNA expression in rat granulosa cells were assessed using semiquantitative RT-PCR. Granulosa cells were cultured in P6 multiwell plates at a density of 2.5×10^6 viable cells/well. After 24 h (for *Cyp1a1*) or 6 h (for *Cyp1b1*) of incubation with the different stimuli, cells were lysed directly in the culture dish with TRIzol Reagent (Invitrogen, Molecular Research Center, Inc.) and total RNA extracted according to the manufacturer's instructions. Complementary DNA was synthesized from deoxyribonuclease I-treated (DNA free, Ambion Inc.) total RNA (1 μg RNA in 10 μl of RT reaction). A blank without RNA was included in each set of RT reactions. A control of RNA that was not subjected to RT was also included in subsequent PCR reactions.

The primer sequences used to amplify the two hydroxylases' target cDNAs were those described by Dasmahapatra et al. [35]. *Cyp1a1* and 18S Classic II primers:competimers (Quantum mRNA Ambion, Inc.; used as internal control for normalization) generated fragments of 509 and 324 bp, respectively. *Cyp1b1* and 18S Classic primers:competimers (Quantum mRNA Ambion, Inc.) generated fragments of 372 and 488 bp, respectively.

One-microliter aliquots of the RT reaction were used to amplify *Cyp1a1* or *Cyp1b1* and 18S fragments in a multiplex reaction. In preliminary experiments, optimum cycle number was determined for each target, so that signals were always in the exponential portion of the amplification curve. All amplification programs included an initial step at 94°C for 3 min and a final step at 72°C for 5 min. Each cycle consisted of 30 sec denaturation at 94°C, 30 sec annealing at the temperature indicated in each case, and 1 min extension at 72°C. Amplification of *Cyp1a1* and 18S Classic II cDNAs was performed for 33 cycles, 2 mM $MgCl_2$ and annealing at 62°C. *Cyp1b1* and 18S Classic cDNAs were amplified for 32 cycles in the presence of 2 mM $MgCl_2$, annealing at 65°C. Ten microliters of the PCR reaction were electrophoresed in 2% agarose gels with subsequent ethidium bromide staining. The relative amount of each mRNA was quantified with ImageQuant software (Amersham Biosciences) and normalized to the 18S ribosomal signal (given by the 18S primers:18S competimers ratio) for each sample.

Estradiol Radioimmunoassay

Granulosa cells were cultured in 96-well plates at a density of 3×10^5 viable cells/cm^2 for 64 h, in the presence of different stimuli and androstenedione (0.35 μM) as aromatizable substrate. Estradiol in the culture medium was determined by a specific radioimmunoassay as previously described [36].

Transient Transfection and Luciferase Assay

Transient transfection of granulosa cells with the reporter construct was performed in suspension by calcium phosphate coprecipitation. Six μg/well of the pTK-Red-ERE-LUC plasmid (kindly given by Dr. M. Beato, Institute for Molecular Biology and Tumor Research, Marburg, Germany) and 6 μg/well of the control reporter plasmid pCMV-β-galactosidase (Clontech) were cotransfected and the cells were then seeded onto P6 multiwell plates at a density of 3×10^6 viable cells/well in phenol red-free medium (DMEM-F12 1:1, 2.2 g/L bicarbonate, pH 7.4 without phenol red) with 10% fetal bovine serum (FBS). Nonattached cells were removed 16 h later and phenol red-free fresh medium without FBS was added. Two hours later, fresh phenol red-free medium without FBS containing the different stimuli was added. The incubation was allowed to proceed for 24 h. Cells were then washed twice with PBS and lysed in 200 μl of 1× reporter lysis buffer (Promega) , and the cleared extract was assayed for luciferase and β-galactosidase activities according to the manufacturer's instructions (Promega). Transfection efficiencies were normalized by β-galactosidase activity in each well, and luciferase activity expressed as relative light units, as percentage of the activity observed in the vehicle (ethanol) controls, which was set arbitrarily to 100%.

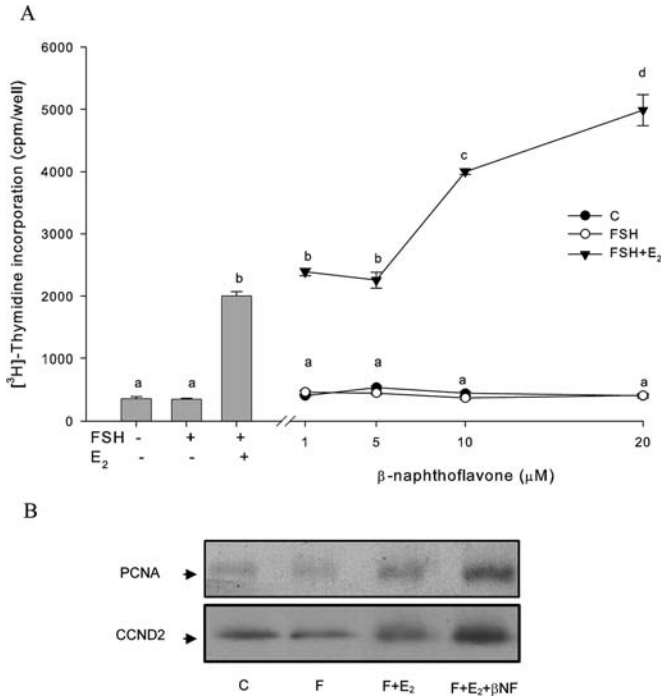

FIG. 1. Effect of β-naphthoflavone on granulosa cells proliferation. **A**) DNA synthesis assay. Granulosa cells were cultured in the absence or with increasing concentrations of β-naphthoflavone in control medium (black circles), with FSH (2 ng/ml, white circles), or with the combination of FSH and E_2 (100 ng/ml, black triangles). [^{3}H]-thymidine incorporation was performed for 24 h starting 24 h after plating. Results (depicted as bars or dose-response curves) are expressed as the mean ± SEM of four independent experiments, each run in quadruplicate. Values not sharing a common letter are significantly different ($P < 0.01$). **B**) PCNA and CCND2 expression. Granulosa cells were cultured for 36 h in control medium (C), with FSH (F, 2 ng/ml), with FSH and E_2 (F+E_2, 100 ng/ml) or with a combination of both hormones and β-naphthoflavone (β-NF, 20 μM). Total protein was extracted and Western blot was conducted as described in *Materials and Methods* using an antibody that recognizes PCNA (upper panel) or CCND2 (lower panel). A representative of three experiments is shown.

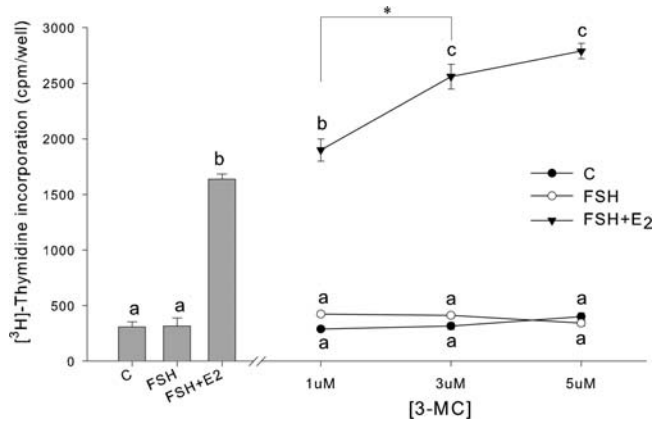

FIG. 2. Effect of 3-methylcholanthrene on granulosa cell proliferation. Granulosa cells were cultured in the absence or with increasing concentrations of 3-methylcholanthrene (3-MC) in control medium (black circles), with FSH (2 ng/ml, white circles), or with the combination of FSH and E_2 (100 ng/ml, black triangles). [^{3}H]-thymidine incorporation was performed for 24 h starting 24 h after plating. Results (depicted as bars or dose-response curves) are expressed as the mean ± SEM of three independent experiments, each run in quadruplicate. Values not sharing a common letter are significantly different ($P < 0.001$, except for the comparison marked with *, where $P < 0.01$).

Statistical Analysis

Treatments were applied to at least duplicate wells in each of three or four separate experiments, unless otherwise indicated. Results are expressed as the mean ± SEM of the independent experiments. Statistical comparisons of the results were made using one-way ANOVA and the Tukey-Kramer test for multiple comparisons after logarithmic transformation of data when necessary [37].

RESULTS

β-Naphthoflavone Amplifies the Comitogenic Effect of FSH and Estradiol in Granulosa Cells

The effect of the well-characterized AHR agonist β-naphthoflavone was assessed on the proliferation of cultured granulosa cells.

No effect was observed on DNA synthesis when this flavone was added either alone or in the presence of FSH (2 ng/ml), even at the maximal dose tested (Fig. 1A). However, when β-naphthoflavone was added in the presence of FSH and estradiol (100 ng/ml), it was capable of amplifying the stimulation elicited by both hormones in a dose-dependent manner, having a significant effect at doses of 10 μM or greater (Fig. 1A). At a dose of 20 μM, this flavone produced a 2.7-fold increase in thymidine incorporation levels when compared to cells treated with a combination of FSH and estradiol.

Thymidine incorporation data were validated by assessing the levels of expression of a marker of proliferating cells, PCNA. As shown in Figure 1B (upper panel), the expression of PCNA increases in cells treated with β-naphthoflavone, FSH, and estradiol, when compared with cells treated only with both hormones.

Because CCND2 is the limiting cyclin for the proliferation of granulosa cells and because FSH and estradiol have a synergistic effect on the expression of this cyclin [38, 39], we assessed the effect of β-naphthoflavone on this protein as well. This flavone added together with FSH and estradiol was able to further increase the expression of CCND2, when compared with the level of expression in cells treated only with both hormones (Fig. 1B, lower panel).

To determine whether the mitogenic actions of β-naphthoflavone in the presence of FSH and estradiol can also be induced by other AHR agonist, we tested the effect of the receptor agonist 3-MC on granulosa cells' DNA synthesis. As shown in Figure 2, 3-MC is also able to amplify the proliferative response elicited by FSH and estradiol, as assessed by uptake of tritiated thymidine.

α-Naphthoflavone Inhibits the Comitogenic Effect of FSH and Estradiol in Granulosa Cells

We then examined the effect of α-naphthoflavone (doses ranging from 1 to 20 μM), alone, in combination with FSH, or in combination with FSH and estradiol on granulosa cells proliferation. As shown in Figure 3A, this AHR ligand had no effect on granulosa cells proliferation when added alone or with FSH, but was able to inhibit in a dose dependent fashion (at doses of 5 μM or greater) the proliferative effect of FSH and estradiol. At the maximal dose tested, it was able to produce a 2.6-fold decrease in the estradiol induced DNA synthesis.

Thymidine incorporation data were validated by assessing the levels of expression of a marker of proliferating cells, PCNA. As shown in Figure 3B (upper panel), the expression of PCNA diminishes in cells treated with α-naphthoflavone, FSH and estradiol, when compared with cells treated only with both hormones.

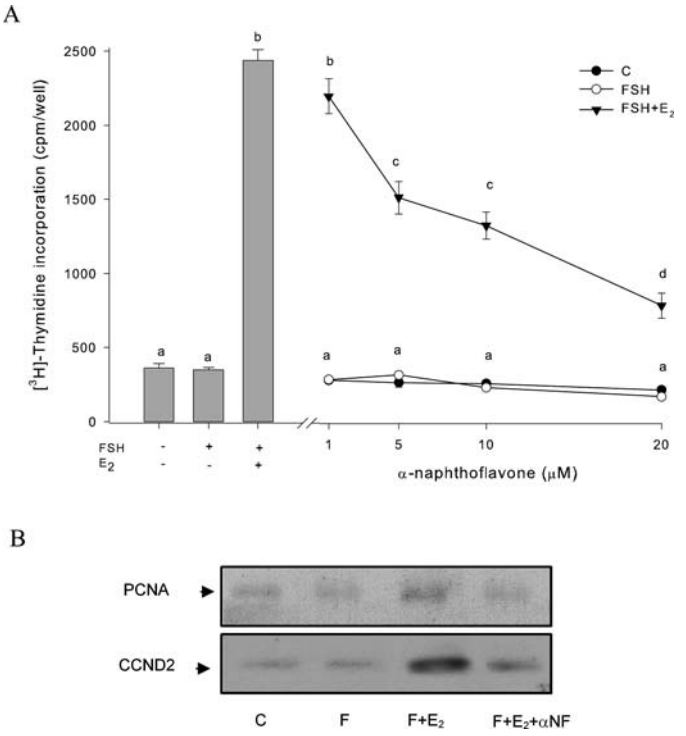

FIG. 3. Effect of α-naphthoflavone on granulosa cells proliferation. **A**) DNA synthesis assay. Granulosa cells were cultured in the absence or with increasing concentrations of α-naphthoflavone in control medium (black circles), with FSH (2 ng/ml, white circles) or with the combination of FSH and E_2 (100 ng/ml, black triangles). [^{3}H]-thymidine incorporation was performed for 24 h starting 24 h after plating. Results (depicted as bars or dose-response curves) are expressed as the mean ± SEM of four independent experiments, each run in quadruplicate. Values not sharing a common letter are significantly different ($P < 0.01$). **B**) PCNA and CCND2 expression. Granulosa cells were cultured for 36 h in control medium (C), with FSH (F, 2 ng/ml), with FSH and E_2 (F+E_2, 100 ng/ml), or with a combination of both hormones and α-naphthoflavone (α-NF, 20 μM). Total protein was extracted and Western blot was conducted as described in *Materials and Methods*, using an antibody that recognizes PCNA (upper panel) or CCND2 (lower panel). A representative of three experiments is shown.

Moreover, α-naphthoflavone added together with FSH and estradiol was able to abolish the stimulation of CCND2 expression exerted by these hormones (Fig. 3B, lower panel).

Specificity of the Comitogenic Action of Estradiol and β-Naphthoflavone

We then tried to determine whether the described synergism observed between estradiol and β-naphthoflavone in the presence of FSH is in fact mediated by the estrogen receptor and the AHR. With this purpose, we decided to test the effect of the antiestrogen ICI 182,780 and the AHR antagonist α-naphthoflavone on this system.

As can be seen in Figure 4A, the estrogen receptor antagonist was able to inhibit the synergistic action of estradiol and β-naphthoflavone.

When cells were cotreated with β-naphthoflavone (20 μM) and α-naphthoflavone (10 μM) in the presence of FSH and estradiol, the comitogenic effect of the former flavone was completely suppressed (Fig. 4B). In the same way, α-naphthoflavone at doses of 1 μM was able to reverse the effect of β-naphthoflavone at doses of 10 μM (Fig. 4B). Thus, β-naphthoflavone is not able to exert its comitogenic effect in the presence of α-naphthoflavone.

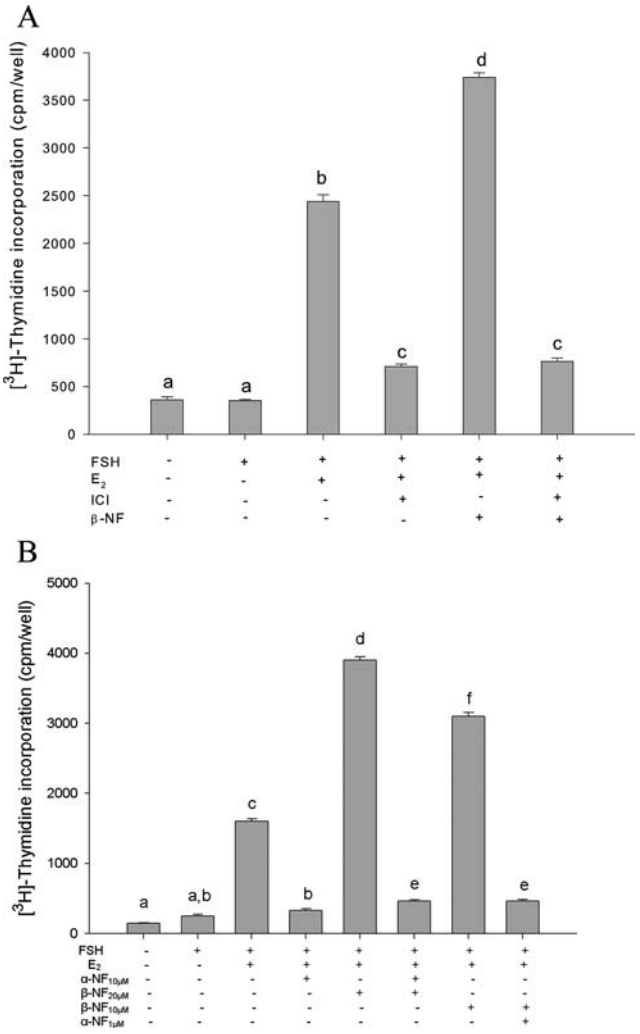

FIG. 4. Specificity of the comitogenic effect of FSH, estradiol and β-naphthoflavone in granulosa cells. **A**) Effect of ICI on the mitogenic action of FSH, estradiol, and β-naphthoflavone. Granulosa cells were cultured in control medium, with FSH (2 ng/ml), with FSH and E_2 (100 ng/ml), or with a combination of both hormones and β-naphthoflavone (β-NF, 10 μM), in the presence or absence of the nuclear estrogen receptor antagonist ICI 182,780 (10 μM). [^{3}H]-thymidine incorporation was performed for 24 h starting 24 h after plating. Results are expressed as the mean ± SEM of three independent experiments, each run in quadruplicate. Values not sharing a common letter are significantly different ($P < 0.001$). **B**) Effect of α-naphthoflavone on the mitogenic action of FSH, estradiol, and β-naphthoflavone. Granulosa cells were cultured in control medium, with FSH (2 ng/ml), with FSH and E_2 (100 ng/ml), or with a combination of both hormones and β-naphthoflavone at doses of 20 μM (β-$NF_{20\ \mu M}$) or 10 μM (β-$NF_{10\ \mu M}$). When indicated, α-naphthoflavone was added at doses of 10 μM (α-$NF_{10\ \mu M}$) or 1 μM (α-$NF_{1\ \mu M}$). [^{3}H]-thymidine incorporation was performed for 24 h starting 24 h after plating. Results are expressed as the mean ± SEM of three independent experiments, each run in quadruplicate. Values not sharing a common letter are significantly different ($P < 0.001$, except c vs. e and d vs. f, $P < 0.01$, and b vs. e, $P < 0.05$).

Overexpression of AHR Leads to an Increase in β-Naphthoflavone Action on Granulosa Cells Proliferation

With the aim of further studying the involvement of the AHR in the mitogenic action exerted by β-naphthoflavone in the presence of FSH and estradiol, we assessed the effect of this flavone on the proliferation of granulosa cells that overexpress the AHR. As can be seen in Figure 5, cells that overexpress the AHR have higher levels of CCND2 expression when treated with FSH, estradiol, and β-naphthoflavone than

those treated with both hormones and the flavone but having only the endogenous levels of AHR. These results were obtained when assessing the level of CCND2 both by immunofluorescence (Fig. 5A) and by immunoblotting analyses (Fig. 5B).

Induction of Cyp1a1 *and* Cyp1b1 *mRNA Levels by* β*-Naphthoflavone*

Because the induction of *Cyp1a1* mRNA is one of the described effects of AHR agonists and a typical characteristic of AH-responsiveness in most cells, we decided to evaluate the effect of β-naphthoflavone on these transcripts levels in rat granulosa cells. As shown in Figure 6A, addition of β-naphthoflavone (10 μM) induced *Cyp1a1* mRNA ~4.5 fold, as measured by semiquantitative RT-PCR. This effect was abolished by cotreatment with α-naphthoflavone at doses of 0.5 μM, and partially reversed by α-naphthoflavone at doses of 1 μM. Addition of α-naphthoflavone alone, at doses of 0.5 μM, had no effect on the induction of *Cyp1a1* mRNA expression, and doses of 1 μM of this flavone produced a modest and marginally significant increase in this hydroxylase mRNA level (Fig. 6A).

The ability of β-naphthoflavone to induce *Cyp1a1* mRNA and the antagonistic effect of α-naphthoflavone on this induction were also verified in the presence of FSH (Fig. 6B). Besides, β-naphthoflavone was also able to cause an increase in *Cyp1b1* mRNA when compared with the basal levels of these transcripts (Fig. 6C). However, this induction was not as marked as that for *Cyp1a1*. The addition of estradiol had no effect on the β-naphthoflavone-induced mRNA levels (Fig. 6, B and C). It is to be noted that basal levels of *Cyp1a1* mRNA in untreated granulosa cells were much lower than those for *Cyp1b1* (Fig. 6D).

Effect of the AHR Ligands on Estradiol Production

To elucidate whether the described effects of the AHR ligands β-naphthoflavone and α-naphthoflavone on granulosa cells' DNA synthesis were a result of changes in estradiol levels caused by these compounds, we assessed the levels of this estrogen in granulosa cells treated with the different stimuli.

As shown in Figure 7, β-naphthoflavone did not significantly alter endogenous estradiol levels, even at doses of 20 μM. In contrast, α-naphthoflavone produced a dose-dependent decrease in estradiol levels, this effect being significant at concentrations of 10 μM or greater (Fig. 7).

Effect of Catecholestrogens on Granulosa Cells DNA Synthesis

Given the fact that the AHR regulates at the transcriptional level the enzymes CYP1A1 and CYP1B1, which hydroxylate estradiol to give catecholestrogens, we assessed the effect of the catecholestrogens 2-hydroxyestradiol and 2-methoxyestradiol on DNA synthesis in cultured granulosa cells.

As shown in Figure 8, neither of the tested catecholestrogens was able to amplify the signal of FSH and stimulate the DNA synthesis of these cells. Furthermore, doses of 100 ng/ml or greater of 2-methoxyestradiol and doses of 1 μg/ml of 2-hydroxyestradiol produced an inhibition of DNA synthesis when compared with FSH-treated cultures (Fig. 8). The addition of the catecholestrogen 4-hydroxyestradiol had no effect, even at the maximal doses tested (data not shown).

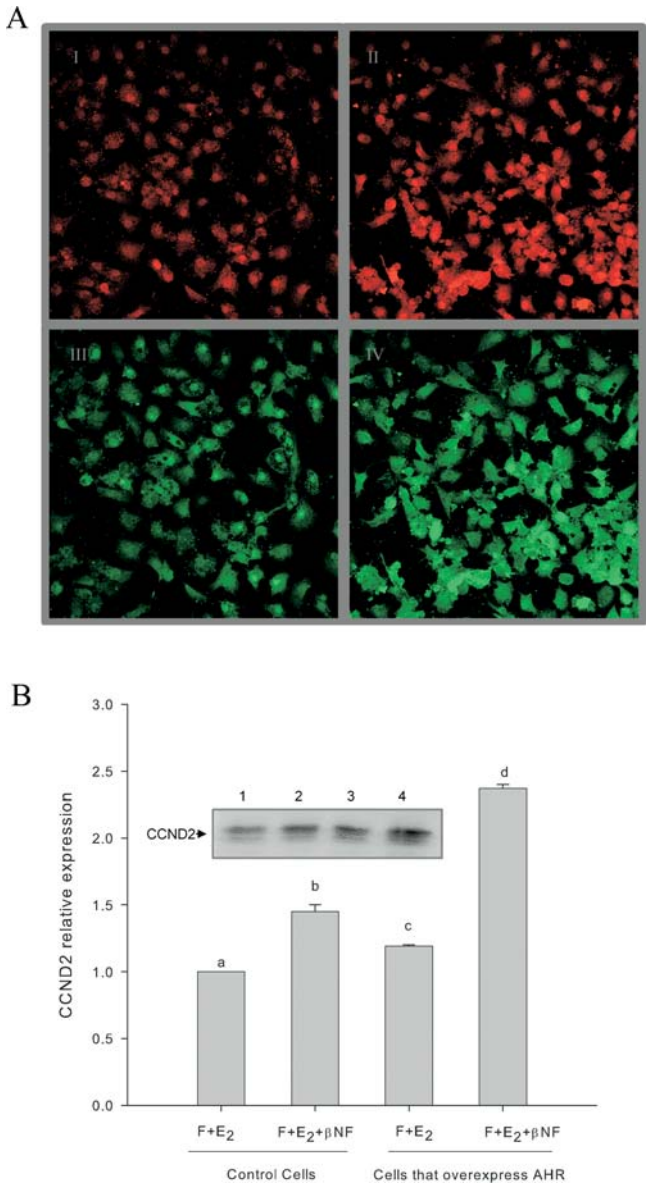

FIG. 5. Overexpression of AHR leads to an increase in β-naphthoflavone action on granulosa cells proliferation. Granulosa cells were transiently transfected with an expression vector encoding the AHR, as explained in *Materials and Methods*. They were cultured in medium containing FSH (2 ng/ml) and E_2 (100 ng/ml), in the presence or absence of β-naphthoflavone (20 μM). **A**) Immunofluorescence for CCND2 and AHR was carried out as described in *Materials and Methods*. Panels I and II: representative immunofluorescence for CCND2 in control cells (I) or cells that overexpress the AHR (II) treated with FSH, E_2 and β-naphthoflavone. Panels III and IV: representative immunofluorescence for AHR in control cells (III) or cells that overexpress the AHR (IV) treated with FSH, E_2 and β-naphthoflavone. The same pattern of response was obtained in two independent experiments, each run in duplicate. Original magnification ×400. **B**) Western blot of CCND2. Control cells and cells that overexpress the AHR were treated with a combination of 2 ng/ml FSH and 100 ng/ml E_2 ($F+E_2$), or with both hormones and β-naphthoflavone 20 μM ($F+E_2+\beta NF$) and then subjected to Western blot of CCND2 as described in *Materials and Methods*. Results are expressed as means ± SEM of two independent experiments, each run in duplicate. Values not sharing a common letter are significantly different ($P < 0.001$, except a vs. b, $P < 0.01$; a vs. c, $P < 0.05$; and b vs. c, $P < 0.05$). Inset: A representative Western Blot of CCND2 is shown. Lane 1: control cells treated with FSH and estradiol; lane 2: control cells treated with FSH, estradiol, and β-naphthoflavone; lane 3: cells that overexpress the AHR treated with FSH and estradiol; lane 4: cells that overexpress the AHR treated with FSH, estradiol, and β-naphthoflavone.

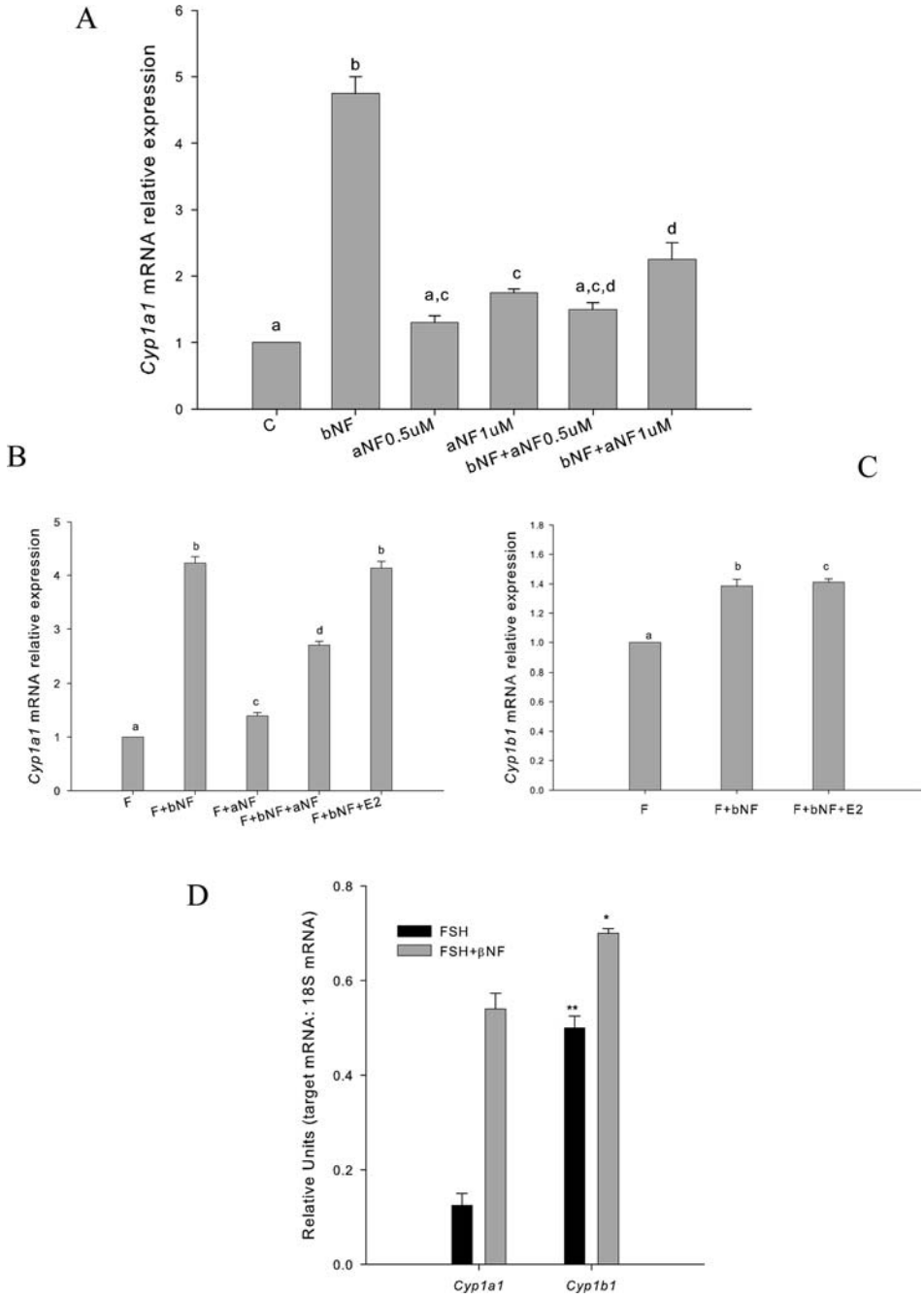

FIG. 6. Effect of β-naphthoflavone on the levels of *Cyp1a1* and *Cyp1b1* transcripts in rat granulosa cells. **A**) Granulosa cells were cultured for 24 h in control medium (C), with β-naphthoflavone (bNF, 10 μM) or α-naphthoflavone at doses of 0.5 μM ($aNF_{0.5uM}$) or 1 μM (aNF_{1uM}), or with a combination of both flavones. Total RNA extraction and semiquantitative RT-PCR for *Cyp1a1* mRNA were performed as described in *Materials in Methods*. The amount of each mRNA was normalized to the 18S ribosomal signal for each sample, and values (relative to control cells) were plotted as the mean ± SEM of seven independent experiments. Values not sharing a common letter are significantly different ($P < 0.001$, except for the following comparisons: C vs. aNF_{1uM}, $P < 0.05$; $aNF_{0.5uM}$ vs. $bNF+aNF_{1uM}$, $P < 0.05$; C vs. $bNF+aNF_{1uM}$, $P < 0.01$). **B**) Granulosa cells were cultured for 24 h in control medium containing 2 ng/ml FSH (F) or treated with β-naphthoflavone (10 μM, F+bNF), with α-naphthoflavone (1 μM, F+aNF), with a combination of both flavones (F+bNF+aNF), or with β-naphthoflavone and E_2 (100 ng/ml, F+bNF+E2). Total RNA extraction and semiquantitative RT-PCR for *Cyp1a1* mRNA were performed as described in *Materials and Methods*. The amount of each mRNA was normalized to the 18S ribosomal signal for each sample, and values (relative to control cells) were plotted as the mean ± SEM of seven independent experiments. Values not sharing a common letter are significantly different ($P < 0.001$, except a vs. c, $P < 0.05$). **C**) Granulosa cells were cultured for 6 h in control medium containing 2 ng/ml FSH in the absence (F) or presence of β-naphthoflavone alone (10 μM, F+bNF) or in combination with E_2 (100 ng/ml, bNF+E2). Total RNA extraction and semiquantitative RT-PCR for *Cyp1b1* mRNA were performed as described in *Materials and Methods*. The amount of each mRNA was normalized to the 18S ribosomal signal for each sample, and values (relative to control cells) were plotted as the mean ± SEM of seven independent experiments. Values not sharing a common letter are significantly different ($P < 0.001$). **D**) Quantification of *Cyp1a1* and *Cyp1b1* mRNA signals in samples run in parallel in the same experiment and each normalized to its corresponding 18S signal are depicted as the mean ± SEM of seven independent experiments. *$P < 0.05$ vs. same treatment in the other group; **$P < 0.01$ vs. same treatment in the other group.

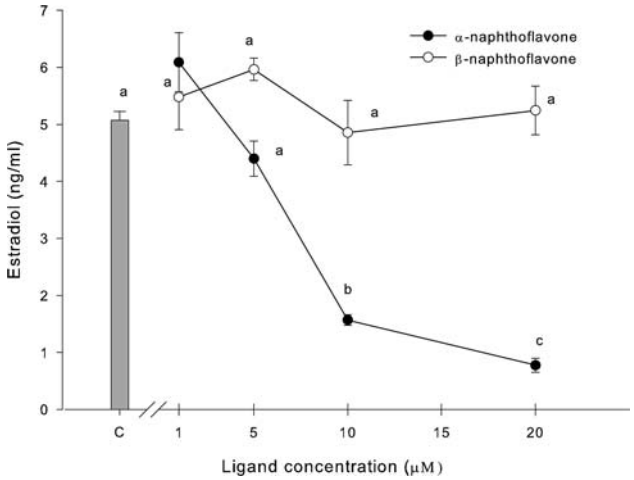

FIG. 7. Effect of β-naphthoflavone and α-naphthoflavone on FSH-stimulated estradiol production by granulosa cells. Granulosa cells were cultured for 64 h in control medium containing FSH 2 ng/ml (C) or with FSH and increasing doses of β-naphthoflavone (white circles) or α-naphthoflavone (black circles). Androstenedione (0.35 μM) was added to cultures as aromatizable androgen. Estradiol in culture media was determined by radioimmunoassay. Results (depicted as bars or dose-response curves) are expressed as means ± SEM of two independent experiments, each run in triplicate. Values not sharing a common letter are significantly different ($P < 0.001$).

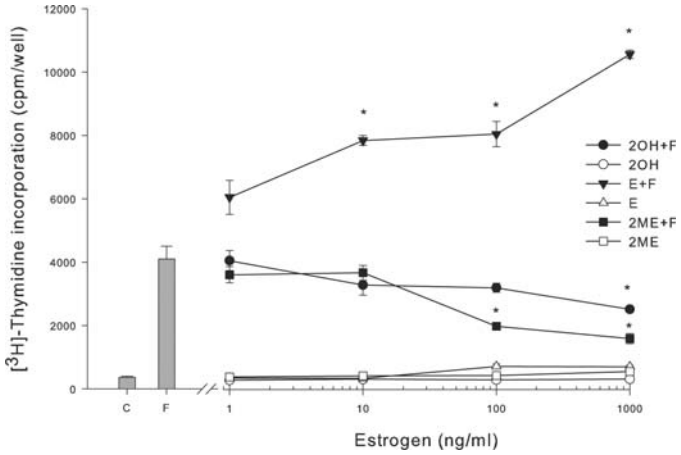

FIG. 8. Effect of catecholestrogens 2-hydroxyestradiol and 2- methoxyestradiol on granulosa cells DNA synthesis. Granulosa cells were cultured in the absence or with increasing concentrations of estradiol (E), 2-hydroxyestradiol (2OHE) or 2-methoxyestradiol (2ME) in control medium (C) or in the presence of FSH (F, 10 ng/ml). [^{3}H]-thymidine incorporation was performed for 24 h starting 24 h after plating. Results (depicted as bars or dose-response curves) are means ± SEM of three independent experiments, each run in quadruplicate. Asterisks denote significant differences when compared with each corresponding control ($P < 0.001$).

β-Naphthoflavone Inhibits the Estrogen Receptor-Mediated Transcriptional Activation

The observed synergism between an AHR agonist and estradiol on the stimulation of DNA synthesis seemed to be in contrast with the antiestrogenic effect of AHR ligands at the genomic level reported in other cell types [4, 9–11]. Therefore, we decided to investigate whether this negative interaction at the genomic level also takes place in rat granulosa cells. With this aim, we transiently transfected these cells with the pTK-Red-ERE-LUC construct, an estradiol-responsive promoter that bears the luciferase gene cloned downstream, and treated the cells with the different stimuli.

As expected, estradiol in the presence of FSH produced a stimulation of 1.5-fold in luciferase activity, which was blocked by cotreatment with ICI 182,780 (Fig. 9). The addition of β-naphthoflavone to the cultured granulosa cells produced a dramatic inhibition of estradiol-induced luciferase expression (Fig. 9), reaching levels even lower than those observed in control cells.

DISCUSSION

The AHR is a transcriptionally regulatory protein that binds upstream DNA response elements in target genes involved in xenobiotic metabolism and cellular proliferation and differentiation (reviewed in [3–6, 17]).

The AHR has been described in the ovary of different species, including the rat, in which the receptor was localized to oocytes, granulosa and thecal cells of growing follicles [40]. Although several lines of evidence indicate that the AHR is implicated in ovarian function, its physiological role has not been clearly established.

The fact that AHR ligands induce destruction of the oocyte, together with the observed increase in primordial oocytes in AHR-null versus wild-type females, has led to the assumption that still unknown ligands of the AHR seem to regulate germ cell death during female gametogenesis [18]. A functional role of the AHR in granulosa cells is also suggested by the finding of a marked induction of its expression after gonadotropin treatment in monkeys [41].

On the other hand, activation of the AHR by binding of ligands such as the environmental contaminants furans, polyhalogenated dioxins, and polychlorinated biphenyls is associated with a wide range of adverse biological actions [21].

In the present study we have shown a dose-dependent stimulatory effect of an AHR agonist on rat granulosa cell proliferation that was evidenced only in the presence of estrogens. Although the AHR agonist increased the steady-state levels of *Cyp1a1* and *Cyp1b1* mRNAs, the described action of this compound was not a result of changes in estradiol endogenous levels caused by increased metabolism, because the radioimmunoassay for this estrogen showed that β-naphthoflavone has no effect on estradiol levels in granulosa cells. Moreover, we have found that the tested catecholestrogens inhibited the hormonally-stimulated DNA synthesis, which rules out the possibility that β-naphthoflavone exerts its action on proliferation through the induction of estradiol metabolism to catecholestrogens. The inhibitory action of 2-hydroxyestradiol and 2-methoxyestradiol on rat granulosa cell proliferation is consistent with similar actions in pig granulosa cells [42], and suggests that the regulation of CYPs enzyme levels by AHR would be an important regulatory point in granulosa cells growth.

On the other hand, we found that α-naphthoflavone markedly decreases endogenous estradiol levels. This action is consistent with its previously demonstrated inhibition of the aromatase activity in other species [43–45], but does not explain the inhibitory action of this flavone on the stimulation of DNA synthesis elicited by exogenous estradiol in our experimental conditions. α-naphthoflavone inhibits hormonally-stimulated proliferation at doses at which it functions as a weak AHR agonist, as assessed by induction of *Cyp1a1* mRNA levels. A possible explanation for this apparent contradictory result, when compared to the actions exerted by β-naphthoflavone or 3-MC on proliferation, is that different AHR agonists can lead to complete different patterns of

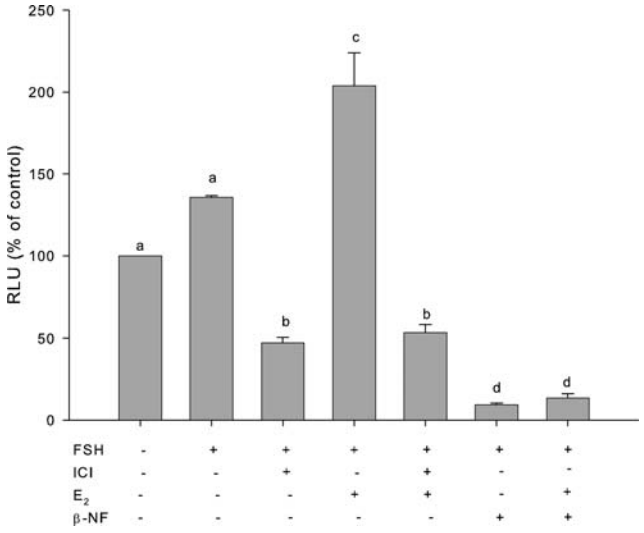

FIG. 9. Effect of β-naphthoflavone on estrogen receptor-stimulated transcription in granulosa cells. Granulosa cells were transiently transfected with the pTK-Red-ERE-LUC reporter construct and the control reporter plasmid pCMV-β-Galactosidase as described in *Materials and Methods*. After transfection (18 h later), cells were treated with vehicle only or with FSH (2 ng/ml), FSH and ICI 182,780 (10 μM), FSH and E_2 (100 ng/ml), both hormones and ICI 182,780, FSH and β-naphthoflavone (β-NF, 10 μM), or a combination of both hormones and β-naphthoflavone for 24 h, and then processed for luciferase reporter assay. Results (normalized to β-Gal activity) are expressed as percentage of relative light units (RLU) when compared to control cells (only vehicle added, taken as 100%). Values are means ± SEM of two independent experiments, each performed in duplicate. Values not sharing a common letter are significantly different ($P < 0.01$).

response even in the same cell type, as demonstrated for the induction of apoptosis by PAH- but not dioxin-activated AHR in murine oocytes [46]. Besides, it has to be taken into consideration that despite the partial induction of *Cyp1a1* transcripts levels elicited by α-naphthoflavone when present alone at high doses, this flavone acts as an AHR antagonist at those doses in the presence of the AHR agonist β-naphthoflavone in the same experiments. Thus, our results point out that the proliferative response induced by the AHR ligands and estrogen in rat granulosa cells cannot be solely explained by the transcriptional activity of the cognate receptors.

The abrogation of the effect of β-naphthoflavone in the presence of the AHR antagonist α-naphthoflavone, together with the observation that overexpression of the AHR in granulosa cells leads to an increase in β-naphthoflavone amplification of estradiol-stimulated proliferation, strongly suggest that the AHR is implicated in the observed synergism between this flavone and estradiol. The antagonist α-naphthoflavone has been previously shown to counteract the action of other AHR agonists. For example, in vivo treatment with this compound has been shown to prevent the oocyte and primordial follicle destruction induced by 7,12-dimethyl benzanthracene [47]. We postulate that the estrogen receptor is also comprised in this synergism, because the estrogen receptor antagonist ICI 182,780 was also able to abolish the comitogenic effect of estradiol and β-naphthoflavone.

In spite of the amplification of estradiol effects on cell proliferation exerted by the AHR agonist β-naphthoflavone, a negative interaction between both ligands was observed in the expression of a reporter gene under control of a promoter containing an estrogen response element, suggesting a negative interaction between the two receptors at the transcriptional level. Interestingly, this negative phenomenon is not bidirectional, because estradiol did not exert any inhibitory effect on the β-naphthoflavone-stimulated expression of the *Cyp1a1* or *Cyp1b1* genes. This unidirectional effect is in agreement with findings in fish hepatocytes and in some cell lines [48–50], but contrasts with reports in other human and mouse cell lines, where estradiol either decreases or enhances TCDD-induced *Cyp1a1* mRNA levels. Thus, the mutual interaction at the transcriptional level appears to be cell-type specific and is most likely regulated by specific protein factors (coactivators or corepressors) restricted to each cell type. Functional and physical interactions between the AHR and the estrogen receptor have been demonstrated in different systems. For example, in MCF-7 cells expressing the AHR, activated AHR inhibits the estrogen receptor mediated responses (reviewed in [51, 52]) and estradiol exerts transrepression of dioxin-inducible gene transcription through estrogen receptor-AHR-ARNT protein-protein interactions [53].

Although a positive interaction between both receptors taking place in the promoter of a key gene regulating cell proliferation in granulosa cells cannot be ruled out, our present hypothesis is that the interaction between the estrogen receptor and the AHR does not occur at the transcriptional level. In fact, the negative interaction between both ligands on the pTK-Red-ERE-LUC reporter assay suggests an alternative (probably nongenomic) mechanism underlying the synergism observed on proliferation. In this regard, increasing amounts of evidence indicate that the mitogenic actions of estrogens are exerted through a nongenomic pathway (reviewed in [54]). It has been reported that the association of agonist activated AHR/ARNT heterodimer with the estrogen receptor results in the recruitment of the unoccupied estrogen receptor and coactivator p300 to estrogen-responsive gene promoters, leading to activation of transcription and estrogenic effects [55]. Our results with the pTK-Red-ERE-LUC reporter assay, the absence of effect of estradiol on the induction of *Cyp1a1* and *Cyp1b1* mRNA elicited by β-naphthoflavone, and the fact that the effect of this flavone on proliferation occurs only in the presence of estradiol, suggest that the above mentioned mechanism is not responsible for the synergism observed between both ligands in our system.

The identity of the AHR endogenous ligand has not been determined. Although tryptophan metabolites [56–58] and the arachidonic acid metabolite lipoxin [59] have been proposed as candidates, the AHR remains an orphan receptor. In this context, a number of studies have reported that AHR-mediated processes occur in the absence of exogenous AHR ligands and suggested a physiological role for this receptor [14, 60–64]. In regard to the hydroxylase CYP1A1, it has been found that its expression is developmentally regulated in porcine ovarian granulosa cells [65] and in the fertilized ovum of the mouse [66]. This has led to the hypothesis that this enzyme, regulated at the transcriptional level by the AHR, may be involved in the inactivation of the endogenous ligand. Moreover, constitutive expression of this enzyme and its induction during maturation has been reported in bovine cumulus-oocyte complexes [67]. In our system of cultured rat granulosa cells, the high constitutive levels of *Cyp1b1* mRNA, together with the fact that AHR signal was found in the nucleus of untreated cells by immunofluorescence (unpublished data from our laboratory), might indicate the presence of an endogenous ligand for this receptor.

In conclusion, the results of this study provide the first demonstration of a synergism between estradiol and an agonist of the AHR in rat granulosa cell proliferation. Although our data strongly suggest that the AHR and the estrogen receptor are implicated in this effect, the level at which the cross-talk between

both pathways takes place remains to be established. Even though most of the reported interactions between both pathways are inhibitory (reviewed in [8, 51]), still undescribed mechanisms might account for the positive interaction between both occupied receptors reported herein. It is reasonable to speculate that the mitogen-activated protein kinases cascade is also implicated in the observed comitogenic effect of β-naphthoflavone and estradiol, because interactions between the AHR and the mitogen-activated protein kinase signaling pathways (reviewed in [17, 68]) and between the latter and the estrogen receptor pathway (reviewed in [53]) have been described.

The effects of the AHR agonist reported herein might reflect the enhanced physiological function of the AHR, the specific actions that environmental contaminants can exert through the xenobiotic-AHR complex, or a combination of both.

Our study suggests that, in addition to the previously proposed action in ovarian physiology, the AHR is involved in the regulation of granulosa cell proliferation through modulation of the mitogenic action of classical hormones.

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REFERENCES

1. Hahn ME. The aryl hydrocarbon receptor: a comparative perspective. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol 1998; 121:23–53.
2. Butler RA, Kelley ML, Powell WH, Hahn ME, Van Beneden RJ. An aryl hydrocarbon receptor (AHR) homologue from the soft-shell clam, *Mya arenaria*: evidence that invertebrate AHR homologues lack 2,3,7,8-tetrachlorodibenzo-p-dioxin and beta-naphthoflavone binding. Gene 2001; 278:223–234.
3. Hankinson O. The aryl hydrocarbon receptor complex. Annu Rev Pharmacol Toxicol 1995; 35:307–340.
4. Safe SH. Modulation of gene expression and endocrine response pathways by 2,3,7,8-tetrachlorodibenzo-p-dioxin and related compounds. Pharmacol Ther 1995; 67:247–281.
5. Schmidt JV, Bradfield CA. Ah receptor signaling pathways. Annu Rev Cell Dev Biol 1996; 12:55–89.
6. Rushmore TH, Kong AN. Pharmacogenomics, regulation and signaling pathways of phase I and II drug metabolizing enzymes. Curr Drug Metab 2002; 5:481–490.
7. Gonzales FK, Fernandez-Salguero P. The aryl hydrocarbon receptor: studies using the AHR-null mice. Drug Metab Dispos 1998; 26:1194–1198.
8. Safe S, Wang F, Porter W, Duan R, McDougal A. Ah receptor agonists as endocrine disruptors: antiestrogenic activity and mechanisms. Toxicol Lett 1998; 102–103:343–347.
9. Porter W, Safe S. Estrogenic and antiestrogenic compounds. In: Puga A, Wallace KB, (eds.), Molecular Biology Approaches to Toxicology. Fairfax, VA: Techbooks;267–283.
10. Wormke M, Castro-Rivera E, Ichen C, Safe S. Estrogen and aryl hydrocarbon receptor expression and crosstalk in human Ishikawa endometrial cancer cells. J Steroid Biochem Molec Biol 2000; 72:197–207.
11. Safe S. Molecular biology of the Ah receptor and its role in carcinogenesis. Toxicol Lett 2001; 120:1–7.
12. Crawford RB, Holsapple MP, Kaminski N. Leukocyte activation induces aryl hydrocarbon receptor up-regulation, DNA binding, and increased Cyp1a1 expression in the absence of exogenous ligand. Mol Pharmacol 1997; 52:921–927.
13. Chang C, Puga A. Constitutive activation of the aromatic hydrocarbon receptor. Mol Cell Biol 1998; 18:525–535.
14. Singh SS, Hord NG, Predew GH. Characterization of the activated form of the aryl hydrocarbon receptor in the nucleus of HeLa cells in the absence of exogenous ligand. Arch Biochem Biophys 1996; 329:47–55.
15. Komura K, Hayashi S, Makino I, Poellinger L, Tanaka H. Aryl hydrocarbon receptor/dioxin receptor in human monocytes and macrophages. Mol Cell Biochem 2001; 226:107–118.
16. Peters JM, Wiley LM. Evidence that murine preimplantation embryos express aryl hydrocarbon receptor. Toxicol Appl Pharmacol 1995; 134:214–221.
17. Puga A, Xia Y, Elferink C. Role of the aryl hydrocarbon receptor in cell cycle regulation. Chem Biol Interact 2002; 141:117–130.
18. Robles R, Morita Y, Mann KK, Perez GI, Yang S, Matikainen T, Sherr DH, Tilly JL. The aryl hydrocarbon receptor, a basic helix-loop-helix transcription factor of the PAS gene family, is required for normal ovarian germ cell dynamics in the mouse. Endocrinology 2000; 141:450–453.
19. Benedict JC, Lin TM, Loeffler IK, Peterson RE, Flaws JA. Physiological role of the aryl hydrocarbon receptor in mouse ovary development. Toxicol Sci 2000; 56:382–388.
20. Benedict JC, Miller KP, Lin TM, Greenfeld C, Babus JK, Peterson RE, Faws JA. Aryl hydrocarbon receptor regulates growth, but not atresia, of mouse preantral and antral follicles. Biol Reprod 2003; 68:1511–1517.
21. Abbott BD, Schmid JE, Pitt JA, Buckalew AR, Wood CR, Geld GA, Diliberto JJ. Adverse reproductive outcomes in the transgenic Ah receptor-deficient mouse. Toxicol Appl Pharmacol 1999; 155:2–70.
22. Gasiewicz TA, Rucci G. Alpha-naphthoflavone acts as an antagonist of 2,3,7, 8-tetrachlorodibenzo-p-dioxin by forming an inactive complex with the Ah receptor. Mol Pharmacol 1991; 40:607–612.
23. Merchant M, Krishnan V, Safe S. Mechanism of action of alpha-naphthoflavone as an Ah receptor antagonist in MCF-7 human breast cancer cells. Toxicol Appl Pharmacol 1993; 120:179–185.
24. Merchant M, Safe S. In vitro inhibition of 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced activity by alpha-naphthoflavone and 6-methyl-1,3,8-trichlorodibenzofuran using an aryl hydrocarbon (Ah)-responsive construct. Biochem Pharmacol 1995; 50:663–668.
25. Brauze D, Crow JS, Malejka-Giganti D. Modulation by beta-naphthoflavone of ovarian hormone dependent responses in rat uterus and liver in vivo. Can J Physiol Pharmacol 1997; 75:1022–1029.
26. Navas JM, Segner H. Antiestrogenicity of beta-naphthoflavone and PAHs in cultured rainbow trout hepatocytes: evidence for a role of the arylhydrocarbon receptor. Aquat Toxicol 2000; 51:79–92.
27. Huang P, Ceccatelli S, Hakansson H, Grandison L, Rannug A. Constitutive and TCDD-induced expression of Ah receptor-responsive genes in the pituitary. Neurotoxicology 2002; 23:783–793.
28. Dong W, Teraoka H, Yamazaki K, Tsukiyama S, Imani S, Imagawa T, Stegeman JJ, Peterson RE, Hiraga T. 2,3,7,8-tetrachlorodibenzo-p-dioxin toxicity in the zebrafish embryo: local circulation failure in the dorsal midbrain is associated with increased apoptosis. Toxicol Sci 2002; 69:191–201.
29. Henry EC, Gasiewicz TA. Agonist but not antagonist ligands induce conformational change in the mouse aryl hydrocarbon receptor as detected by partial proteolysis. Mol Pharmacol 2003; 63:392–400.
30. Granberg L, Ostergren A, Brandt I, Brittebo EB. CYP1A1 and CYP1B1 in blood-brain interfaces: CYP1A1-dependent bioactivation of 7,12-dimethylbenz(a)anthracene in endothelial cells. Drug Metab Dispos 2003; 31:259–265.
31. Bley MA, Saragüeta PE, Barañao JL. Concerted stimulation of rat granulosa cell deoxyribonucleic acid synthesis by sex steroids and follicle-stimulating hormone. J Steroid Biochem Mol Biol 1997; 62:11–19.
32. Bley MA, Simón JC, Saragüeta PE, Barañao JL. Hormonal regulation of rat granulosa cell deoxyribonucleic acid synthesis: effects of estrogens. Biol Reprod 1991; 44:880–888.
33. Bley MA, Simón JC, Estévez AG, Jiménez de Asúa L, Barañao JL. Effect of follicle-stimulating hormone on insulin-like growth factor-I-stimulated rat granulosa cell deoxyribonucleic acid synthesis. Endocrinology 1992; 131:1223–1229.
34. Lanuza GM, Fischman ML, Barañao JL. Growth promoting activity of oocytes on granulosa cells is decreased upon meiotic maturation. Dev Biol 1998; 197:129–139.
35. Dasmahapatra AK, Wimpee BA, Trewin AL, Hutz RJ. 2,3,7,8-Tetrachlorodibenzo-p-dioxin increases steady-state estrogen receptor-beta mRNA levels after CYP1A1 and CYP1B1 induction in rat granulosa cells in vitro. Mol Cell Endocrinol 2001; 182:39–48.
36. Barañao JL, Bley MA, Batista FD, Glikin GC. A DNA topoisomerase I inhibitor blocks the differentiation of rat granulosa cells induced by follicle-stimulating hormone. Biochem J 1991; 277:557–560.
37. Sokal RR, Rohlf FJ. Biometry. New York: WH Freeman and Co; 1995.
38. Sicinski P, Donaher JL, Geng Y, Parker SB, Gardner H, Park MY, Robker RL, Richards JS, McGinnis LK, Biggers JD, Eppig JJ, Bronson RT, et al. Cyclin D2 is an FSH-responsive gene involved in gonadal cell proliferation and oncogenesis. Nature 1996; 384:470–474.
39. Robker RR, Richards JS. Hormone-induced proliferation and differentiation of granulosa cells: a coordinated balance of the cell cycle regulators cyclin D2 and p27Kip1. Mol Endocrinol 1998; 12:924–940.
40. Davis BJ, Mccurdy EA, Miller BD, Lucier GW, Tritscher AM. Ovarian

tumors in rats induced by chronic 2,3,7,8-tetrachlorodibenzo-p-dioxin treatment. Cancer Res 2000; 60:5414–5419.
41. Chaffin CL, Stouffer RL, Duffy DM. Gonadotropin and steroid regulation of steroid receptor and aryl hydrocarbon receptor messenger ribonucleic acid in macaque granulosa cells during the periovulatory interval. Endocrinology 1999; 140:4753–4760.
42. Spicer LJ, Hammond JM. Catecholestrogens inhibit proliferation and DNA synthesis of porcine granulosa cells in vitro: comparison with estradiol, 5 alpha-dihydrotestosterone, gonadotropins and catecholamines. Mol Cell Endocrinol 1989; 64:119–126.
43. Kellis JT Jr, Vickery LE. Inhibition of human estrogen synthetase (aromatase) by flavones. Science 1984; 225:1032–1034.
44. Campbell DR, Kurzer MS. Flavonoid inhibition of aromatase enzyme activity in human preadipocytes. J Steroid Biochem Mol Biol 1993; 46: 381–388.
45. Pelissero C, Lenczowski MJ, Chinzi D, Davail-Cuisset B, Sumpter JP, Fostier A. Effects of flavonoids on aromatase activity, an in vitro study. J Steroid Biochem Mol Biol 1996; 57:215–223.
46. Matikainen T, Perez GI, Jurisicova A, Pru JK, Schlezinger JJ, Ryu HY, Laine J, Sakai T, Korsmeyer SJ, Casper RF, Sherr DH, Tilly JL. Aromatic hydrocarbon receptor-driven Bax gene expression is required for premature ovarian failure caused by biohazardous environmental chemicals. Nat Genet. 2001; 28(4):355–360.
47. Shiromizu K, Mattison DR. Murine oocyte destruction following intraovarian treatment with 3-methylcholanthrene or 7,12-dimethylbenz(a)anthracene: protection by alpha-napthoflavone. Teratog Carcinog Mutagen 1985; 5(6):463–472.
48. Navas JM, Segner H. Estrogen-mediated suppression of cytochrome P4501A (CYP1A) expression in rainbow trout hepatocytes: role of estrogen receptor. Chem Biol Interact 2001; 138:285–298.
49. Bemanian V, Male R, Goksoyr A. The aryl hydrocarbon receptor-mediated disruption of vitellogenin synthesis in the fish liver: Cross-talk between AHR- and ERalpha-signalling pathways. Comp Hepatol 2004; 3(1):2.
50. Hoivik D, Willett K, Wilson C, Safe S. Estrogen does not inhibit 2,3,7,8-tetrachlorodibenzo-*p*-dioxin mediated effects in MCF-7 and Hepa1c17 cells. J Biol Chem 1997; 272:30270–30274.
51. Safe S, Wormke M, Samudio I. Mechanisms of inhibitory aryl hydrocarbon receptor-estrogen receptor crosstalk in human breast cancer cells. J Mammary Gland Biol Neoplasia 2000; 5:295–306.
52. Kohle C, Hassepass I, Bock-Hennig BS, Walter Bock K, Poellinger L, McGuire J. Conditional expression of a constitutively active aryl hydrocarbon receptor in MCF-7 human breast cancer cells. Arch Biochem Biophys 2002; 402:172–179.
53. Beischlag TV, Perdew GH. ERα-AHR-ARNT protein-protein interactions mediate estradiol-dependent transrepression of dioxin inducible gene transcription. J Biol Chem 2005; 280:21607–21611. Published 18 April 2005, doi: 10.1074/jbc.C500090200.
54. Lösel R, Wehling M. Nongenomic actions of steroid hormones. Nat Rev Mol Cell Biol 2003; 4:46–56.
55. Ohtake F, Takeyama K, Matsumoto T, Kitagawa H, Yamamoto Y, Nohara K, Tohyama C, Krust A, Mimura J, Chambon P, Yanagisawa J, Fujii-Kuriyama Y, et al. Modulation of estrogen receptor signalling by association with the activated dioxin receptor. Nature 2003; 423:545–550.
56. Heath-Pagliuso S, Rogers WJ, Tullis K, Seidel SD, Cenijn PH, Brouwer A, Denison MS. Activation of the Ah receptor by tryptophan and tryptophan metabolites. Biochemistry 1998; 37:11508–11515.
57. Adachi J, Mori Y, Matsui S, Takigami H, Fujino J, Kitagawa H, Miller CA 3rd, Kato T, Saeki K, Matsuda T. Indirubin and indigo are potent aryl hydrocarbon receptor ligands present in human urine. J Biol Chem 2001; 276:31475–31478.
58. Song J, Clagett-Dame M, Peterson RE, Hahn ME, Westler WM, Sicinski RR, DeLuca HF. A ligand for the aryl hydrocarbon receptor isolated from lung. Proc Natl Acad Sci USA 2002; 99:14694–14699.
59. Schaldach CM, Riby J, Bjeldanes LF. Lipoxin A4: a new class of ligand for the Ah receptor. Biochemistry 1999; 38:7594–7600.
60. Sadek CM, Allen-Hoffmann BL. Cytochrome P450IA1 is rapidly induced in normal human keratinocytes in the absence of xenobiotics. J Biol Chem 1994; 269:16067–16074.
61. Mufti NA, Bleckwenn NA, Babish JG, Shuler ML. Possible involvement of the Ah receptor in the induction of cytochrome P-450IA1 under conditions of hydrodynamic shear in microcarrier-attached hepatoma cell lines. Biochem Biophys Res Commun 1995; 208:144–152.
62. Zaher H, Fernandez-Salguero PM, Letterio J, Sheikh MS, Fornace AJ, Roberts AB, Gonzalez FJ. The involvement of aryl hydrocarbon receptor in the activation of transforming growth factor-beta and apoptosis. Mol Pharmacol 1998; 54:312–321.
63. Elizondo G, Fernandez-Salguero P, Sheikh MS, Kim GY, Fornace AJ, Lee KS, Gonzalez FJ. Altered cell cycle control at the G(2)/M phases in aryl hydrocarbon receptor-null embryo fibroblast. Mol Pharmacol 2000; 57: 1056–1063.
64. Monk SA, Denison MS, Rice RH. Transient expression of CYP1A1 in rat epithelial cells cultured in suspension. Arch Biochem Biophys 2001; 393: 154–162.
65. Leighton JK, Canning S, Guthrie HD, Hammond JM. Expression of cytochrome P450 1A1, an estrogen hydroxylase, in ovarian granulosa cells is developmentally regulated. J Steroid Biochem Mol Biol 1995; 52:351–356.
66. Dey A, Nebert DW. Markedly increased constitutive CYP1A1 mRNA levels in the fertilized ovum of the mouse. Biochem Biophys Res Commun 1998; 251:657–661.
67. Pocar P, Augustin R, Fischer B. Constitutive expression of CYP1A1 in bovine cumulus oocyte-complexes in vitro: mechanisms and biological implications. Endocrinology 2004; 145:1594–1601.
68. Yim S, Oh M, Choi SM, Park H. Inhibition of the MEK-1/p42 MAP kinase reduces aryl hydrocarbon receptor-DNA interactions. Biochem Biophys Res Commun 2004; 322:9–16.