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Interaction between the aryl hydrocarbon receptor and transforming growth factor-β signaling pathways: Evidence of an asymmetrical relationship in rat granulosa cells

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ABSTRACT

The aryl hydrocarbon receptor (AHR) mediates toxic responses to environmental contaminants and plays pivotal physiological roles in various biological processes as well, particularly in ovarian function. It is well documented that expression and function of the AHR is negatively regulated by transforming growth factor-β (TGF-β) in many cell types. In addition, several studies indicate that AHR activity inhibits TGF-β expression and function in some systems. However, the interplay between these two signals is highly dependent upon the cell type being studied, precluding a generalization about the outcome of such interaction. Therefore, the goal of the present study was to determine the effect of TGF-β on AHR expression and activation in granulosa cells, an ovarian cell type where the growth factor is mitogenic and AHR activation has been associated with promotion of proliferation as well. In addition, we conducted experiments aimed at evaluating the effect of AHR ligands on TGF-β action in our system. Results presented herein demonstrate that AHR expression is not regulated by TGF-β in rat granulosa cells, neither at the mRNA level nor at the protein level. Moreover, we find that the growth factor does not alter the transcriptional function of the AHR. Conversely, we show that activation of AHR by an agonist deregulates TGF-β function in granulosa cells, inhibiting its transcriptional activity and its mitogenic action. The described one-sided interplay between TGF-β and AHR signaling pathway may help provide a mechanistic explanation to some of the physiological outcomes of AHR or TGF-β activation in granulosa cells.

1. Introduction

The aryl hydrocarbon receptor (AHR) is a highly conserved member of the basic helix-loop-helix/Per-Arnt-Sim family of transcription factors, which are activated by binding of specific ligands. This receptor mediates most of the toxic and biological responses elicited by different aromatic compounds, such as 2,3,7,8-tetrachlorodibenzeno-*p*-dioxin (TCDD), β-naphthoflavone (5,6-benzophlavone) or dibenzofurans ([1], and references therein). It plays key roles in the adaptive metabolic response to

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Abbreviations: AHR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon nuclear translocator; CYP1A1, cytochrome P450 1A1; CYP1A2, cytochrome P450 1A2; DES, diethylstilbestrol; DMEM, Dulbecco modified Eagle medium; FGF, fibroblast growth factor; FSH, follicle stimulating hormone; MAPKs, mitogen activated protein kinases; PAI-1, plasminogen activator inhibitor type I; PDGF, platelet derived growth factor; TCDD, 2,3,7,8-tetrachlorodibenzeno-*p*-dioxin; TGF-β, transforming growth factor-β; TGIF, 5′-TG-3′-interacting factor; XREs, xenobiotic response elements.

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these compounds and in the toxic mechanisms triggered by them. Several of the described AHR ligands are widespread and persistent environmental contaminants, which exert diverse effects on reproductive, developmental, nervous and immune systems [2]. Besides its function as a receptor for xenobiotics, the AHR is also responsive to natural compounds and endogenous physiological signals; it is actually involved in normal development of several organs and plays multiple roles in fundamental cell biology and physiology [1,3,4].

Upon binding agonists, the AHR translocates from the cytoplasm to the nucleus where it binds its dimerization partner, 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 the AHR target genes such as the xenobiotic metabolizing enzymes CYP1A1 (Cytochrome P450 1A1, EC 1.14.14.1) and CYP1A2 (Cytochrome P450 1A2, EC 1.14.14.1), and many other genes involved in cellular function [5].

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 [6]. AHR activation has been shown to produce important alterations in female reproduction but also to be implicated in normal ovarian function [7,4]. 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, leading for instance to enhanced proliferation of the ovarian cells [8,9].

Several studies have focused on the regulation of the AHR and have shown that the receptor levels and activity are modulated by the receptor's own ligands and by many other different chemical agents [10,11]. In addition, there is solid evidence that AHR expression and function is subjected to physiological regulation, and it has been previously demonstrated that this receptor can be regulated by endogenous biological factors, such as serum, hormones, glucocorticoids and growth factors [10–12]. It has also been reported that AHR levels vary widely across different tissues and among different cell types, and that its expression fluctuates in ovarian tissue depending on the reproductive status [13,14]. Regarding regulation of AHR function, the interaction between AHR and TGF-β is of special interest [3,10].

Transforming growth factor-beta is a member of a large family of structural related ligands that regulates a variety of cellular processes, including cell-cycle progression, cell differentiation, reproductive function and development, among others [15]. After interaction of TGF-β with membrane-bound serine–threonine receptors, activation of one or more intermediaries known as Smads takes place, and then the activated complex of different Smads proteins translocates to the nucleus to promote altered gene expression [16,17]. Transforming growth factor-β plays a key role in the regulation of ovarian function, since it exerts crucial paractine/autocrine actions that impact on granulosa cell proliferation and differentiation [16–18]. In particular, TGF-β is a potent inducer of rat immature granulosa cell proliferation, being its co-mitogenic action with FSH the most remarkable effect [19].

Several lines of evidence indicate that the AHR is a negative regulator of TGF-β levels in various systems [10]. Conversely, it has been shown that TGF-β downregulates AHR expression and function in a cell-specific manner [10]. Besides, both ligand-independent and ligand-dependent AHR activity has been shown to regulate TGF-β signaling in some systems [3,20]. Interestingly, depending on the cell type, tissue or system under study, the mutual interaction between the TGF-β and the AHR signaling pathways is distinct, existing both stimulatory and inhibitory effects of TGF-β on AHR expression and function, as well as reports of no effect of the growth factor on the dioxin receptor signal [10,21,22].

The disparity of effects elicited by TGF-β on AHR function depending on cell type, hormonal status and experimental conditions, prompted us to evaluate a possible regulation of AHR function by this factor in the ovary. Thus, the main goal of the present study was to assess the effect of TGF-β on the receptor expression levels and on its activation in rat granulosa cells. This is of special interest because to our knowledge there are no reports on the regulation of AHR expression and activity by TGF-β in ovarian cells. Additionally, we hypothesized that AHR activation would exert an effect on TGF-β action in these cells. The ovarian granulosa cells are a mesenchyme derived cell type whose function and regulation have been comprehensively studied. The defined primary culture system used in this study has been extensively characterized in terms of the hormonally regulated and growth factor-promoted cell proliferation [23,19] and has been shown to express AHR and respond to its activation [8]. Our culture system therefore represents a useful cell model for the study of the influence of TGF-β on the expression of AHR and genes of the aryl hydrocarbon gene battery in cells where TGF-β stimulates proliferation. We found that neither AHR expression nor its typical transcriptional function is regulated by TGF-β in granulosa cells. Conversely, evidence is provided indicating that AHR is capable of regulating TGF-β action, impairing the action of the growth factor on these ovarian cells. This study suggests that there might be an interaction between AHR and TGF-β signaling pathways in granulosa cells that impinges on TGF-β action.

2. Materials and methods

2.1. *Hormones and chemicals*

Ovine FSH (NIDDK-oFSH-20) was obtained from the National Hormone and Pituitary Program. Transforming growth factor-β1 from porcine platelets was obtained from R&D Systems (Minneapolis, MN, USA) and [methyl-^{3}H]-thymidine from Dupont NEN Research Products. Percoll™ was purchased by Amersham Bioscience (Upsala, Sweden). 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, USA), unless otherwise indicated. Collagen was prepared from rat tails as previously described [24]. PCR and RT reagents and enzymes were purchased by Invitrogen (Carlsbad, CA, USA).

2.2. 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 [25]. Briefly, the ovaries were punctured with a 30-gauge needle and incubated in Dulbecco modified Eagle medium (DMEM, 4.5 g glucose/l)-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 two 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 or 96-well 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, unless otherwise indicated. In those experiments where cells received both TGFβ and β-naphthoflavone as stimuli, cultures were pretreated with the growth factor for 2 h and then cotreated with the flavone for 24 h.

2.3. RNA extraction and semiquantitative RT-PCR

Levels of AHR and CYP1A1 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, Molecular Research Center Inc., Carlsbad, CA, USA) and total RNA extracted according to the manufacturer's instructions (the organic phase of each sample was saved for protein extraction). Complementary DNA was synthesized from 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 hydroxylase and the receptor target cDNA were those described by Dasmahapatra et al. [26] and Timsit et al. [27], respectively. CYP1A1 and 18S Classic II primers:competimers (Quantum mRNA Ambion Inc., Austin, TX, USA, used as internal control for normalization) generated fragments of 509 and 324 bp, respectively. AHR primers generated fragments of 917 bp.

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. That means this is not an "end-point PCR" since samples are being quantified exclusively around the midpoint of the exponential portion of the amplification curve, which renders a sensitive PCR with a quite wide dynamic range. 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 s denaturation at 94 °C, 30 s annealing at 62 °C and 1 min extension at 72 °C. Amplification of AHR and 18S Classic II cDNAs was performed for 24 cycles in the presence of 1.5 mM $MgCl_2$, each cycle consisting of 20 s denaturation at 94 °C, 20 s annealing at 54 °C and 40 s 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, Sunnyvale, CA, USA) and normalized to the 18S ribosomal signal (given by the 18S primers:18S competimers ratio) for each sample.

2.4. 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., Carlsbad, CA, USA) and protein extracted from the organic phase according to the manufacturer's instructions. 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, USA, 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 chemioluminiscence kit (Amersham ECL™ Western Blotting Analysis System, GE Healthcare UK Limited, Little Chalfont, Buckinghamshire, UK) as specified by the manufacturer. Quantification of protein bands was performed with ImageQuant software (Amersham Biosciences, Sunnyvale, CA, USA). Densitometric units obtained for the AHR band intensities were normalized to densitometric units obtained for the intensities of the internal control, and results expressed relative to the values obtained for control cells.

2.5. Transient transfection and luciferase assay

Granulosa cells were cultured in P6 multiwell plates at a density of 2×10^6 viable cells/well in DMEM-F12 1:1 medium (2.2 g/l bicarbonate, pH 7.4) with 10% fetal bovine serum. After

18 h, cells were preincubated in OPTI-MEM®I (Gibco™, Invitrogen Corporation, Auckland, NZ) for 30 min and then transiently transfected with the reporter construct 3TP-Lux [28] in OPTI-MEM®I medium. The transfection was made using Lipofectamine 2000 (Invitrogen, Molecular Research Center Inc., Carlsbad, CA, USA) at 1 μl liposomes/cm^2 and 500 ng DNA/cm^2. The TGF-β inducible reporter construct p3TP-Lux (kindly given by Dr. J. Massague, Howard Hughes Medical Institute, New York, USA) was cotransfected with the control reporter plasmid pCMV-β-galactosidase (Clontech, Palo Alto, CA, USA). Sixteen hours post-transfection, fresh media (DMEM-F12 1:1 without FBS) containing the different stimuli were added. After 24 h of stimuli, cells were washed twice with PBS, lysed in 200 μl of 1× reporter lysis buffer (Promega, Madison, WI, USA), and the cleared extract was assayed for luciferase and β-galactosidase activities according to the manufacturer's instructions (Promega, Madison, WI, USA). 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%.

2.6. DNA synthesis assay

DNA synthesis was determined by [^{3}H]-thymidine incorporation according to a method previously validated in these culture conditions [23–25,8]. 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, Roskilde, Denmark) and radioactivity was measured in a scintillation counter.

2.7. 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 ± S.E.M. of the independent experiments. Statistical comparisons of the results were made using one-way ANOVA and Tukey–Kramer's test for multiple comparisons after logarithmic transformation of data when necessary [29].

3. Results

3.1. AHR transcript levels are not regulated by TGF-β in granulosa cells

With the aim of determining if TGF-β can regulate AHR mRNA expression in granulosa cells, we assessed the steady-state levels of those transcripts by RT-PCR after treatment with 2.5 ng/ml TGF-β. Comparable doses of the growth factor have been used extensively to study TGF-β actions on cultured granulosa cells (for example, see [19,30]) and have been shown to modulate AHR expression and function in other systems [21,22,31,32]. It has been shown that minor changes in AHR

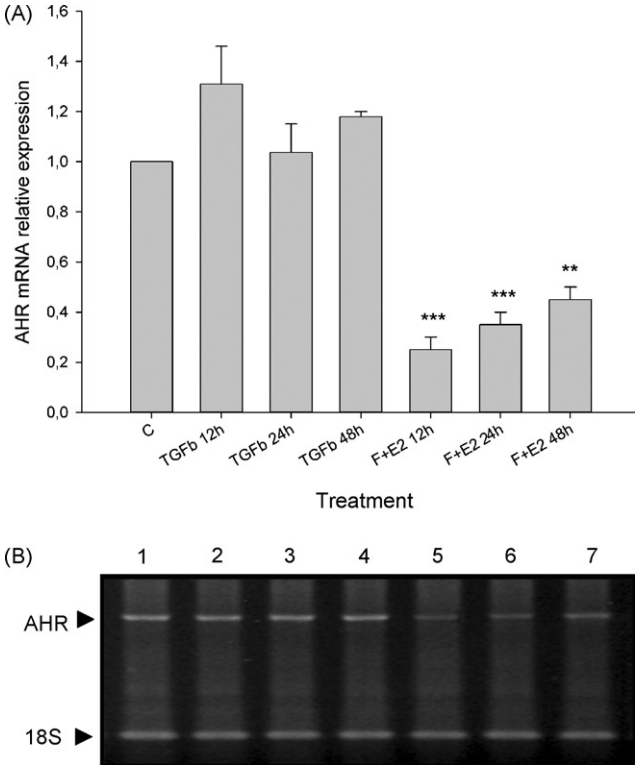

Fig. 1 – Levels of AHR transcripts are not altered by TGF-β treatment. Granulosa cells were cultured in control medium (C), with TGF-β (2.5 ng/ml, TGFb) or with a combination of FSH (2 ng/ml) and estradiol (100 ng/ml) (F + E2). After 12, 24 or 48 h of incubation with the different stimuli, total RNA was extracted and semiquantitative RT-PCR for AHR mRNA performed as described in Section 2. (A) 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 ± S.E.M. of three independent experiments. Asterisks denote statistically significant differences (*$P < 0.001$ vs. control group; **$P < 0.01$ vs. control group). (B) A representative electrophoretic separation of the semiquantitative RT-PCR products is shown. Lane 1: cells cultured in control medium; lane 2: cells cultured in the presence of 2.5 ng/ml TGF-β for 12 h; lane 3: cells cultured in the presence of 2.5 ng/ml TGF-β for 24 h; lane 4: cells cultured in the presence of 2.5 ng/ml TGF-β for 48 h; lane 5–7: cells cultured with FSH and estradiol for 12, 24 and 48 h, respectively.**

mRNA levels are certainly detectable by means of RT-PCR in our system [11]. As can be seen in Fig. 1, treatment with the growth factor for 12 h produced an apparent increase on AHR transcript levels, but this effect was not statistically significant. TGF-β had no effect on AHR mRNA levels when incubation was allowed to proceed for either 24 or 48 h (Fig. 1). Due to the known inhibitory action of FSH and estradiol on AHR mRNA in granulosa cells [11], those hormones were also tested for comparison. As shown in Fig. 1, those stimuli were able to reduce the receptor mRNA at levels comparable

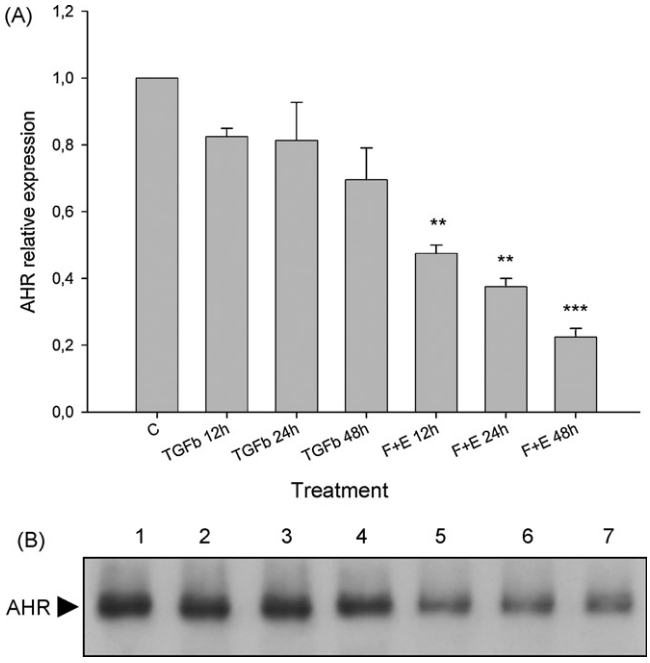

Fig. 2 – TGF-β does not regulate AHR protein expression. Granulosa cells were cultured for 12, 24 or 48 h in control medium (C), with TGF-β (2.5 ng/ml, TGFb) or with a combination of FSH (2 ng/ml) and estradiol (100 ng/ml) (F + E). Total protein was isolated from whole extracts at each time point and Western blot was conducted as described in Section 2 using an antibody that recognizes the AHR. (A) 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 ± S.E.M. of three independent experiments. Asterisks denote statistically significant differences (*$P < 0.001$ vs. control group; **$P < 0.01$ vs. control group). (B) A representative Western blot of AHR is shown. Lane 1: cells cultured in control medium; lane 2: cells cultured in the presence of 2.5 ng/ml TGF-β for 12 h; lane 3: cells cultured in the presence of 2.5 ng/ml TGF-β for 24 h; lane 4: cells cultured in the presence of 2.5 ng/ml TGF-β for 48 h; lane 5–7: cells cultured with FSH and estradiol for 12, 24 and 48 h, respectively.**

to the reported ones. This latter result serves as a positive control that ensures that changes in transcript levels are being detected in our system.

3.2. *AHR protein expression is not regulated by TGF-β in granulosa cells*

Despite a lack of effect on AHR transcript levels, TGF-β would regulate the receptor expression by inducing inhibition or stimulation of AHR expression at the protein level. Therefore, in order to further explore a possible regulation of the receptor expression by TGF-β, we tested the effect of the growth factor on AHR protein total cellular content. Treatment of cultured granulosa cells with TGF-β during 12 h produced a slight reduction on the receptor protein expression level, this effect was however not statistically significant (Fig. 2). Similarly, when cells were incubated with the stimulus during a period of 24 or 48 h and content of AHR protein in total cell lysates was measured by immunoblotting, no significant effect of TGF-β on AHR expression was evident (Fig. 2). As can be seen also in Fig. 2, treatment of granulosa cells with the combination of the hormones FSH and estradiol exerted the reported inhibitory effect on the receptor protein expression [11].

3.3. *Neither constitutive nor induced CYP1A1 gene expression is regulated by TGF-β in granulosa cells*

Even with no changing levels of AHR expression, a possible regulation of AHR function by TGF-β in ovarian cells cannot be ruled out. Therefore, we evaluated if this growth factor was capable of regulating the activity of this receptor in non-stimulated conditions or when its activity is induced by an agonist. *CYP1A1* gene transcription is considered a hallmark of Ah-response in most cells (included granulosa cells), constituting a model for the study of the AHR as transcription factor. Thus, we tested the effect of TGF-β on the expression levels of CYP1A1 mRNA, an endogenous gene that bears naturally occurring XREs. After treatment with TGF-β, no changes in the hydroxylase mRNA levels were evident in our system, as assessed by RT-PCR (Fig. 3). We subsequently examined the effect of TGF-β on the induction of *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 [1,33]. In the same way, TGF-β also failed to modulate the magnitude of the β-naphthoflavone-induced activation of AHR pathway (Fig. 3).

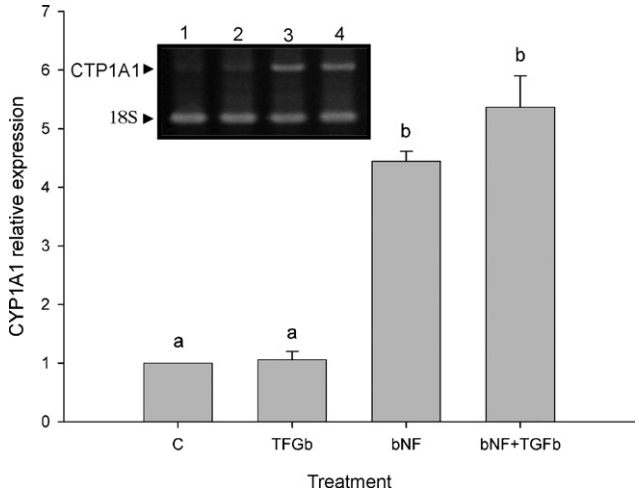

Fig. 3 – TGF-β has no effect on the levels of CYP1A1 transcripts. Granulosa cells were cultured for 24 h in control medium (C) or in medium containing TGF-β (2.5 ng/ml, TGFb). Beta-naphthoflavone (10 μM, bNF) was added to the corresponding wells after 2 h pretreatment with TGF-β (bNF + TGFb). Total RNA extraction and semiquantitative RT-PCR for CYP1A1 mRNA were performed as described in Section 2. 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 ± S.E.M. of three independent experiments. Values not sharing a common letter are significantly different ($P < 0.001$). Inset: A representative electrophoretic separation of the semiquantitative RT-PCR products is shown. Lane 1: cells cultured in control medium; lane 2: cells cultured in the presence of 2.5 ng/ml TGF-β; lane 3: cells cultured in the presence of 10 μM β-naphthoflavone; lane 4: cells cultured in the presence of 2.5 ng/ml TGF-β added as pretreatment and 10 μM β-naphthoflavone.

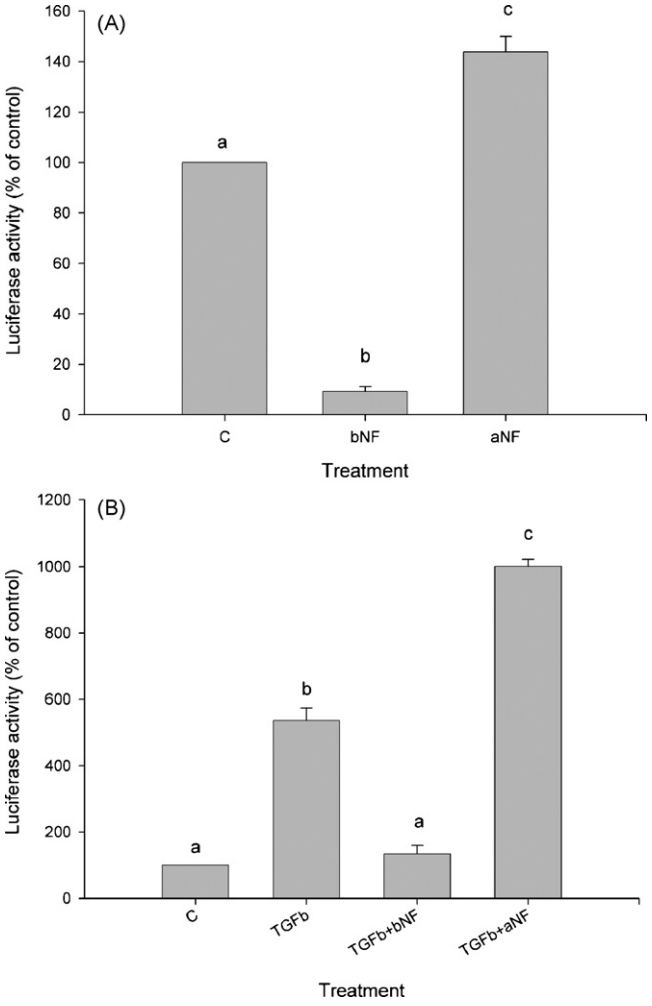

Fig. 4 – Effect of β-naphthoflavone on TGF-β-driven transcription. Granulosa cells were transiently transfected with the p3TP-Lux reporter construct and the control reporter plasmid pCMV-β-Galactosidase as described in Section 2. (A) After transfection (16 h later) cells were treated with vehicle only (C) or with the AHR ligands β-naphthoflavone (10 μM, bNF) or α-naphthoflavone (0.5 μM, aNF). (B) After transfection (16 h later) cells were treated with vehicle only (C), with TGF-β (2.5 ng/ml, TGFb), with TGF-β and β-naphthoflavone (10 μM, TGFb + bNF) or with TGF-β and α-naphthoflavone (0.5 μM, TGFb + aNF). After 24 h of incubation with the different stimuli, cells were harvested as described in Section 2 and processed for luciferase reporter assay. Results (normalized to β-Gal activity) are expressed as percent of relative luciferase units, when compared to control cells (only vehicle added, taken as 100%). Values are means ± S.E.M. of three independent experiments, each performed in duplicate. Values not sharing a common letter are significantly different ($P < 0.001$).

3.4. *Beta-naphthoflavone inhibits the constitutive activity of a TGFβ-responsive promoter in granulosa cells*

Although the activation of TGF-β pathway has no evident effect on either AHR expression or AHR-driven transcriptional activity, it is still plausible an interaction between both signals that impacts exclusively on TGF-β action. A possible effect of AHR pathway activation on TGF-β mediated response in granulosa cells was addressed in first place by evaluating the expression of the TGF-β inducible luciferase reporter plasmid 3TP-Lux when cells are treated with β-naphthoflavone. We have previously demonstrated that this AHR ligand acts as an agonist in our system, being able to activate the receptor and induce a typical cellular response [8]. Treatment of transiently transfected granulosa cells for 24 h with β-naphthoflavone led to a marked inhibition of luciferase constitutive activity, compared to the vehicle-treated controls (Fig. 4A). When cell cultures were treated with the AHR antagonist α-naphthoflavone, a ~40% induction on luciferase activity was observed, compared to the vehicle-treated controls (Fig. 5A). Alpha-naphthoflavone has been demonstrated to act as an antagonist of the AHR in our system at doses of 0.5 and 1 μM [8].

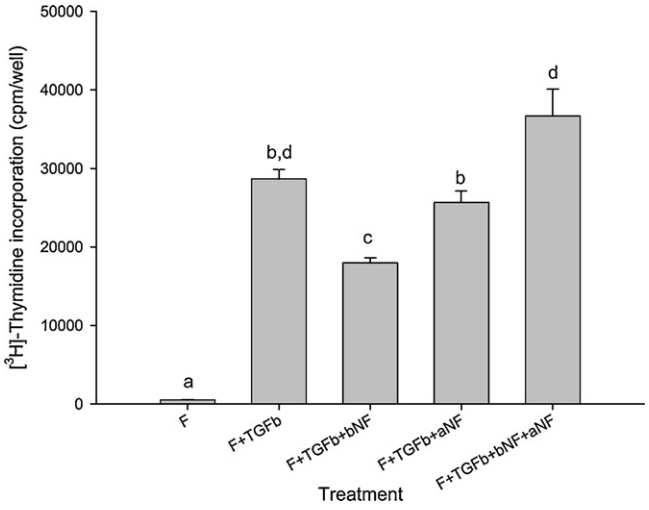

Fig. 5 – Effect of β-naphthoflavone on TGF-β-stimulated granulosa cells proliferation. A) Granulosa cells were cultured in medium containing 2 ng/ml FSH (F) with (F + TGFb) or without the addition of TGF-β 2.5 ng/ml. Cells were treated either with β-naphthoflavone 10 μM (F + TGFb + bNF), with α-naphthoflavone 0.5 μM (F + TGFb + aNF) or with a combination of both ligands (F + TGFb + bNF + aNF). [3H]-thymidine incorporation was performed for 24 h starting 24 h after plating. Results (depicted as bars) are expressed as the mean ± S.E.M. of three independent experiments, each run in quadruplicate. Values not sharing a common letter are significantly different ($P < 0.001$, except for the comparison between F + TGFb and F + TGFb + bNF, where $P < 0.01$).

3.5. *Beta-naphthoflavone inhibits TGF-β-induced transcription in granulosa cells*

The effect of AHR activation on TGF-β-driven transcriptional activity was further investigated by evaluating the effect of the AHR agonist on the stimulation of gene expression exerted by the growth factor. As expected, when granulosa cells transiently transfected with the reporter construct p3TP-Lux were treated with TGF-β, an induction of luciferase activity was observed (~5-fold induction compared to control cells) (Fig. 4B). This induction was completely reversed by cotreatment with β-naphthoflavone (Fig. 4B). On the contrary, treatment of TGF-β stimulated cultures with the AHR antagonist α-naphthoflavone led to a superinduction of luciferase activity (~2-fold induction compared to TGF-β treated cells) (Fig. 4B).

3.6. *Beta-naphthoflavone inhibits TGF-β-induced proliferation in granulosa cells*

In order to further evaluate the action of AHR activation on TGF-β-mediated cellular response, we assessed the known mitogenic effect of the growth factor on these ovarian cells [19] in the presence of an agonist of AHR. Consequently, we tested the effect of β-naphthoflavone on TGF-β-stimulated DNA synthesis in granulosa cells. The induction of DNA synthesis triggered by TGF-β in the presence of FSH was partially inhibited by treatment with β-naphthoflavone (Fig. 5). Treatment with the AHR antagonist α-naphthoflavone had no effect on the magnitude of the induction of proliferation elicited by the growth factor (Fig. 5). Moreover, the inhibitory effect of β-naphthoflavone on TGF-β-stimulated DNA synthesis was reversed by co-treatment with the AHR antagonist α-naphthoflavone (Fig. 5).

4. Discussion

Besides mediating toxic responses, the AHR plays pivotal roles in normal biological processes such as those related with female reproductive physiology [1,7,4]. Of special interest is the fact that AHR is involved in growth processes in the ovary, and particularly its activation has been associated with promotion of proliferation in granulosa cells [7,8,34]. An unusually large number of agents and biological factors alter the level and/or activity of the AHR, thereby affecting normal physiologic functions as well as responses to toxic environmental chemicals [10,35]. An interesting example of the complexity of the interactions among biological factors is the interaction between TGF-β and the AHR [3,10]. This background prompted us to study a possible interaction between the AHR and the TGF-β signals in ovarian cells, focusing on a possible regulation of AHR expression and activity triggered by the growth factor.

We have found that mRNA levels of AHR are neither diminished nor induced by 12-h treatment with TGF-β in granulosa cells. This time of incubation was chosen because it has been previously used to test the ability of hormones to reproduce in vitro the reduction in AHR transcripts observed on the evening of proestrus in ovarian tissue of cycling rats [14,11]. Treatment with the growth factor for 24 or 48 h did not change AHR transcripts either. These results are of great interest, since they are opposed to those found in A549 cells (where TGF-β led to repression of AHR mRNA expression after 24 h of incubation [22]) and to those reported in HepG2 cells (where TGF-β leads to induction of *AHR* promoter activity [32]). The disparity in TGF-β effects can be ascribed to intrinsic differences between cell types. Actually, proliferation of the human lung cancer cell line is inhibited by TGF-β [21], while this factor does not alter the hepatoma cell line growth ([36], and references therein). This is opposed to granulosa cells, where the growth factor is mitogenic [19]. Further in accordance with this notion, it has been shown that TGF-β has no effect on AHR mRNA expression in MDA-MB 231 human breast cancer cells, a cell type whose proliferation is not inhibited by the growth factor either [21]. It has been proposed that the low level of the corepressor TGIF found in MDA-MB 231 cells might be responsible for the lacking response of AHR mRNA expression toward TGF-β in this cell line [32]. This would be also the case for rat granulosa cells, what makes future experiments in this regard warranted. Concerning the diverse responses of different cell types toward TGF-β action on AHR mRNA observed by us and others, it is tempting to speculate that the previously reported inhibition of AHR expression elicited by TGF-β would take place only in cells where the factor inhibits cell growth. Although there is evidence suggesting that in fibroblasts the

signal transduction pathway mediating progression through the cell cycle and those that regulate AHR expression are distinct [37], an indirect relationship between both phenomena in granulosa cells cannot be ruled out.

There is no previous data on the effect of TGF-β on AHR protein expression, a feature that we consider of great interest, as protein levels can change irrespective of mRNA levels either due to altered protein transduction or degradation. Our data show that expression of AHR is not altered significantly by treatment with TGF-β. These results might indicate that there are differences between TGF-β and other mitogenic growth factors in terms of their influence on AHR expression, since it has been reported that serum, PDGF and FGF are able to induce AHR protein levels in murine 3T3 fibroblast [37]. However, inherent differences between murine Swiss 3T3 fibroblast and rat granulosa cells cannot be excluded, raising the possibility that the dissimilarity between both cell types regarding the effect of the mitogenic factors is due to differences in their physiology and cellular characteristics. In fact, as opposed to fibroblast, PDGF for instance has been shown not to be a mitogenic factor in our system conditions [29].

A possible regulation of the hydroxylase CYP1A1 expression by TGF-β was of interest because results in some cell lines indicate that the regulation of agonist-induced as well as basal CYP1A1 mRNA expression exerted by TGF-β can be independent of the regulation of AHR mRNA [22]. Our results on CYP1A1 mRNA expression indicate that AHR function as transcription factor is not regulated by TGF-β in granulosa cells, which contrasts with observations made in A549 cells, rat and human primary hepatocytes and MDA-MB 231 cells, where the growth factor diminishes the expression of the hydroxylase gene [22,31,38,21]. Again, the observed differences may be ascribed to distinct intrinsic characteristics of each cell type, mainly to differences in the signal triggered by TGF-β and by AHR activation and to the existence of regulation by specific protein factors (coactivators or corepressors) restricted to each cell type. This might explain their unique interaction and the effect of the activation of those pathways on the physiology of the cell. The exact mechanisms which lead to regulation of AHR expression and function in other cell types that are not verified in granulosa cells remain to be established.

Several studies conducted to evaluate the outcome of *Ahr* deletion or exogenous AHR stimulation on TGF-β isoforms expression indicate that the AHR is a negative regulator of TGF-β levels, which in turn evidently alters TGF-β action [39,3,10]. However, relatively less attention has focused on the direct effect of AHR activation on TGF-β transcriptional activity or TGF-β-mediated proliferative cellular response. The results presented herein attempt to provide data on this item. We found that TGF-β-driven constitutive transcription is strongly inhibited by treatment with the AHR agonist β-naphthoflavone, as indicated by a ~90% reduction in basal activity of a TGF-β responsive promoter. Our results suggest that this effect would be mediated by activation of the AHR, since the antagonist α-naphthoflavone was not able to reproduce the described action on TGF-β driven transcriptional activity. Similar results were obtained when we assessed the effect of β-naphthoflavone on TGF-β-stimulated transcription. The AHR agonist inhibited the induction of the promoter activity elicited by TGF-β, effect that could not be exerted by the antagonist α-naphthoflavone, suggesting that AHR activation is needed in order to accomplish the inhibition. Of further interest is the finding that treatment with the antagonist produced an induction of both basal and TGF-β stimulated activity of the TGF-β responsive promoter, when compared to the values obtained for the corresponding controls. This effect would be explained in terms of the existence of endogenous activation of the AHR in granulosa cells, for example by an endogenous ligand, which is physiologically inhibiting the basal or stimulated promoter activity and is being antagonized by the addition of α-naphthoflavone in our experiments. There is evidence that point to the existence of physiological activation of AHR in these ovarian cells [40,41,8] and our data add further support to this notion. The inhibition of the TGF-β responsive promoter activity exerted by the AHR agonist in this study is in agreement with the well documented notion that AHR activation alters TGF-β action, but contrasts with the effect reported in Hepa1c1c7 cells, where the agonist TCDD induced PAI-1 transcription via an AHR dependent mechanism [42]. Differences in the constructs used in the experiments, in the nature of the AHR agonists or in the transcriptional machinery and cellular milieu between rat granulosa cells and the mouse hepatoma cell line may account for the dissimilar findings. In the above-mentioned report, it was demonstrated that the AHR dependent induction of TGF-β responsive gene transcription is distinctly different from TGF-β-driven PAI-1 transcription. It can be speculated that the inhibition described in the present experiments would implicate interaction between the AHR-, the MAPKs- and the TGF-β pathways, since there are previous reports of interactions of the AHR and the MAPK pathways [7,43,44] and of inhibition of Smad activity after phosphorylation in their MAPK sites in the ovary [18]. Future work is needed to determine the basis and mechanisms mediating the observed inhibition of TGF-β responsive promoters that exerts β-naphthoflavone in our system. Additionally, we found that a complex cellular response elicited by TGF-β in granulosa cells, as it is the induction of proliferation, is also negatively regulated by an AHR agonist, most probably via an AHR-dependent mechanism, since the effect is only elicited by a ligand that is capable of activating the receptor and is blocked by an antagonist of the receptor. Our data on TGF-β activity deregulation after treatment with AHR exogenous ligands is of additional interest, since disruption of TGF-β activity has been implicated in a variety of human diseases. Besides, from a physiological perspective and in absence of exogenous ligand for the AHR, our results suggest that in normal conditions AHR and TGF-β should be expressed and activated 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. The mechanisms leading to the reported inhibition of TGF-β mitogenic action by AHR activation in granulosa cells might involve interaction of the receptor with other growth factors that display autocrine loops, factors or proteins that mediate extracellular matrix remodeling, MAPKs pathways, protein kinase A, and/or the calcium dependent protein kinase Calmodulin, since those have been

proposed as possible mediators of TGF-β mitogenic actions [45]. The potential engagement of those pathways as mechanisms underlying the reported negative interaction between AHR and TGF-β found in this study remains to be elucidated.

Besides interactions with TGF-β, estrogen receptor or MAP kinases pathways, AHR ligands such as β-naphthoflavone or other polycyclic aromatic hydrocarbons have been also reported to exert effects on a variety of other pathways. For instance, there are reports of alterations in gonadotropin-stimulated steroidogenesis, apoptosis process and signaling via retinoblastoma protein, NFκB and protein kinase C [46,47]. These effects might account for the disruptive action of AHR exogenous activation in diverse systems and are plausible to take place in ovarian cells as well.

Our findings are of special interest when trying to understand AHR function and regulation across different tissues or among different cell types and may provide clues to understanding ovarian function deregulation in the presence of AHR exogenous agonists.

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