BIOLOGY OF REPRODUCTION **75,** 360–369 (2006)
Published online before print 31 May 2006.
DOI 10.1095/biolreprod.106.053017

Regulation of Aryl Hydrocarbon Receptor Expression in Rat Granulosa Cells[1]

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ABSTRACT

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that mediates most of the toxic and endocrine-disruptive actions of aromatic compounds in the ovary. Paradoxically, this receptor has been shown to play important roles in normal female reproductive function as well. Although knowledge of AHR expression regulation in the ovary is of crucial significance to understand the receptor biology and its function in reproductive physiology, there are only limited data in this area. The purpose of the present study was to establish the possible regulation that AHR might undergo in ovarian cells. Here we show that the hormones FSH and estradiol are able to reduce AHR protein and transcript levels in granulosa cells in a way that parallels the changes observed in ovarian tissue across the rat estrous cycle. These findings suggest that estradiol and FSH would be cycle-associated endogenous modulators of AHR expression. In addition, we show that in granulosa cells the receptor is rapidly downregulated via proteasomal degradation following treatment with AHR ligands. However, prolonged treatment with an agonist caused an increase in *Ahr* mRNA levels. These actions would constitute a regulatory mechanism that both attenuates AHR signal rapidly and replenishes the cellular receptor pool in the long term. In conclusion, our results indicate that AHR expression is regulated by classical hormones and by its own ligands in granulosa cells.

estradiol, follicle-stimulating hormone, granulosa cells, ovary, toxicology

INTRODUCTION

The AHR is a highly conserved member of the basic helix-loop-helix/Per-Arnt-Sim (bHLH-PAS) family of transcription factors. It mediates most of the toxic and biological responses elicited by different aromatic compounds, such as 2,3,7,8-tetrachlorodibenzeno-p-dioxin (TCDD), or the heteropolynuclear aromatic hydrocarbon β-naphthoflavone (5,6-benzophlavone) (reviewed in [1, 2]). Several of the described AHR ligands are widespread and persistent environmental contaminants which exert diverse effects on reproductive, immune, developmental, and nervous systems (reviewed in [3]). In particular, the endocrine-disruptive, teratogenic, and antiestrogenic effects of AHR activation, as well as the role of the receptor in carcinogenesis, have been well studied (reviewed in [4–7]).

[1]Supported by grants form the ANPCYT PICT 05–09044 and UBACYT X218. J.L.B. is an established investigator from the CONICET.
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Received: 10 April 2006.
First decision: 3 May 2006.
Accepted: 23 May 2006.

ISSN: 0006-3363. http://www.biolreprod.org

The AHR has been described in the ovary of different species, including the rat, where the receptor was localized to oocytes and granulosa cells in the rat ovarian follicles [8]. AHR activation has been shown to produce important alterations in female reproduction. For example, exposure of female rodents to AHR ligands or their metabolites can cause reductions of FSH and LH secretion during the preovulatory period, ovarian follicle loss, oocyte and granulosa cell apoptosis, inhibition of proliferation, alteration in steroidogenesis and differentiation, or premature transition to reproductive senescence (reviewed in [9]) [10, 11]. Moreover, besides the well-known antiestrogenic effects of AHR ligands, we and others have shown that activation of this receptor can also lead to a positive modulation of estrogen receptor-elicited responses in rat granulosa cells or in different cell lines [12, 13].

In addition to the toxicological actions that AHR activation exerts in numerous systems, this receptor may play important roles in the maintenance of homeostatic function (reviewed in [4, 9, 14]) [15–24]. In particular, the AHR is proposed to be implicated in ovarian function, mainly in follicle growth and recruitment, in ovulation, and in steroidogenesis (reviewed in [9]) [10].

Numerous aspects regarding AHR-mediated signaling are already well established. 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 (XREs), and upregulates the transcription of a battery of xenobiotic metabolizing enzymes as well as many other genes involved in cellular function (reviewed in [2, 9, 25–29]).

Relatively less emphasis has been placed on the fate of the AHR following its nuclear binding to XREs and the way in which the signaling pathway is turned off. Nevertheless, in the past few years several studies have focused on the regulation of the AHR upon exposure to its ligands. It has been shown in several systems that AHR agonists induce degradation of their own receptor; this downregulation is proposed to be mediated via the 26S proteasome pathway following ubiquitylation [30–32], and its physiological role may be to modulate AHR-mediated gene regulation (reviewed in [29, 33]). However, it has also been demonstrated that different AHR agonists upregulate the expression of the receptor in various systems [34–38].

Little is currently known about the physiological regulation of AHR, and there are few studies concerning AHR expression in the absence of exogenous ligands. Serum and growth factors have been demonstrated to regulate AHR expression in some systems [39–41], and it has been reported that cell stage of differentiation modulates the expression of the receptor as well [42–44]. Besides, *Ahr* mRNA levels have been shown to increase during the periovulatory interval in macaque granulosa cells [45]. It has also been demonstrated that *Ahr* transcript levels fluctuate across the estrous cycle in liver and ovarian

tissue, dropping significantly on the evening of proestrus [46]. However, the factors associated with the rat reproductive cycle responsible for AHR regulation remain to be determined. These observations point to the presence of endogenous modulators of AHR expression, supporting the idea of an endocrine regulation of the receptor.

In order to determine possible physiological regulators of AHR expression in the ovary, we evaluated the effect of hormones on the receptor expression levels in a defined culture system of rat granulosa cells that has been extensively characterized [47]. We found that FSH and estradiol, classical stimuli that regulate the function of these cells and whose levels fluctuate across the estrous cycle, are able to decrease AHR expression, suggesting that these hormones would be endogenous AHR modulators associated with the reproductive cycle. In addition, we sought to contrast our system with the already existing data in terms of agonist-regulated AHR expression, since those compounds have been shown to have opposite effects depending on their nature and, mainly, the system under study. We found that AHR protein levels are rapidly downregulated in granulosa cells after treatment with an agonist, whereas transcript levels for this receptor are increased after prolonged stimulation. Our findings are of special interest when trying to understand AHR function and the regulation of the responses elicited by activation of this receptor in ovarian cells.

MATERIALS AND METHODS

Hormones and Chemicals

Ovine FSH (NIDDK-oFSH-20) was obtained from the National Hormone and Pituitary Program. Lactacystin was purchased from Calbiochem, EMD Biosciences, Inc. Tissue culture reagents, 17β-estradiol (estradiol), 5,6-benzoflavone (β-naphthoflavone), 7,8-benzoflavone (α-naphthoflavone), and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated. Collagen was prepared from rat tails as previously described [48].

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 Institutes of Health. Granulosa cells were prepared and cultured as previously described [49]. Briefly, the ovaries were punctured with a 30-gauge needle and incubated in Dulbecco Modified Eagle medium (DMEM, 4.5 g glucose/litre)-Ham F12 (1:1, Gibco, Gaithersburg, MD), 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, Geneva, Switzerland). To eliminate contaminating theca/interstitial cells, the crude granulosa cell suspension was layered over a 40% Percoll solution in saline and centrifuged at $400 \times g$ 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). Cells were seeded on P6 multiwell plastic plates (Nunc, Roskilde, Denmark) precoated with collagen at a density of 2.5×10^6 viable cells/well. 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.

Exposure to the Different Stimuli

Granulosa cells were treated in vitro with FSH, estradiol, β-naphthoflavone, α-naphthoflavone, or lactacystin, added alone or in combination, depending on the experimental design. Lactacystin and FSH were prepared in PBS, and estradiol, β-naphthoflavone, and α-naphthoflavone were dissolved in absolute ethanol (Merck KGaA, Darmstadt, Germany). Control cells were treated with vehicle only (final ethanol concentration in each well was $\leq 0.6\%$). Cells were incubated with the stimuli for 4, 12, 24, 36, or 48 h, depending on the experiment. Thereafter, cells were lysed directly in the culture dish, and protein and RNA were extracted following standard procedures.

For experiments involving removal of β-naphthoflavone from culture media, cells incubated with the flavone during 4 h were washed free of the compound by a series of five 20-min washes in 2 ml PBS-BSA 0.25% followed by a final 20-min wash in 2 ml PBS. Fresh medium containing vehicle was added to each medium, and incubation was allowed to proceed for 46 h.

Protein Extraction and Western Blot Analysis

Granulosa cells were cultured in P6 multiwell plates at a density of 2.5×10^6 viable cells/well. At different times of incubation with the stimuli, cells were lysed directly in the culture dish with TRIzol Reagent (Invitrogen, Molecular Research Center, Inc.) and protein extracted from the organic phase according to the manufacturer's instructions. The use of total cell lysates instead of subcellular fractions in the analysis of AHR expression allows us to evaluate the entire cellular pool of the receptor, without the concern that reduced levels of protein are due to the subcellular fractionation procedures. Forty micrograms of total protein were electrophoresed under reducing conditions in 8% polyacrylamide gels and electrotransferred to nitrocellulose membranes. To corroborate equal protein load in each lane, membranes were stained after transfer with Ponceau S. Aryl hydrocarbon receptor expression was assessed with standard Western blot techniques using an antibody against AHR raised in rabbit (SA-210, Biomol Research Laboratories Inc, PA, 1.5 μg/ml) and the appropriate peroxidase-conjugated second antibody (Amersham Biosciences, UK). The AHR antibody cross-reacted with a low molecular weight band of approximately 60 kDa, whose expression level did not vary significantly across the different treatments and was therefore used as internal control. Detection was performed with a chemioluminescence kit (DuPont NEN). Quantification of protein bands was performed with ImageQuant software (Amersham Biosciences, Sunnyvale, CA). Densitometric units obtained for the AHR band intensities were normalized to densitometric units obtained for the intensities of the internal control, and results were expressed relative to the values obtained for control cells.

Semiquantitative RT-PCR

Levels of *Cyp1a1* and *Ahr* 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 the indicated periods of time (for *Ahr*) of incubation with the different stimuli, cells were lysed directly in the culture dish with TRIzol Reagent (Invitrogen) and total RNA extracted according to the manufacturer's instructions (the organic phase of each sample was saved for protein extraction). Total RNA quantitation and purity determination was assessed by spectral absorption (A260/280) prior to RT reactions. The quality of the RNA was examined by assessing the integrity and ratio of the 28S and 18S rRNA bands after running an aliquot of the sample on a denaturing agarose gel stained with EtBr. Complementary DNA was synthesized from total RNA (1μg 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 hydroxylase and the receptor target cDNA were those described by Dasmahapatra et al. and Timsit et al., respectively [50, 51]. *Cyp1a1* and 18S Classic II primers:competimers (Quantum mRNA Ambion, Inc., used as internal control for normalization) generated fragments of 509 and 324 base pairs (bp), respectively. *Ahr* and 18S Classic primers:competimers (Quantum mRNA Ambion, Inc.) generated fragments of 917 and 488 bp, respectively.

One-microliter aliquots of the RT reaction were used to amplify *Cyp1a1* or *Ahr* 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. Amplification of *Cyp1a1* and 18S Classic II cDNA was performed for 33 cycles in the presence of 2 mM $MgCl_2$, each cycle consisting of 30 sec denaturation at 94°C, 30 sec annealing at 62°C, and 1 min extension at 72°C. Amplification of *Ahr* and 18S Classic cDNAs was performed for 24 cycles in the presence of 1.5 mM $MgCl_2$, each cycle consisting of 20 sec denaturation at 94°C, 20 sec annealing at 54°C, and 40 sec extension at 72°C. Ten microliters of the PCR reaction were electrophoresed in 2% (for *Cyp1a1*) or 1.5 % (for *Ahr*) 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.

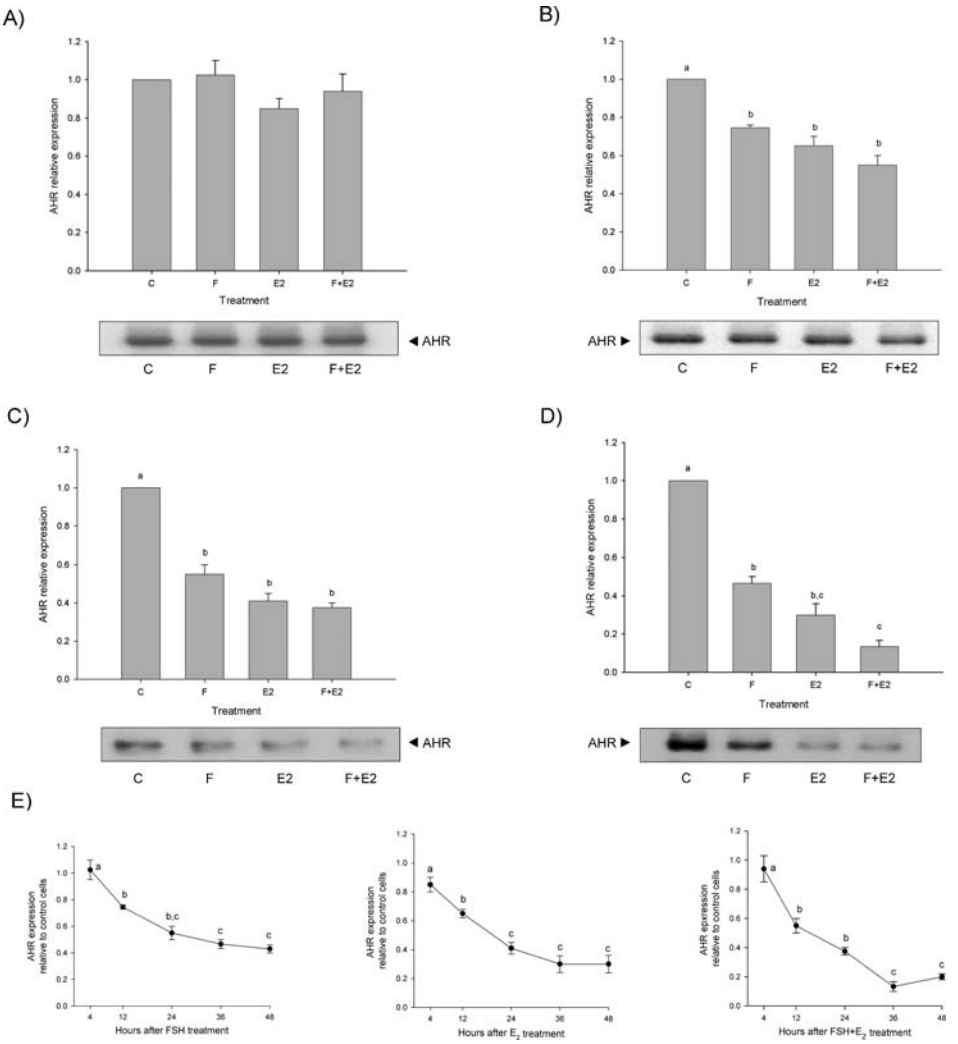

FIG. 1. AHR protein expression is decreased by FSH and estradiol in granulosa cells. Granulosa cells were cultured for 4 (**A**), 12 (**B**), 24 (**C**) or 36–48 h (**D**) in control medium (C), with FSH (2 ng/ml, F), with estradiol (100 ng/ml, E2), or with a combination of FSH and estradiol (F+E2). Total protein was isolated from whole extracts, and Western blot was conducted as described in Materials and Methods using an antibody that recognizes the AHR. Densitometric units obtained for the AHR band intensities (normalized to the values obtained for the internal control) were expressed relative to control cells and plotted as the mean ± SEM of three independent experiments. Values not sharing a common letter are significantly different: **B**) $P < 0.05$; **C**) $P < 0.001$, except for C vs. F, where $P < 0.01$; **D**) $P < 0.001$, except for C vs. F, where $P < 0.01$, and F vs. F+E2, where $P < 0.05$. Representative Western blots of AHR are shown below each corresponding graph. **E**) Changes in AHR protein content 4, 12, 24, 36, or 48 h after stimulation with FSH (left panel), estradiol (E_2, middle panel), or a combination of FSH and estradiol ($F+E_2$, right panel). Values not sharing common letters are statistically different, with the following *P* values: left panel: $P < 0.05$ for 4 h vs. 12 h and 12 h vs. 36, $P < 0.01$ for 12 h vs. 48 h, and $P < 0.001$ for all other comparisons; middle panel: $P < 0.01$ for a vs. c and a vs. d, $P < 0.05$ for all other comparisons; right panel: $P < 0.05$ for 24 h vs. 36 h, $P < 0.01$ for 4 h vs. 12 h, 12 h vs. 36 h, and 12 h vs. 48 h, and $P < 0.001$ for all other comparisons.

Statistical Analysis

Treatments were applied to at least duplicate wells in each of three 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 Tukey-Kramer test for multiple comparisons after logarithmic transformation of data when necessary [52].

RESULTS

Total Cellular Content of AHR Protein Is Reduced by FSH and Estradiol in Granulosa Cells

With the aim of determining if FSH and estradiol can regulate the expression of AHR in granulosa cells, we assessed by immunoblotting the content of AHR protein in total cell lysates after treatment with the gonadotropin, the estrogen, or a combination of both hormones. As can be seen in Figure 1A, 4 h treatment with the hormones had no effect on AHR protein levels. Same results were obtained when incubation was allowed to proceed for 6 h (data not shown). However, treatment with FSH or estradiol for 12 h was able to induce a ~25% or ~30% reduction in total AHR protein levels, respectively (Fig. 1B). Treatment with both hormones reduced by approximately 40% the total protein content of AHR in granulosa cells (Fig. 1B). Nevertheless, no significant differences were found among the effects elicited by the gonadotropin, the estrogen, or the combination of both hormones. The reduction in AHR protein content induced by FSH and estradiol was also verified after 24 h of treatment (Fig. 1C). When incubation was allowed to proceed for 36–48 h, the combination of FSH and estradiol decreased AHR protein levels to ~20% of control cells. Addition of the gonadotropin alone produced ~60% reduction in the receptor levels (Fig. 1D). Figure 1E shows the time course of changes in AHR protein levels following stimulation with the hormones.

Effect of FSH and Estradiol on Ahr mRNA Levels

To find out if the inhibition of AHR expression elicited by FSH and estradiol is caused, at least in part, by reduced *Ahr* mRNA levels, the steady-state levels of those transcripts were determined by semiquantitative RT-PCR. We observed that treatment with FSH for 12 h, both when added alone and in combination with estradiol, produces a marked reduction in the receptor mRNA levels (Fig. 2A). The inhibition elicited by FSH or a combination of FSH and estradiol on *Ahr* transcript levels was also verified after 24 or 36–48 h of treatment, though its magnitude was not as great as that observed when incubation was performed for 12 h (Fig. 2, B and C). Addition of estradiol alone had no effect on *Ahr* transcripts steady-state levels after 12 h treatment (Fig. 2A). However, 24 or 36–48 h of treatment with the estrogen induced a marked decrease in *Ahr* levels (Fig. 2, B and C).

Proteasomal Degradation Mediates the Reduction in AHR Protein Levels induced by 12 h Treatment with Estradiol

In order to determine which mechanism would be responsible for the reduced levels of AHR protein observed after 12 h treatment with estradiol, we investigated whether enhanced proteasomal degradation was taking place in estrogen-treated cells. Figure 3 shows the results of experiments using the 26S proteasome specific inhibitor lactacystin in granulosa cells cultured for 12 h in control conditions or with estradiol, either added alone or in the presence of FSH. When proteasomal activity was inhibited, the AHR degradation elicited by estradiol was almost completely blocked (Fig. 3B). On the other hand, lactacystin was not able to reverse the reduction of AHR levels elicited by FSH or the combination of both hormones (Fig. 3, A and C, respectively).

Total Cellular Content of the AHR Protein Is Rapidly Reduced by β-Naphthoflavone in Granulosa Cells

In order to determine if AHR protein expression levels are regulated by its own agonists in granulosa cells, we measured by immunoblotting the content of AHR protein in total cell lysates after treatment with 10 μM β-naphthoflavone. We have previously corroborated that the flavone at this dose acts as an AHR agonist in our system [12]. As shown in Figure 4, 4 h treatment with β-naphthoflavone produced a marked reduction in total immunodetectable AHR protein. Interestingly, cotreatment with α-naphthoflavone at doses at which it functions as an AHR antagonist in this system [12] was not able to reverse the decrease in total AHR protein content elicited by β-naphthoflavone. Moreover, addition of α-naphthoflavone alone at doses of 1 μM or 0.5 μM reproduced the effect of β-naphthoflavone, causing a clear reduction in total AHR content as well (Fig. 4B). The described action of β-naphthoflavone was also verified in the presence of FSH and estradiol (Fig. 4A).

As can be seen in Figure 5A, the levels of total cellular AHR remained depressed even after 36 h of incubation with the agonist, at which time the reduction in protein content was even more dramatic. Alpha-naphthoflavone was also not able to reverse the effect of the agonist. The depletion of AHR protein caused by β-naphthoflavone after 36 h of incubation was verified both in control conditions and in the presence of FSH and estradiol (Fig. 5A). Reversal of agonist-induced downregulation of AHR protein was verified by assessing recovery of protein levels after ligand removal. Removal of β-naphthoflavone from the culture media after 4 h of stimulation produced a ~3-fold recovery in AHR protein after 50 h of incubation (Fig. 5B).

Ahr *mRNA Steady-state Levels Are Induced by β-Naphthoflavone in Granulosa Cells*

In those systems where AHR ligands reduce the expression of their own receptor, the downregulation of the protein has been shown not to be due to reductions in *Ahr* mRNA [33]. In order to determine if this is also the case for the described effect of β-naphthoflavone in granulosa cells, we assessed by means of semiquantitative RT-PCR the steady-state levels of *Ahr* mRNA in our system. As can be seen in Figure 6A, 4 h treatment with β-naphthoflavone had no effect on *Ahr* transcript levels, whether added alone or in the presence of FSH and estradiol. On the contrary, treatment with the AHR agonist for 48 h resulted in an increase in *Ahr* mRNA steady-state levels, an effect that was also verified in the presence of FSH and estradiol when compared with the corresponding control (Fig. 6B).

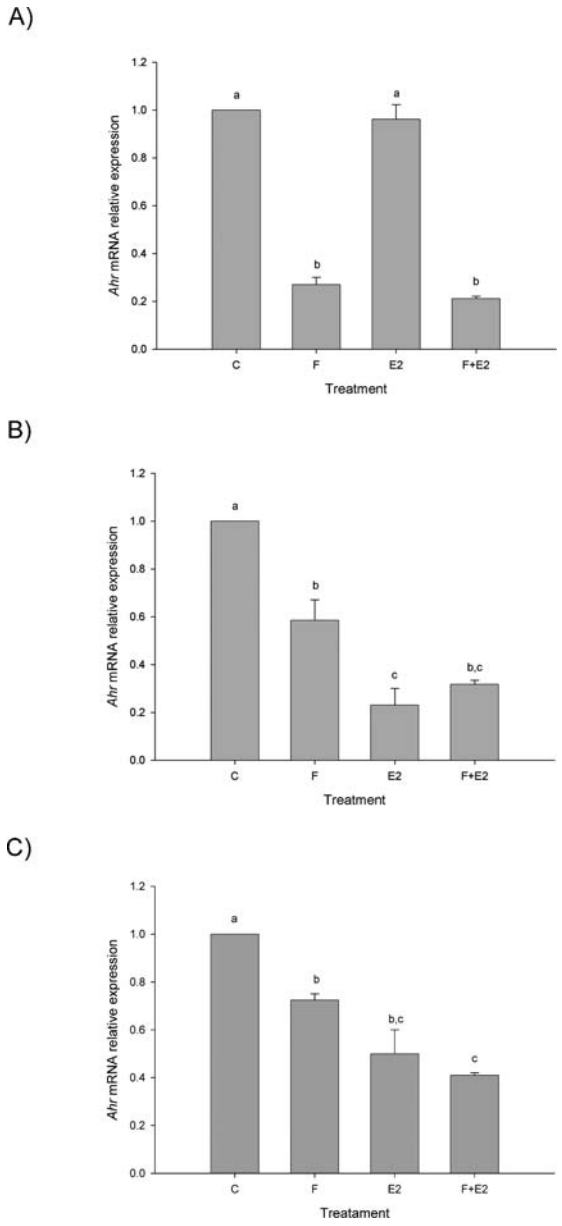

FIG. 2. Levels of *Ahr* transcripts are reduced by treatment with FSH and estradiol. Granulosa cells were cultured in control medium (C), with FSH (2 ng/ml, F), with estradiol (100 ng/ml, E2), or with a combination of FSH and estradiol (F+E2). After 12 (**A**), 24 (**B**), or 36–48 h (**C**) of incubation with the different stimuli, total RNA was extracted and semiquantitative RT-PCR for *Ahr* mRNA 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 three independent experiments. Values not sharing a common letter are significantly different: **A**) $P < 0.001$; **B**) $P < 0.01$, except for C vs. F and F vs. E2, where $P < 0.05$; **C**) $P < 0.01$, except for C vs. F+E2, where $P < 0.001$.

Proteasomal Degradation Mediates the Reduction in AHR Protein Levels Induced by β-Naphthoflavone in Granulosa Cells

It has been reported that the AHR downregulation observed in a number of systems after ligand exposure is due to 26S proteasome degradation [29, 33]. In order to establish if that mechanism is also responsible for the reduction in AHR protein levels found in granulosa cells after treatment with β-naphthoflavone, we assessed total AHR protein levels in cell cultures

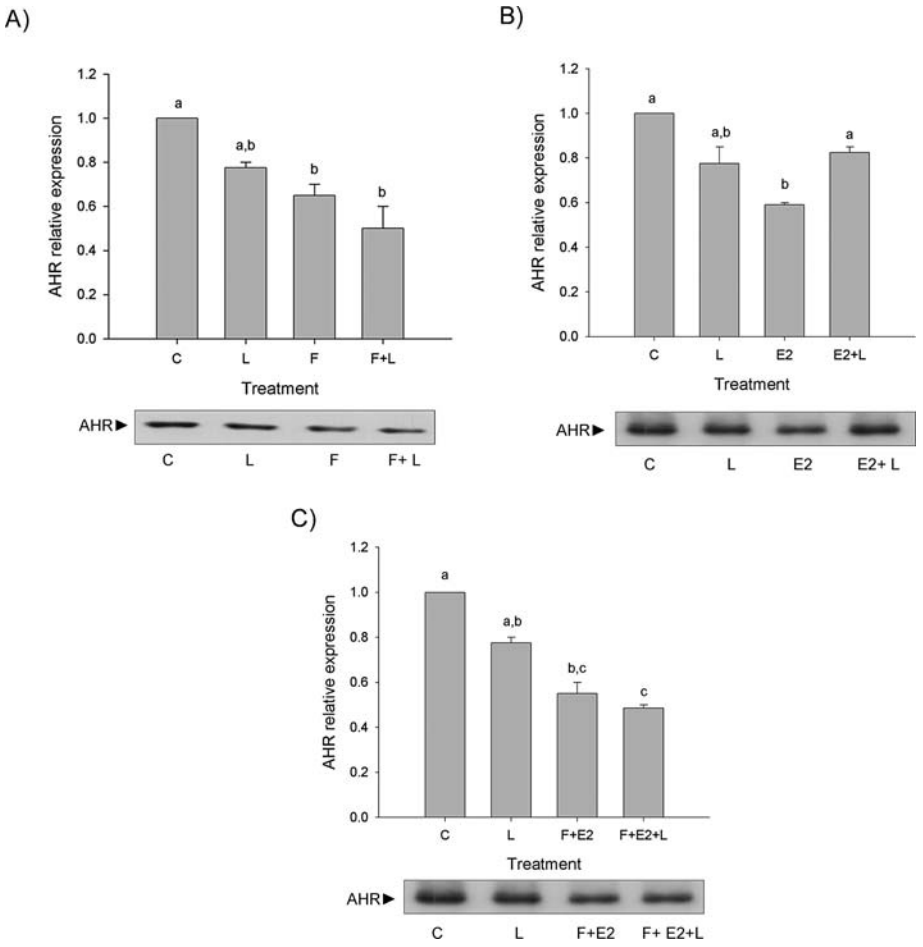

FIG. 3. Proteasomal inhibition reverses the decrease in AHR protein levels induced by 12 h treatment with estradiol. Granulosa cells were cultured in control medium (C), with the 26S proteasome inhibitor lactacystin (20 μM, L), with FSH (2 ng/ml, F), with estradiol (100 ng/ml, E2), or with a combination of FSH and estradiol (F+E2). Hormones were added either alone or in the presence of 20 μM lactacystin (F+L, E2+L, F+E2+L, respectively). After 12 h of incubation with the different stimuli, total protein was isolated from whole extracts and Western blot was conducted as described in *Materials and Methods* using an antibody that recognizes the AHR. Densitometric units obtained for the AHR band intensities (normalized to the values obtained for the internal control) were expressed relative to control cells and plotted as the mean ± SEM of three independent experiments. Values not sharing a common letter are significantly different ($P < 0.05$, except for the following comparisons: **B**) C vs. E2, $P < 0.01$; **C**) C vs. F+E2 and C vs. F+E2+L, $P < 0.01$). Representative Western blots of AHR are shown below each graph.

stimulated with the agonist in the presence of the 26S proteasome inhibitor lactacystin. As shown in Figure 7A, lactacystin completely abolishes the reduction in protein levels elicited by β-naphthoflavone after 4 h of incubation. Thus, the agonist is not able to reduce AHR protein levels when proteasomal activity is blocked. When incubation was allowed to proceed for 24 h, the proteasome inhibitor partially reversed the effect of the AHR agonist (β-naphthoflavone induced 50% of the reduction elicited in the absence of lactacystin, Figure 7B).

Inhibition of AHR Proteasomal Degradation Superinduces Cyp1a1 *mRNA Levels*

In those systems where AHR agonists elicit their own receptor proteasomal degradation, this mechanism has been shown to serve as a means of controlling the activity of ligand-activated AHR in the nucleus [33]. The fact that β-naphthoflavone induces both activation and degradation of the AHR in granulosa cells would indicate that downregulation of the AHR would serve a role in the attenuation of the gene regulatory response elicited by the agonist in our system as well. To verify the validity of this model in granulosa cells, we examined the effect of the proteasome inhibitor on the induction of the endogenous *Cyp1a1* gene expression by β-naphthoflavone, a well-characterized transcriptional response mediated by the AHR that has been extensively utilized to assess the activation of AHR-mediated signal transduction (reviewed in [1, 29, 53, 54]). As shown in Figure 8, while β-naphthoflavone induced *Cyp1a1* mRNA ~4.5-fold, cotreatment with the AHR agonist and lactacystin enhanced the induction to nearly 4-fold higher, as assessed by semiquantitative RT-PCR (~4-fold higher levels of *Cyp1a1* mRNA in cells exposed to the agonist and lactacystin compared to agonist-treated cells in which the AHR degradation was not blocked). Interestingly, treatment with lactacystin alone produced a ~2.5-fold increase in *Cyp1a1* mRNA steady-state levels when compared to control cells (Fig. 8).

DISCUSSION

The AHR is a ligand-activated transcription factor responsive to both natural compounds and exogenous contaminants. This receptor and orthologs are widely and ubiquitously expressed in diverse tissues throughout dissimilar groups of vertebrates and invertebrates [1, 55]. While several lines of evidence suggest that the AHR plays a pivotal role in reproduction (for instance, in follicular development) (reviewed in [9]) [10], its complex physiological function remains still largely elusive.

Activation of the AHR by binding of exogenous ligands such as environmental contaminants is associated with a wide range of adverse biological actions; particularly, the toxicity on the reproductive system (ovarian follicle loss, alteration of proliferation and steroidogenesis, oocyte apoptosis, etc.) is well documented (reviewed in [3, 56]) [11]. There are also several reports of endogenous AHR activation, and numerous studies strongly suggest the presence of an endogenous ligand for this receptor [9, 15–24]. However, the identity of the physiological ligand has not been yet unequivocally identified.

Although information regarding physiological regulation of AHR expression is of crucial importance for understanding its function, there are only limited data in this area. The existing reports point to the presence of endogenous modulators and

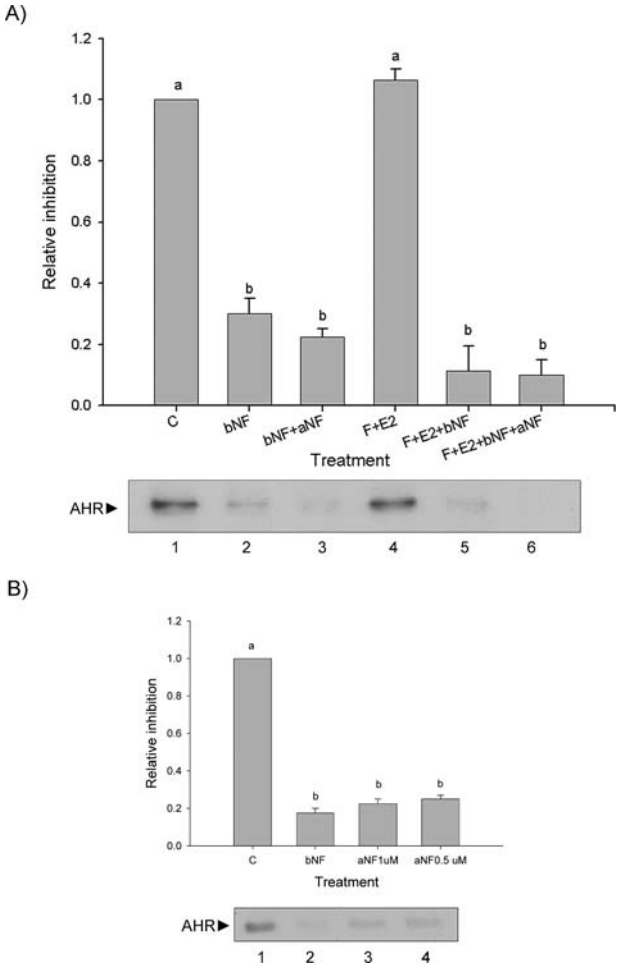

FIG. 4. Total cellular content of the AHR protein drops rapidly after exposure of granulosa cells to β-naphthoflavone. **A**) Granulosa cells were cultured for 4 h in control medium (C), with 10 μM β-naphthoflavone, added either alone (bNF) or in combination with 1 μM α-naphthoflavone (bNF+aNF), or in medium containing FSH and estradiol (2 ng/ml and 100 ng/ml, respectively) either in the absence (F+E2) or in the presence of β-naphthoflavone 10 μM added alone (F+E2+bNF) or in combination with 1 μM α-naphthoflavone (F+E+bNF+aNF). **B**) Granulosa cells were cultured for 4 h in control medium (C), with 10 μM β-naphthoflavone (bNF), with 1 μM α-naphthoflavone (aNF1uM), or with 0.5 μM α-naphthoflavone (aNF0.5uM). Total protein was isolated from whole extracts, and Western blot was conducted as described in *Materials and Methods* using an antibody that recognizes the AHR. Densitometric units obtained for the AHR band intensities (normalized to the values obtained for the internal control) were expressed relative to control cells and plotted as the mean ± SEM of four independent experiments. Values not sharing a common letter are significantly different ($P < 0.001$). **A**) Lower panel: a representative Western blot of AHR is shown. Lane 1: granulosa cells cultured in control medium; lane 2: granulosa cells cultured in the presence of 10 μM β-naphthoflavone; lane 3: granulosa cells cultured in the presence of 10 μM β-naphthoflavone and 1 μM α-naphthoflavone; lane 4: granulosa cells cultured with FSH and estradiol; lane 5: granulosa cells cultured in medium containing 10 μM β-naphthoflavone in the presence of FSH and estradiol; lane 6: granulosa cells cultured in medium containing 10 μM β-naphthoflavone and 1 μM α-naphthoflavone in the presence of FSH and estradiol. **B**) Lower panel: a representative Western blot of AHR is shown. Lane 1: granulosa cells cultured in control medium; lane 2: granulosa cells cultured in the presence of 10 μM β-naphthoflavone; lane 3: granulosa cells cultured in the presence of 1 μM α-naphthoflavone; lane 4: granulosa cells cultured in the presence of 0.5 μM α-naphthoflavone.

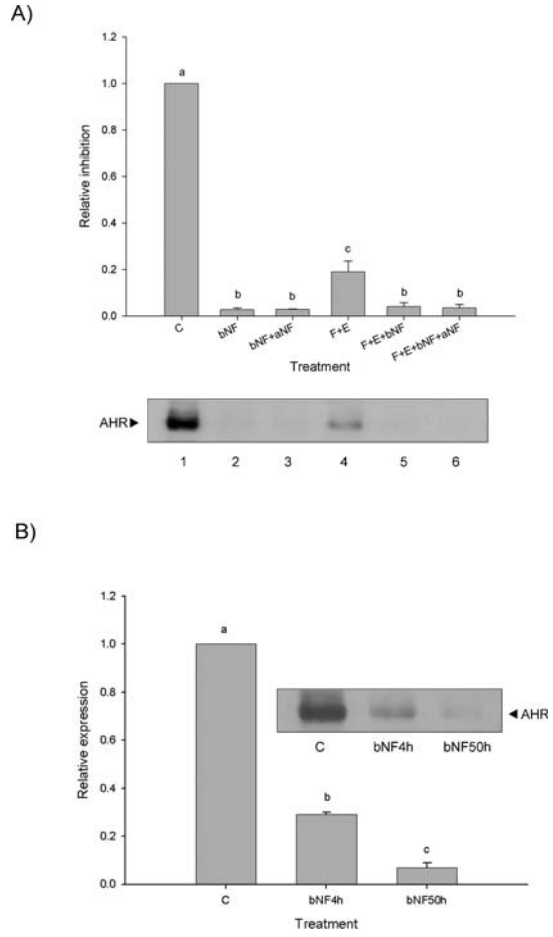

FIG. 5. **A**) β-naphthoflavone-induced AHR depletion persists after 36 h of incubation. Granulosa cells were cultured in control medium (C), with 10 μM β-naphthoflavone, added either alone (bNF) or in combination with 1 μM α-naphthoflavone (bNF+aNF), or in medium containing FSH and estradiol (2 ng/ml and 100 ng/ml, respectively) either in the absence (F+E2) or in the presence of β-naphthoflavone 10 μM added alone (F+E2+bNF) or in combination with 1 μM α-naphthoflavone (F+E+bNF+aNF). After 36 h of incubation, cells were lysed, total protein was isolated from whole extracts, and Western blot was conducted as described in *Materials and Methods* using an antibody that recognizes the AHR. Densitometric units obtained for the AHR band intensities (normalized to the values obtained for the internal control) were expressed relative to control cells and plotted as the mean ± SEM of five independent experiments. Values not sharing a common letter are significantly different ($P < 0.001$, except b vs. c, $P < 0.05$). Lower panel: A representative Western blot of AHR is shown. Lane 1: granulosa cells cultured in control medium; lane 2: granulosa cells cultured in the presence of 10 μM β-naphthoflavone; lane 3: granulosa cells cultured in the presence of 10 μM β-naphthoflavone and 1 μM α-naphthoflavone; lane 4: granulosa cells cultured with FSH and estradiol; lane 5: granulosa cells cultured in medium containing 10 μM β-naphthoflavone in the presence of FSH and estradiol; lane 6: granulosa cells cultured in medium containing 10 μM β-naphthoflavone and 1 μM α-naphthoflavone in the presence of FSH and estradiol. **B**) Recovery of AHR protein levels after ligand removal. Granulosa cells were cultured in control medium (C) or treated with 10 μM β-naphthoflavone during 4 h (bNF4h) or during 50 h (bNF50h). After 50 h of incubation, cells were lysed, total protein was isolated from whole extracts, and Western blot was conducted as described in *Materials and Methods* using an antibody that recognizes the AHR. Densitometric units obtained for the AHR band intensities (normalized to the values obtained for the internal control) were expressed relative to control cells and plotted as the mean ± SEM of three independent experiments. Values not sharing a common letter are significantly different ($P < 0.001$, except b vs. c, $P < 0.01$). Inset: A representative Western blot of AHR is shown.

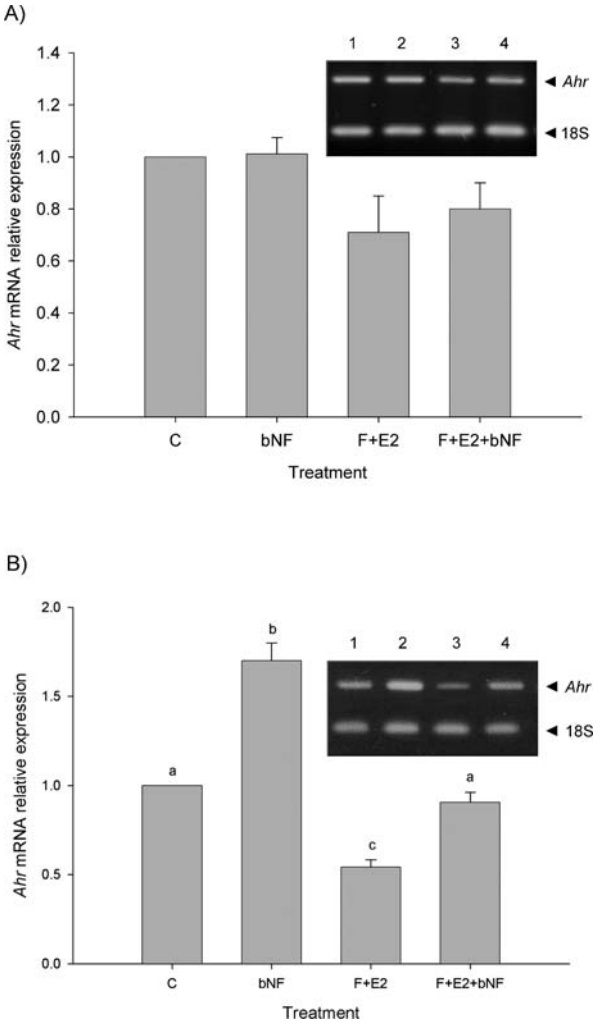

FIG. 6. Effect of β-naphthoflavone on *Ahr* transcript levels in granulosa cells. Granulosa cells were cultured in control medium in the absence (C) or presence of 10 μM β-naphthoflavone (bNF), or in medium containing FSH and estradiol (2 ng/ml and 100 ng/ml, respectively) either alone (F+E2) or in the presence of β-naphthoflavone 10 μM (F+E2+bNF). After 4 h (**A**) or 48 h (**B**) of incubation, cells were lysed and total RNA extraction and semiquantitative RT-PCR for *Ahr* 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 three independent experiments. Values not sharing a common letter are significantly different ($P < 0.01$, except a vs. c, $P < 0.05$; b vs. c, $P < 0.001$). Insets: A representative electrophoretic separation of the semiquantitative RT-PCR products is shown. Lane 1: granulosa cells cultured in control medium; lane 2: granulosa cells cultured in the presence of 10 μM β-naphthoflavone; lane 3: granulosa cells cultured with FSH and estradiol; lane 4: granulosa cells cultured in medium containing 10 μM β-naphthoflavone in the presence of FSH and estradiol.

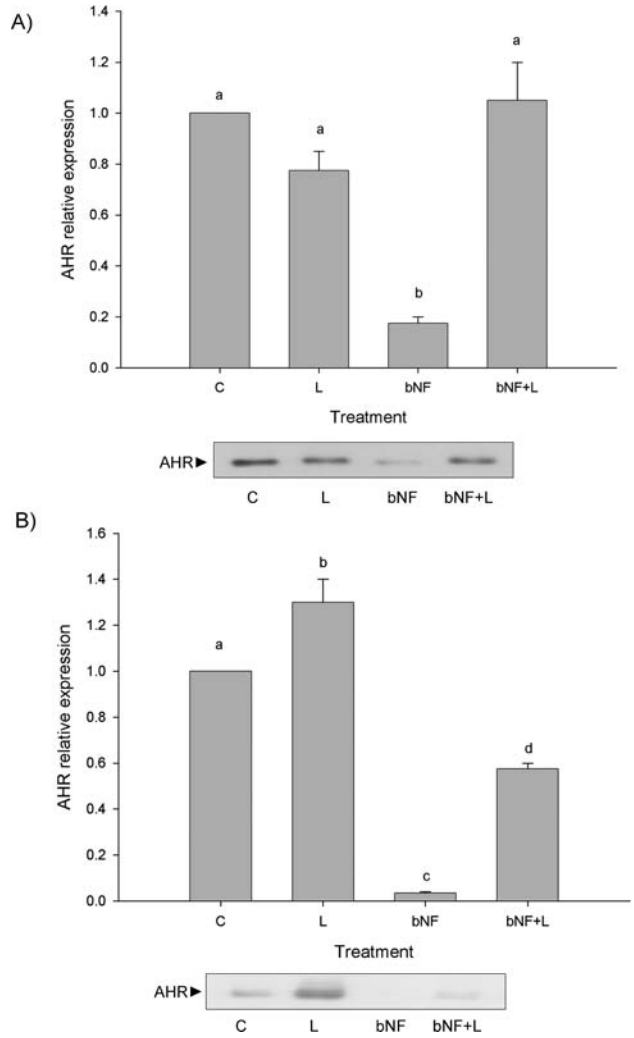

FIG. 7. Proteasomal inhibition reverses β-naphthoflavone-induced AHR degradation in granulosa cells. Granulosa cells were cultured in control medium (C), with the 26S proteasome inhibitor lactacystin (20 μM, L) or with 10 μM β-naphthoflavone either added alone (bNF) or in the presence of 20 μM lactacystin (bNF+L). After 4 (**A**) or 24 h (**B**) of incubation, cells were lysed, total protein isolated from whole extracts, and Western blot conducted as described in Materials and Methods using an antibody that recognizes the AHR. Densitometric units obtained for the AHR band intensities (normalized to the values obtained for the internal control) were expressed relative to control cells and plotted as the mean ± SEM of three independent experiments. Values not sharing a common letter are significantly different: **A**) L vs. bNF, $P < 0.05$; bNF vs. bNF+L, $P < 0.01$; **B**) $P < 0.01$, except for a vs. b, $P < 0.05$; b vs. c, $P < 0.001$; c vs. d, $P < 0.05$. Lower panels: a representative Western blot of AHR is shown.

support the idea of an endocrine regulation of the receptor. This background prompted us to study the effect of the classical hormones FSH and estradiol on AHR expression in granulosa cells isolated from immature female rats. As these animals are not yet cycling, estrogen and gonadotropin levels can be manipulated experimentally, making this model useful for our study.

We have found that both FSH and estradiol, key stimuli for granulosa cell function and for follicular development, induce a decrease in AHR protein expression. This inhibitory effect was modest but statistically significant within 12 h of stimulation, increasing thereafter in a time-dependent fashion. The decrease in AHR protein expression observed after 12 h treatment with FSH or after 24 or 36 to 48 h of treatment with the different hormones or with a combination of them can be attributed, at least in part, to reduced *Ahr* transcript levels (due to inhibition of *Ahr* gene transcription or reduced mRNA stability). However, estradiol added alone had no effect on *Ahr* mRNA steady-state levels within 12 h of stimulation. This is in agreement with findings in the rat liver, where administration of the estrogen in vivo does not alter hepatic *Ahr* mRNA, despite a dramatic drop of these transcripts observed on the evening of proestrus [46]. The results of the experiments using lactacystin indicate that the reduction in AHR protein levels elicited by 12 h treatment with estradiol would be ascribed to proteasomal degradation triggered by the estrogen. This finding seems to be in contrast with reports in MCF-7 cells, where

estradiol did not affect AHR expression levels in the cell line [57]. A possible explanation for these apparent opposing results is that in the above-mentioned study stimulation with estradiol was performed for 3 h, at which time point there was no evidence of AHR protein degradation in our system either (data not shown). Hence, estrogen-elicited proteasomal degradation would be a phenomenon which does not take place immediately. Besides, differences between actions of estradiol mediated by estrogen receptor beta (present in granulosa cells) and estrogen receptor α cannot be ruled out. Our results suggest that the decrease in *Ahr* transcripts observed in ovarian tissue on the evening of proestrus [46] could be ascribed to actions elicited by FSH. The inhibitory action elicited by FSH in our system is in agreement with the effect described in seminiferous tubules of the rat testis, where the gonadotropin reverses the upregulation of these transcripts observed when cells are cultured in serum-free medium [58].

The results regarding hormonal regulation of AHR expression indicate that FSH and estradiol are able to modulate AHR expression in granulosa cells in a way that mirrors the changes observed in the ovary across the rat estrous cycle [46]. Therefore, it can be speculated that these hormones may be the endogenous physiological modulators of AHR expression responsible for the changes observed in vivo. However, regulatory actions of locally produced ovarian growth factors, progestins, glucocorticoids, or any other factor whose levels change through the reproductive cycle cannot be ruled out. In any case, our study indicates that FSH and estradiol, either through direct or indirect actions, might be important regulators of AHR function in the ovary. As there is abundant evidence of the existence of physiological activation of the AHR and for the presence of endogenous ligands for this receptor [9, 15–24], it is reasonable to hypothesize that the attenuation of AHR expression exerted by the hormones would counteract the well-established endocrine-disruptive effects that the activation of this receptor might have in the ovary (mainly alteration of proliferation and steroidogenesis, as mentioned above). Thus, our findings on AHR regulation by FSH and estradiol may be of important physiological significance, since the described declines in AHR expression would allow appropriate granulosa cell growth and differentiation, which would lead to proper follicular development. However, several studies show that AHR plays central roles in ovarian physiology and that its expression is critical for accurate ovarian function (reviewed in [9]) [10]. Indeed, deletion of *Ahr* gene results in slower follicular growth in *Ahr* -/- mice [59]. Since FSH and estradiol are known survival and proliferation hormones for granulosa cells, their inhibitory effect on AHR expression reported herein would seem to contrast with the findings obtained from female mice lacking the receptor. This apparent potential discrepancy would be explained in terms of the level of AHR expression needed for accurate follicular growth or the time-course and extent of the response required for proper development of the follicle. In fact, enhanced or sustained activation of AHR can lead for instance to ovarian follicle loss, inhibition of proliferation and steroidogenesis, or blockade of ovulation, as mentioned above. Therefore, AHR should be expressed at certain levels and at appropriate times and cell types during follicular development, allowing a coordinated interplay between the different signal pathways that regulate ovarian function. Knowledge of the exact mechanisms by which the AHR regulates granulosa cell proliferation in the absence of exogenous ligands will help in understanding this issue. Future in vivo studies are warranted to further examine the regulation of AHR expression by FSH and estradiol over the course of folliculogenesis.

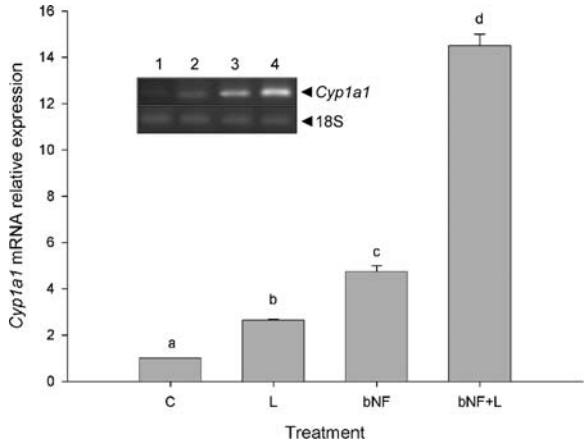

FIG. 8. Proteasomal inhibition enhances β-naphthoflavone induction of *Cyp1a1* transcripts. Granulosa cells were cultured for 24 h in control medium (C), with the 26S proteasome inhibitor lactacystin (20 μM, L) or with 10 μM β-naphthoflavone either added alone (bNF), or in the presence of 20 μM lactacystin (bNF+L). 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 three independent experiments. Values not sharing a common letter are significantly different ($P < 0.001$, except a vs. b and b vs. c, $P < 0.05$; a vs. c, $P < 0.01$). Inset: A representative electrophoretic separation of the semiquantitative RT-PCR products is shown. Lane 1: granulosa cells cultured in control medium; lane 2: granulosa cells cultured in the presence of 20 μM lactacystin; lane 3: granulosa cells cultured in the presence of 10 μM β-naphthoflavone; lane 4: granulosa cells cultured in the presence of 10 μM β-naphthoflavone and 20 μM lactacystin.

Regarding the regulation of AHR expression by its own ligands, the results reported herein show that AHR protein is rapidly downregulated in granulosa cells after treatment with the receptor agonist β-naphthoflavone, both when added alone and in combination with FSH and estradiol. It was of special interest to evaluate the effect of the agonist added together with the hormones, since they are present in granulosa cell milieu in vivo and could have modulated the ligand-induced regulation of AHR expression. The described reduction in AHR protein levels exerted by β-naphthoflavone was prolonged over time, resulting in agonist-treated cells having almost no immunodetectable receptor protein after 36 h of treatment. Moreover, our results strongly suggest that ligand-induced reduction in AHR total cellular content is not due to decreased *Ahr* transcript levels but to protein degradation. This degradation would be mediated via the 26S proteasome pathway, since the effect was completely abolished by 4 h cotreatment with a specific proteasome inhibitor, and partially reversed when exposure was allowed to proceed for 24 h. This latter observation would be due to degradation of lactacystin in the culture medium after longer periods of time, or to excessive degradation that cannot be counteracted by the doses of proteasome inhibitor used in the experiments. Our findings are in agreement with observations made in a variety of systems after exposure to different AHR agonists, where the receptor protein is rapidly degraded via proteasomal activity (reviewed in [29, 33]) [60]. Yet, positive regulation of the receptor expression by AHR ligands has been also shown, both in vivo and in vitro [30–34]. These differences would arise from the nature of the ligand, from the doses and time used in each experimental protocol, or from intrinsic differences in the cell type or species under study. In addition, we demonstrate that besides downregulating AHR protein, the receptor agonist induces an increase in the receptor

transcript levels when treatment is prolonged, which would constitute a regulatory mechanism to replenish the AHR cellular pool in granulosa cells. In this sense, we observed a ~3-fold recovery in AHR protein content after 50 h of incubation when the agonist was removed form the culture media following 4 h of stimulation.

Our results with the use of the receptor antagonist α-naphthoflavone add further support to the notion that the proteolytic machinery present in the cytoplasmic compartment would be sufficient to degrade the AHR, and that nuclear translocation, binding with ARNT, or DNA binding are not necessary for efficient degradation of the AHR [61]. The proteolytic degradation of transcription factors is an established mechanism of regulating signal transduction pathways (reviewed in [62]). The analysis of the induction of endogenous *Cyp1a1* mRNA shows that inhibition of the proteasome enhances the induction of those transcripts by β-naphthoflavone. This would indicate that ligand-induced AHR protein degradation in granulosa cells would serve a role in regulating the activity of the receptor in the nucleus by controlling the amount of ligand-activated AHR so that transcription of the target genes can be maintained at a certain level. It has been demonstrated in other systems that this phenomenon serves for turning off AHR signal as well [33]. We found that inhibition of proteasome in the absence of exogenous AHR ligands induces *Cyp1a1* mRNA levels. This would be considered further evidence for the presence of an endogenous ligand, which would be maintaining AHR transcriptional activity at a determined level in granulosa cells thorough ligand-induced degradation.

The effects of AHR activation on the receptor expression levels reported herein might reflect the enhanced normal AHR function; might be specific actions that environmental contaminants can exert through the xenobiotic-AHR complex, unrelated to endogenous AHR function; or might represent a combination of endogenous and exogenous functions.

Collectively, the results of the present study indicate that FSH and estradiol are important modulators of AHR expression in granulosa cells, suggesting that these hormones would be important cycle-associated endogenous factors that regulate AHR levels in vivo. This modulation might be needed in order to accomplish correct follicular growth and differentiation. Moreover, we report that while ligands of this receptor rapidly degrade AHR protein in granulosa cells, attenuating the signal, they induce the mRNA levels for the receptor when incubation is allowed to proceed longer.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the exceptional and generous contribution of Dr. Leonardo E. Bussmann; we thank him for his selfless support and valuable advice during the course of this work. Special thanks to Juan Manuel Pérez Sáez for his excellent and unconditional assistance during experiments and for his helpful comments and suggestions throughout this study.

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