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# Aryl hydrocarbon receptor activation leads to impairment of estrogen-driven chicken vitellogenin promoter activity in LMH cells

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ABSTRACT

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that mediates most of the toxic effects of environmental contaminants. Among the multiple pleiotropic responses elicited by AHR agonists, the antiestrogenic and endocrine-disrupting action of the receptor activation is one of the most studied. It has been demonstrated that some AHR agonists disrupt estradiol-induced vitellogenin synthesis in the fish liver *via* a mechanism that involves crosstalk between the AHR and the estrogen receptor (ER). Chicken hepatocytes have become a model for the study of AHR action in birds and the induction of the signal and its effect in these cells are well established. However, the impact of AHR activation on estradiol-regulated responses in the chicken liver remains to be demonstrated. The aim of the present study was, therefore, to determine the effect of AHR action on ER-driven transcription in a convenient model of chicken liver cells. For this purpose, we designed a reporter construct bearing the 5′ regulatory region of the chicken vitellogenin II gene and used it to transfect chicken hepatoma LMH cells. We found that β-naphthoflavone represses ER-driven vitellogenin promoter activity and that this action is mediated by the AHR. This inhibitory crosstalk between both pathways appears to be unidirectional, since estradiol did not alter the transcript levels of an AHR target gene. Besides, and highly relevant, we show that LMH cell line transfected with a reporter construct bearing the chicken vitellogenin promoter sequence is a useful and convenient model for the study of AHR–ER interaction in chicken liver-derived cells.


## 1. Introduction

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factors with a very complex and intricate biology. This member of the basic helix–loop–helix/Per–ARNT–Sim family has long been recognized as sensor of contaminants and pollutants present in the environment and as the regulator of the cellular responses elicited by those xenobiotic substances (Mandal, 2005; White and Birnbaum, 2009). Several of the described AHR ligands (such as halogenated aromatics or polycyclic aromatic hydrocarbons) are widespread and persistent environmental contaminants that, as we and others have demonstrated, exert pleiotropic responses and diverse effects on reproductive, developmental, nervous and immune systems (Hernández-Ochoa et al., 2009; Stockinger et al., 2011; King-Heiden et al., 2012). In addition to the toxicological actions of AHR activation, this receptor is also responsive to natural compounds and endogenous physiological signals and plays important roles in the maintenance of homeostatic function (Bock and Köhle, 2009; Hernández-Ochoa et al., 2009; Fujii-Kuriyama and Kawajiri, 2010).

Essential steps in AHR signal transduction include binding of ligand to the receptor in the cytoplasm of cells, translocation of the receptor to the nucleus, dimerization with the AHR nuclear translocator (ARNT), and binding of this heterodimeric transcription factor plus a set of co-activators and/or co-repressors to xenobiotic-responsive elements (XREs) located upstream in the promoter of target genes involved in xenobiotic metabolism (*e.g.* cytochrome P4501A, the hallmark of AHR responsiveness) or involved in any of the other genomic responses elicited by AHR activation (Beischlag et al., 2008; Denison et al., 2011).

The crosstalk existing between the AHR and different signaling pathways in various systems has been described by our lab and others (Bussmann et al., 2006; Bussmann and Barañao, 2008; Ma et al., 2009). In particular, the inhibitory interaction between AHR and ER signals and the endocrine-disruptive and antiestrogenic

*Abbreviations:* AHR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; CEH, chicken embryo hepatocyte; CYP1A1, cytochrome P450 1A1; CYP1A4, cytochrome P450 1A4; DMEM, Dulbecco Modified Eagle medium; ER, estrogen receptor; ERE, estrogen response element; iDRE, inhibitory dioxin response element; iXRE, inhibitory xenobiotic response element; LMH, Leghorn strain M hepatoma; XREs, xenobiotic response elements.

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effects of AHR activation have been the subject of study by many groups over the years (Swedenborg and Pongratz, 2010; Shanle and Xu, 2011).

The AHR and orthologs are widely and ubiquitously expressed in diverse tissues throughout the different groups of vertebrates and invertebrates (Hahn, 2002). Particularly in the chicken (*Gallus gallus*), the AHR has been well characterized and the expression of this protein has been described in the liver and heart of embryos and adult exemplars (Walker et al., 2000). Chicken liver or chicken hepatocyte cultures have been used extensively and AHR responsiveness in these models are well documented (for example, see Kennedy et al., 1996; Head and Kennedy, 2007; Watanabe et al., 2009; Hervé et al., 2010). However, no studies have been conducted in chicken liver cells regarding the modulation that AHR activation exerts on estrogen-regulated genes. This fact makes specific research in the chicken of interest, since the reported diversity concerning mutual regulation of both signals depending on the cell type or species under study prevents from predicting the outcome of such interactions (Swedenborg and Pongratz, 2010; Denison et al., 2011; Shanle and Xu, 2011). The leghorn male hepatoma (LMH) cell line is a chicken hepatocellular carcinoma cell line that shows many of the known hepatocyte properties (Kawaguchi et al., 1987) and has been used as a homologous cell line for studies of the expression of some avian liver genes in specific culture conditions (Berkowitz and Evans, 1992). However, there are no data regarding agonistic or antagonistic effects of classical AHR ligands in LMH cells, or concerning the responsiveness of these cells to the activation of the receptor. This prevents from using the LMH cell line as an alternative model to chicken embryo hepatocyte for the study of AHR action. In particular, the use of this cell line to study AHR–ER crosstalk remains elusive, since it has been postulated the need to over-express ER due to a low content of receptor in these cells with a consequent development of the LMH/2A cell line (Binder et al., 1990; Sensel et al., 1994).

The aim of the present study, therefore, was to study the action of AHR activation on ER-driven transcription in LMH cells. For this purpose, we designed a reporter construct bearing the 5′ regulatory region of the chicken vitellogenin II gene, a prototypic gene that is exclusively transcribed in hepatocytes in response to estrogens. We demonstrate that the AHR agonist β-naphthoflavone impairs the estrogen-stimulated transcription of the chicken vitellogenin promoter, and that this action is mediated by the AHR. This crosstalk between the AHR and the ER signaling pathways in LMH cells, however, appears to be unidirectional, since estradiol does not interfere with β-naphthoflavone-induced CYP1A gene expression. The description of the existence of an AHR–ER inhibitory crosstalk in chicken liver-derived cells constitutes the first report in this species. Besides, we show that the AHR ligands β-naphthoflavone and α-naphthoflavone can act as agonist and antagonist of the receptor, respectively, in LMH cells and that this cell line can be a useful model for the study of AHR–ER interactions in chicken hepatocytes.

## 2. Materials and methods

### 2.1. Hormones and chemicals

Tissue culture reagents, 17β-estradiol (estradiol), fulvestrant/ICI182,780, 5,6-benzoflavone (β-naphthoflavone), 7,8-benzoflavone (α-naphthoflavone), and all other chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), unless otherwise indicated. PCR and RT reagents and enzymes were purchased from Invitrogen (Carlsbad, CA, USA).

### 2.2. Chicken embryo hepatocyte preparation and culture

Fertilized eggs from a Cobb breeding flock (*Gallus gallus*) were facilitated by Tres Arroyos Farm (Buenos Aires, Argentina), incubated at 38.5 °C with a relative humidity of 60%, treated *in ovo* during 48 h with 1.25 mg of 17β-estradiol dissolved in propylene glycol in order to induce estrogen receptor expression (Haché et al., 1987), and killed on day 14 of incubation by decapitation. Induction of estrogen receptor expression in the embryo by means of estradiol treatment of the egg is needed in order to detect changes in classical estrogen responsive genes like vitellogenin or apoVLDLII genes (otherwise, CEH levels of estrogen receptor are too low). 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, USA.

Livers were isolated and hepatocytes were prepared as previously described (Zhou and Zhang, 2005), with some minor modifications introduced in order to improve purity and yield. Briefly, livers were mechanically and chemically disaggregated by passing them through a 40-mesh stainless steel sieve (Sigma) and incubating them with a DMEM: Hepes solution containing 0.1% collagenase (type III, Worthington Biochemical Corporation), 0.05% hyaluronidase (Worthington) and 0.05% DNase (Worthington) at 37 °C in a shaking water bath (90 cycles/min). Incubation was performed for 30 min and included frequent pipetting to facilitate cell dissociation. The preparation was then passed through an 80-mesh stainless steel sieve (Sigma) and then centrifugated at 405 g for 10 min. Hepatocytes were further purified (mainly from red blood cells and adipocytes) by Nycodenz density gradient centrifugation (18% Nycodenz, centrifugation at 400 g for 40 min). Resulting purified and isolated hepatocytes were recovered from the top of the Nycodenz gradient and resuspended in DMEM:Hepes containing 5% heated fetal bovine serum, 5% normal chicken serum and antibiotics. Cell viability was confirmed to be over 90%, as determined by trypan blue exclusion assay. For immunofluorescence experiments cells were seeded on LAB-TEK® Chamber Slide™ (Nalge Nunc International Corp., Naperville, IL. USA) precoated with collagen, at a density of $2.5\times10^5$ viable cells/cm$^2$ and maintained in serum-free medium. For transfection experiments cells were seeded on collagen-precoated P6 multiwell plastic plates (Orange Scientific NV/SA., Belgium) at a density of $2.0\times10^5$ viable cells/cm$^2$ and maintained in serum free medium. Cells were cultured at 38 °C with 5% $CO_2$.

### 2.3. Culture of LMH cells

Chicken hepatocellular carcinoma LMH cells were obtained from the ATCC (catalog no. 2117-CRL) and thawed immediately after arrival. They were cultivated in Waymouth's MB 752/1 medium supplemented with 10% fetal bovine serum and antibiotic–antimycotic (Gibco) on 0.1% gelatin-coated dishes and maintained at 38 °C with 5% $CO_2$.

For transfection experiments and for mRNA analysis experiments, cells were seeded on P6 multiwell plastic plates (Orange Scientific) at a density of $2.0\times10^5$ viable cells/cm$^2$ and maintained in serum-free medium throughout the experiment. For immunofluorescence experiments cells were seeded on LAB-TEK® Chamber Slide™ (Nalge Nunc International Corp., Naperville, IL. USA), at a density of $2.5\times10^5$ viable cells/cm$^2$ and maintained in serum-free medium.

### 2.4. Immunofluorescence for estrogen receptor

Culture media of chicken embryo hepatocytes or LMH cells were removed and cells were washed twice with PBS and fixed in 4% P-formaldehyde for 10 min. After washing cells with PBS, cells were permeabilized for 30 min in 0.25% Triton X100, washed afterwards with PBS and blocked for 60 min with 5% goat normal serum in PBS-Tween 20 (0.05%). Incubation with first antibody against estrogen receptor (Ab-10 Thermo Fisher Scientific Inc., diluted 1:1000) was done during 1 h at RT. Thereafter, cells were washed with PBS and incubated with Alexa Fluor®-546 goat anti-mouse IgG (Invitrogen, CA, USA, diluted 1:1000) for 1 h at RT. After washing with PBS, samples were allowed to dry and then mounted with Mowiol mounting media. Images were then analyzed by confocal microscopy.

### 2.5. RNA extraction and RT-PCR

Levels of CYP1A4 mRNA expression in LMH cells were assessed using RT-PCR. After 22 h of incubation with the different stimuli (β-naphthoflavone or α-naphthoflavone, either added alone or in the presence of estradiol), cells were directly lysed in the culture dish with TRIzol reagent (Invitrogen) and total RNA extracted according to the manufacturer's instructions. 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 PCRs. One-microliter aliquots of the RT reaction were used to amplify CYP1A4 and 18S fragments in a multiplex reaction. The primer sequences used to amplify the CYP1A4 target cDNA were: forward primer 5′CCGTGACAAC CGCCCTGTCC3′; and reverse primer 5′GAGTTCGGTGCCGGCTGCAT3′. CYP1A4 and 18S classic II primers:competimers (Quantum mRNA Ambion, Inc., Austin, TX, USA, used as internal control for normalization) generated fragments of 359 and 324 bp, respectively. 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. We have previously demonstrated that minor changes in CYP1A mRNA levels are certainly detected with this technique (Bussmann et al., 2006; Bussmann and Barañao, 2008). Amplification of CYP1A4 and 18S classic II cDNA was performed for 23 cycles in the presence of 1 mM $MgCl_2$, each cycle consisting of 30 s denaturation at 94 °C, 30 s annealing at 62 °C and 40 s extension at 72 °C. Amplification program included an initial step at 94 °C for 3 min and a final step at 72 °C for 5 min. 10 μL of the PCR reaction was electrophoresed in 2% 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.

### 2.6. Cloning of chicken vitellogenin II promoter region and construction of the reporter vector VTG-Luc

A ~2.2-kilobase (kb) fragment of the 5′-flanking region of the chicken vitellogenin II gene (RefSeq accession number: NW_001471740, *Gallus gallus* chromosome 8) was PCR amplified from genomic DNA isolated from chicken fibroblast. The amplified fragment spans nucleotides from −2147 to +14 (relative to the start of transcription of the vitellogenin gene), a vast region that contains the functional estrogen response elements (EREs), the upstream activator sequences, and all other reported set of positive and negative control elements within the chicken vitellogenin II promoter (Seal et al., 1991; Davis and Burch, 1996; Burch et al., 1998). This fragment was first subcloned into the pGEM-T Easy cloning vector (Promega, Madison, WI) and subsequently inserted into the *SacI and BglII* restriction sites of the *pGL3-Basic* Vector (Promega, Madison, WI). The resulting plasmid, after confirmation of the identity and integrity of the cloned fragment into the luciferase vector, was named VTG-Luc. It is to be noted that, given the restriction sites used for the cloning of the insert, the recently reported XRE located in the multiple cloning site of the pGL3-Basic Vector (Ochs et al., 2012) is not present in our VTG-Luc construct.

### 2.7. Transient transfection and luciferase assay

LMH cells or chicken embryo hepatocytes cultured for 24 h in P6 multiwell plates were preincubated in OPTI-MEM® GlutaMax™ (Gibco™, Invitrogen Corporation, Auckland, NZ) for 30 min and then transiently transfected with the reporter control construct pTK-Red-ERE-LUC (kindly given by Dr. M. Beato) or the reporter construct VTG-Luc in OPTI-MEM® GlutaMax™ medium. The strong estrogen responsive control construct pTK-Red-ERE-LUC is a validated synthetic reporter vector consisting of a constitutive minimal promoter bearing estrogen response elements inserted in front of it and the firefly luciferase gene cloned downstream (Di Croce et al., 1999). The transfection was made using Lipofectamine™ 2000 Reagent (Invitrogen) at 0.3 μL liposomes/$cm^2$ and 50 ng DNA/$cm^2$. Along with the estradiol-inducible reporter constructs, cells were cotransfected with the control reporter plasmid pRL-SV40 (Promega, Madison, WI, USA) which expresses Renilla luciferase as the second reporter. Sixteen hours post-transfection, fresh media (DMEM-F12 1:1 without phenol red and without serum) containing the different stimuli were added. After 32 h of stimuli, cells were washed twice with PBS, lysed in 200 μL of 1X passive lysis buffer (Promega), and the cleared extract was assayed for luciferase activities using the Promega's Dual-Luciferase™ Reporter Assay System (Promega) and according to the manufacturer's instructions. Transfection efficiencies were normalized by Renilla luciferase activity in each well, and firefly luciferase activity was expressed as relative light units, as percentage of the activity observed in the vehicle (ethanol) controls, which were set arbitrarily to 100%.

### 2.8. 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's test for multiple comparisons after logarithmic transformation of data when necessary (Sokal and Rohlf, 1995).

## 3. Results

### 3.1. LMH cells express substantial amount of functional estrogen receptor

The chicken cell line LMH has been used as a homologous cell line for studies of the expression of some avian liver genes in specific culture conditions (Berkowitz and Evans, 1992). Some authors, however, have shown that the expression of estradiol-responsive genes in LMH cells is dependent on co-transfection with an estrogen receptor expression vector due to the low content of functional receptor in these cells (Binder et al., 1990; Sensel et al., 1994). Consequently, our first goal was to determine if LMH cells cultured in our experimental conditions express significant amount of ER and if this quantity is sufficient to elicit an estradiol-dependent response comparable to that generated in CEH. As can be seen in Fig. 1A, the cell line used for our study expresses appreciable quantity of ER, as assessed by immunofluorescence. Moreover, the receptor is able to transduce an estradiol-elicited signal and activate the transcription of a promoter under the regulation of estrogens, as demonstrated by means of transient transfection of LMH cells with the control reporter construct pTK-Red-ERE-LUC (Fig. 1B, left panel). This synthetic estrogen responsive reporter vector is utilized in these experiments because its use is well established and it has a high expression rate (Di Croce et al., 1999; Bussmann et al., 2006). The estrogen-induced transcription observed in LMH cells is comparable to that obtained in CEH, and is reversed by co-treatment with the pure anti-estrogen ICI 182,780 as well (Fig. 1B).

### 3.2. Beta-naphthoflavone acts as an AHR agonist in LMH cells

In order to determine if the AHR ligand β-naphthoflavone is able to act as an agonist of the receptor in LMH cells, we studied the ability of this compound to induce AHR transcriptional activity in our system. The induction of CYP1A gene expression (an endogenous gene that bears naturally occurring XREs) is a well-characterized transcriptional response mediated by the AHR that has been extensively utilized to

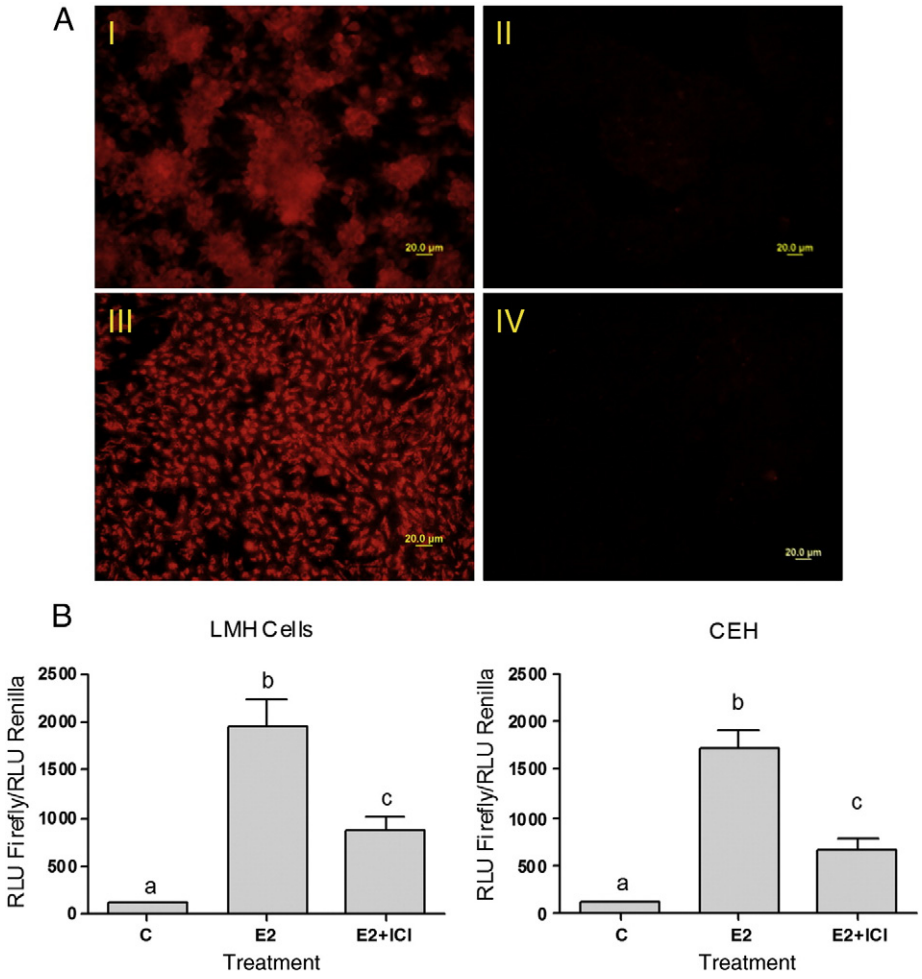


**Fig. 1.** Expression of functional ER in LMH cells. A) LMH cells or chicken embryo hepatocytes (CEH) were cultured during 24 h and subsequently subjected to immunofluorescence for ER as described in Materials and methods. Panels I and II: representative immunofluorescence for ER in LMH cells (II: negative control). Panels III and IV: representative immunofluorescence for ER in primary cultures of CEH (IV: negative control). The same pattern of response was obtained in two independent experiments, each run in duplicate. B) LMH cells (left panel) or CEH (right panel) were transiently transfected with the estrogen responsive reporter construct pTK-Red-ERE-LUC and the control reporter plasmid pRL-SV40 as described in Materials and methods. 16 h after transfection cells were treated with estradiol alone (E2, 1 µM) or in combination with the antiestrogen ICI 182,780 10 µM (E2 + ICI). After 32 h of incubation with the stimuli, cells were harvested as described in Materials and methods and processed for Dual-Luciferase Reporter Assay. Results are expressed as percent of relative luciferase units, when compared to control cells (only vehicle added, taken as 100%). Values are means ± SEM of three independent experiments, each performed in duplicate. Values not sharing a common letter are significantly different (left panel: C *vs.* E2 $P<0.001$; E2 *vs.* E2 + ICI $P<0.01$; C *vs.* E2 + ICI $P<0.05$. Right panel: C *vs.* E2 $P<0.01$; E2 *vs.* E2 + ICI $P<0.05$).

assess the activation of AHR-mediated signal transduction (Köhle and Bock, 2007; Beischlag et al., 2008; Fujii-Kuriyama and Kawajiri, 2010). Particularly in chicken hepatocytes, the CYP1A4 isoform is the one that is mainly induced by AHR agonists (Gilday et al., 1996; Mahajan and Rifkind, 1999; Head and Kennedy, 2007; Hervé et al., 2010). Thus, we tested the effect of different doses of the ligand on CYP1A4 mRNA. As can be seen in Fig. 2A, β-naphthoflavone induces the transcription of CYP1A4 gene in a dose dependent manner, causing a rise in these transcripts of about 4 times at doses of 6 µM or 10 µM. This effect was also verified in the presence of estradiol (Fig. 4); in this latter condition β-naphthoflavone exerts AHR activation at levels comparable to those observed in the absence of estrogen. On the other hand, the AHR ligand α-naphthoflavone had no effect on CYP1A4 mRNA, even when doses as high as 12 µM were used (Fig. 2B). The same results were obtained when α-naphthoflavone was added to LMH culture media in combination with estradiol (Fig. 4).

### 3.3. Alpha-naphthoflavone acts as an AHR antagonist in LMH cells

Since the AHR ligand α-naphthoflavone can act either as agonist or antagonist of the receptor depending on the dose used and the system under study (Gasiewicz and Rucci, 1991; Wilhelmsson et al., 1994; Bussmann et al., 2006; Henry and Gasiewicz, 2008), we decided to investigate the effect of this flavone regarding AHR activation in LMH cells. As mentioned above, no changes in CYP1A4 mRNA levels were evident in our system when cells were treated with different doses of α-naphthoflavone (Figs. 2B and 4). This flavone, however, when added at doses of 0.5 µM or 12 µM was able to reverse the induction of CYP1A4 transcripts exerted by β-naphthoflavone on LMH cells (Fig. 3). No antagonism on the transcription induction was observed when α-naphthoflavone was used at 0.07 µM or 0.1 µM (Fig. 3). Similar results were obtained when the AHR ligand was added in the presence of estrogens (Fig. 4).

### 3.4. Beta-naphthoflavone impairs estradiol-induced vitellogenin promoter activity in LMH cells

The result of AHR activation on ER-driven transcriptional activity was investigated by evaluating the effect of the AHR agonist on the stimulation of gene expression exerted by the estrogen on the vitellogenin promoter. As expected, when LMH cells transiently transfected with the reporter construct VTG-Luc were treated with estradiol, induction of luciferase activity was observed (~7 fold induction compared to control cells) (Fig. 5). This activation was reversed by

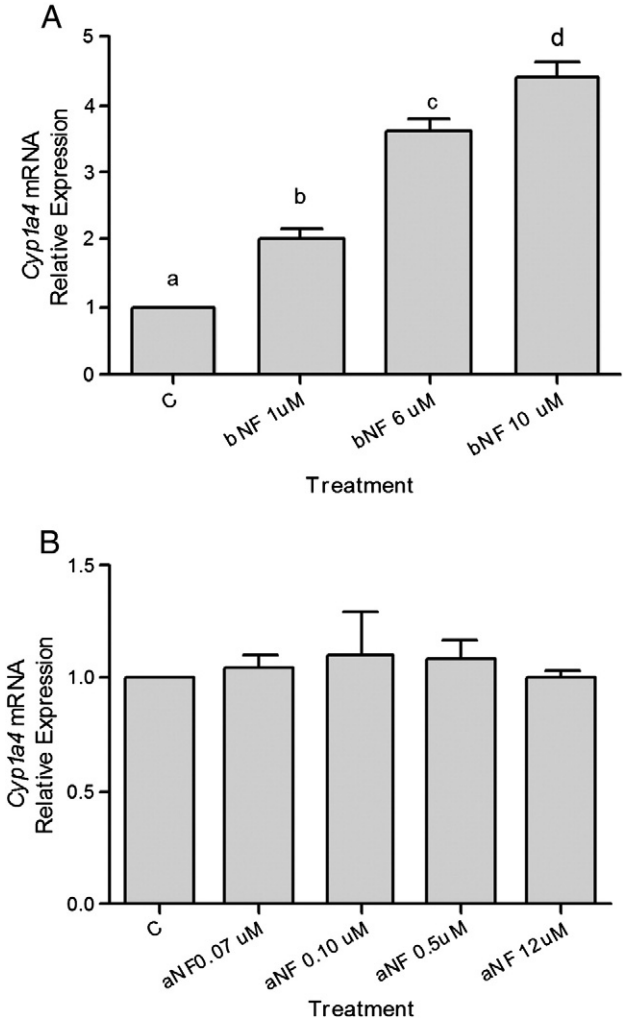


**Fig. 2.** Beta-naphthoflavone acts as AHR agonist in LMH cells. LMH cells were cultured for 24 h in control medium (C) or in the presence of different doses of A) the AHR ligand β-naphthoflavone (bNF, 1–10 μM) or B) the AHR ligand α-naphthoflavone (aNF, 0.07–12 μM). Total RNA extraction and RT-PCR for CYP1A4 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 for a *vs.* b and c *vs.* d, where $P<0.05$).

concomitant treatment with the pure antiestrogen ICI 182,780 (Fig. 5B). As can be seen in Fig. 5A, the observed induction of ER-driven transcription exerted by estradiol was completely inhibited by co-treatment with β-naphthoflavone, blockade that could be partially and significantly reversed by the AHR antagonist α-naphthoflavone. It is to be noted that α-naphthoflavone neither inhibited the transactivation elicited by estradiol nor affected the antiestrogenic action of ICI 182,780 (Fig. 5B). Besides, when the AHR antagonist was added alone, no changes in basal luciferase activity were observed (Fig. 5B).

## 4. Discussion

Activation of the AHR by binding of exogenous ligands such as environmental contaminants is associated with a wide range of adverse biological actions. The analysis of the biology of the receptor and the study of the toxic and physiologic responses mediated by this regulatory protein have been extensive over the past decades (Denison et al., 2011). The domestic chicken has become a typical avian model for the study of AHR in birds, and there are *in ovo*, *in vivo* and *in vitro* experiments that

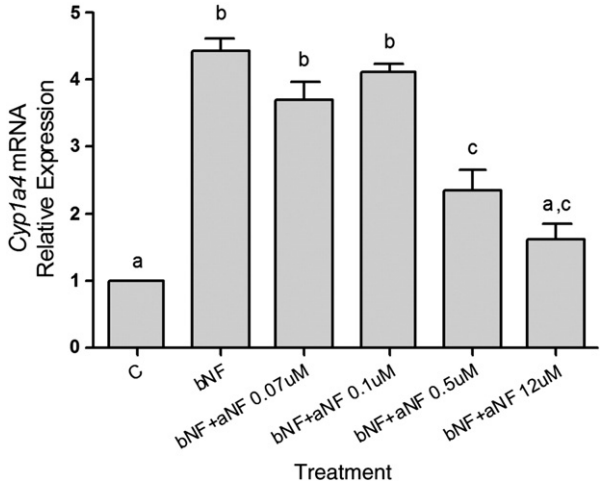


**Fig. 3.** Alpha-naphthoflavone acts as AHR antagonist in LMH cells. LMH cells were cultured for 24 h in control medium (C) or in the presence of β-naphthoflavone (bNF 6 μM) and different doses of the AHR ligand α-naphthoflavone (aNF, 0.07–12 μM). Total RNA extraction and RT-PCR for CYP1A4 mRNA were 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 for C *vs.* bNF + aNF 0.5 μM, and bNF + aNF 0.07 μM *vs.* bNF + aNF 0.5 μM, where $P<0.01$).

have characterized the system and described the effects of the activation of the receptor by dioxin and related compounds, mainly in hepatocytes (for example, see Kennedy et al., 1996; Walker et al., 2000; Head and Kennedy, 2007; Watanabe et al., 2009; Hervé et al., 2010). While the interaction with the ER signaling is perhaps the most well documented crosstalk between the AHR and any other pathway (Swedenborg and Pongratz, 2010; Shanle and Xu, 2011), no experiments have been conducted in chickens regarding the potential antiestrogenic effects of AHR agonists. This background prompted us to study the effect of AHR activation on estradiol-induced responses in chicken liver-derived cells.

In search of a more convenient culture system alternative to primary chicken hepatocytes, we tested the LMH cell line as a candidate

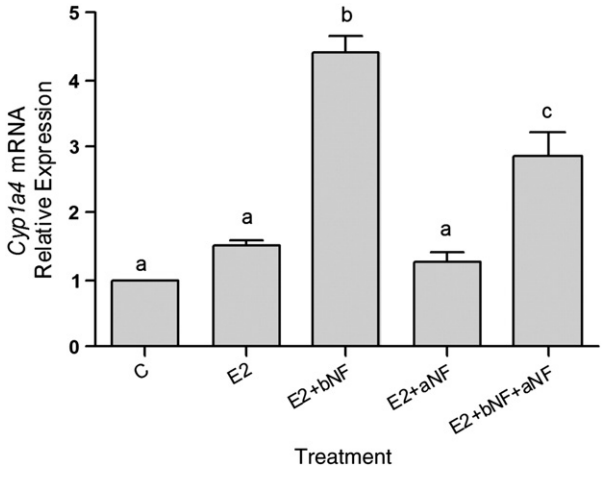


**Fig. 4.** AHR activation in the presence of estradiol in LMH cells. LMH cells were cultured for 24 h in control medium (C) or in the presence of estradiol (E2, 1 μM) added alone or in combination with β-naphthoflavone (bNF 6 μM) and α-naphthoflavone (aNF, 0.5 μM). Total RNA extraction and RT-PCR for CYP1A4 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 for E2 *vs.* E2 + bNF + aNF, and E2 + aNF *vs.* E2 + bNF + aNF, where $P<0.01$).

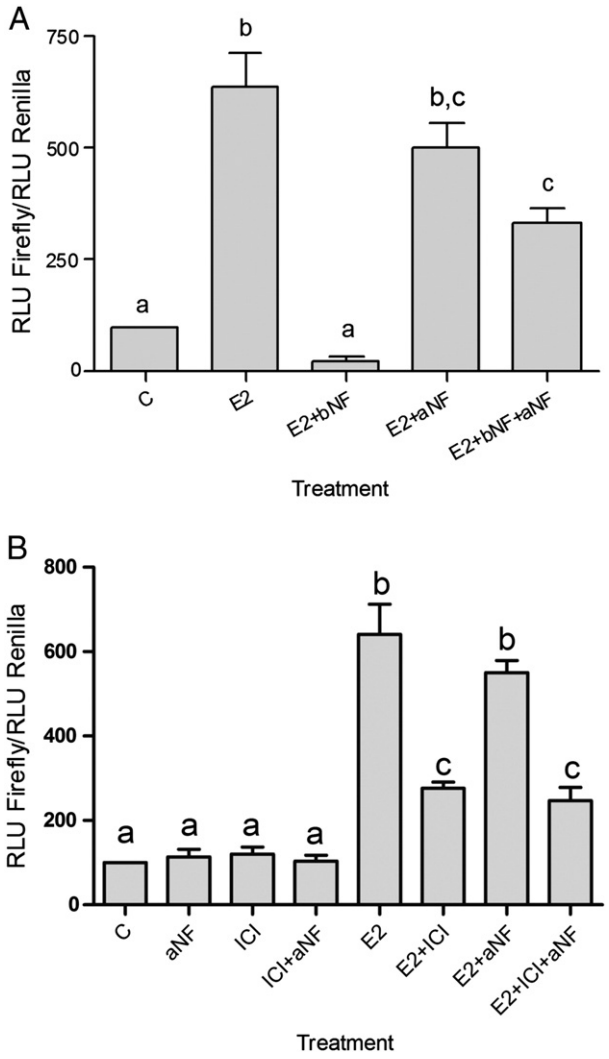


**Fig. 5.** Beta-naphthoflavone impairs estrogen-driven VTG promoter activity in LMH cells. LMH cells were transiently transfected with the VTG-Luc reporter construct and the control reporter plasmid pRL-SV40 as described in Materials and methods. A) After transfection (16 h later) cells were treated with estradiol alone (E2, 1 μM) or with estradiol plus the AHR ligands β-naphthoflavone (6 μM, bNF) or α-naphthoflavone (0.5 μM, aNF), either added separately or in combination. B) 16 h after transfection, cells were treated with ICI 182,780 (10 μM, ICI), estradiol (E2, 1 μM) or estradiol plus ICI 182,780 (10 μM, E2 + ICI), either in the absence or in the presence of α-naphthoflavone (0.5 μM, aNF). After 32 h of incubation with the different stimuli, cells were harvested as described in Materials and Methods and processed for Dual-Luciferase Reporter Assay. Results are expressed as percent of relative luciferase units, when compared to control cells (C, only vehicle added, taken as 100%). Values are means ± SEM of four independent experiments, each performed in duplicate. Values not sharing a common letter are significantly different ($P<0.001$, except for E2 + bNF *vs.* E2 + bNF + aNF, E2 *vs.* E2 + bNF + aNF and E2 + ICI *vs.* E2 + aNF, where $P<0.01$; and C *vs.* E2 + ICI, where $P<0.05$).

for our study. We found that these cells express significant amount of immunoreactive ER and that they are able to transduce an estradiol-elicited signal and activate the transcription of a heterologous reporter gene in a way comparable to that observed in CEH. This is in accordance with the observations made by Berkowitz and Evans, who found that definite culture conditions for LMH can support estrogen-dependent expression of endogenous genes in a serum-free medium in the absence of exogenous estrogen receptors (Berkowitz and Evans, 1992). Our results indicate that the LMH cell line in our specific experimental culture conditions does not require ER over-expression, what makes it an ideal and promising model for the study of AHR–ER interaction in chicken hepatocytes. This experimental system, in comparison to primary hepatocytes, is easier and more practical to work with and does not involve animal sacrifice; and in comparison to the derived LMH/2A cell line, the LMH culture system used here has the added advantage of not requiring ER over-expression.

Since there is no demonstration of the ability of AHR ligands to function as agonist or antagonist of the receptor in LMH cells, we decided to determine if β-naphthoflavone, the well known AHR ligand described as agonist of this receptor in a wide variety of systems, is able to activate the AHR and induce its transcriptional activity in our system. As expected, we found that this flavone induces CYP1A4 mRNA in a dose dependent manner, what is considered a hallmark of Ah-response in most cells and constitutes a model of activation of the AHR as transcription factor. In addition, we tested the effect of α-naphthoflavone on CYP1A4 transcript levels in the system under study in order to establish the doses at which this compound is able to act as antagonist of the receptor. We tested the doses reported to antagonize TCB-stimulated ethoxyresorufin-O-deethylase activity *in vitro* in chicken embryo microsomes (Lorr et al., 1992) or to antagonize β-naphthoflavone-stimulated CYP1A1 expression in fish hepatocytes (Navas and Segner, 2000). Interestingly, we found that in LMH cells α-naphthoflavone acts as antagonist of AHR at doses as high as 12 μM, which is in accordance with the results obtained for CYP1A1 expression in fish hepatocytes (Navas and Segner, 2000). We found that in our system α-naphthoflavone also antagonizes AHR-driven transcription at a dose of 0.5 μM, which is in agreement with results in a variety of species and different experimental models (Gasiewicz and Rucci, 1991; Wilhelmsson et al., 1994; Bussmann et al., 2006; Henry and Gasiewicz, 2008). We decided to choose the dose of 0.5 μM in future experiments in order to maintain a minimal amount of solvent in the culture media.

The chicken major vitellogenin II gene is expressed in hepatocytes in response to estradiol as a result of the presence of EREs within its promoter region (Burch et al., 1998). Although the antiestrogenic effect of AHR activation on the transcriptional regulation of the vitellogenin gene in the fish is well documented (Navas and Segner, 2000; Bemanian et al., 2004; Gräns et al., 2010), there are no specific data regarding aryl hydrocarbons impact on vitellogenin production in the chicken. In particular, the effect of AHR agonists on the promoter activity of the chicken vitellogenin gene has not been explored yet, and existing studies involving promoter analysis have been conducted either with the fish gene or with constructs that bear the *Xenopus* vitellogenin A2 regulatory sequence (for example, see Nodland et al., 2007; Minh et al., 2008). Since there are differences in the nucleotide sequence of the vitellogenin orthologs (Walker et al., 1983; Bouter et al., 2010) and given the variability in AHR action depending on the species, system or even the ligand under study (Denison et al., 2011), it is of interest to evaluate the effect of specific ligands of the AHR on the regulation of the chicken vitellogenin gene expression. We found that the AHR agonist β-naphthoflavone has a drastic antiestrogenic effect on vitellogenin transcription that leads to repression of ER transcription, as assessed by evaluating the ER-driven promoter transactivation. Evidence is provided that this effect is mediated by the AHR, since the receptor antagonist α-naphthoflavone (ligand that is not able to induce AHR and blocks its activation) was able to reverse the inhibitory action of the agonist β-naphthoflavone on estradiol-driven transcriptional activity. In addition, the antagonist alone did not reproduce the described inhibitory effect, did not affect the antiestrogenic action of ICI 182,780 and did not induce the expression of the reporter gene. These facts illustrate the specificity of α-naphthoflavone and indicate that the nuclear AHR complex is required for the described antiestrogenic response in LMH cells. Besides, the transcriptional activity of the estrogen-responsive promoter observed in LMH cells was reversed by co-treatment with the pure anti-estrogen ICI 182,780, corroborating the specificity of the response of LMH to estradiol and the involvement of ER in the phenomenon. Our findings in the chicken are in

accordance with those observed in fish hepatocytes, where the AHR agonist β-naphthoflavone also behaves as an antiestrogenic compound impairing vitellogenin synthesis (Navas and Segner, 2000; Bemanian et al., 2004; Palumbo et al., 2009; Gräns et al., 2010). Interestingly, the inhibitory crosstalk between ER and AHR reported in the present study appears to be unidirectional, since estradiol treatment did not alter significantly CYP1A4 mRNA levels in LMH cells. The lack of effect of ER activation on an AHR target gene is a novel description in the chicken and seems to be species-specific and highly variable, since it is also the case for example in salmon hepatocytes (Bemanian et al., 2004) but contrasts with the results obtained in rainbow trout or goldfish (Navas and Segner, 2000; Yan et al., 2012). The observed differences may be ascribed to distinct intrinsic characteristics of each species, probably due to diversity in the signal triggered by estradiol and by AHR activation or to the existence of regulation by specific protein factors restricted to each species.

The antiestrogenicty of AHR agonists can be exerted by different mechanisms and one of them is the impairment of ER mediated transcription due to the direct binding of agonist-activated AHR to ER target gene promoters (Denison et al., 2011). This can occur when the core-binding nucleotides required for a XRE (namely GCGTG ) are present within the regulatory regions needed for ER transactivation (termed inhibitory XRE or inhibitory DRE — iXRE or iDRE) and has been demonstrated for various estradiol-regulated genes like c-fos and cathepsin D (Krishnan et al., 1995; Duan et al., 1999). Nodland et al. (2007) studied the antiestrogenic action of AHR on the estradiol-stimulated *Xenopus* vitellogenin A2 gene promoter activity and analyzed the existence of iXRE in the 5′ regulatory sequence of the gene to account for their findings. They reported that the 5′-promoter region of the vitellogenin A2 gene does not contain a perfect iXRE and that an imperfect one located in the region does not play a role in AHR responsiveness. Therefore, they suggested the existence of unidentified cis-acting genomic sequences or induced trans-acting factors involved in the phenomenon (Nodland et al., 2007). Likewise, although no iXRE has been reported yet in the fish vitellogenin gene promoter, experiments in salmon and trout hepatocytes point out to the notion that β-naphthoflavone exerts its antiestrogenic effect through the interaction of the activated AHR with specific sites present in the regulatory regions of the gene that interfere with ER binding to DNA (Navas and Segner, 2000; Bemanian et al., 2004). In the chicken vitellogenin gene, in contrast to the *Xenopus* ortholog, we identified a perfect core XRE overlapping with the ERE located between nucleotides −617 and −621 of the 5′-regulatory region, which could account for the disruption of estradiol-induced vitellogenin promoter transcriptional activity. Additional experiments are warranted in order to determine if the identified GCGTG motif is a functional iXRE. It is to be noted, nevertheless, that the existence of mechanisms other than the direct inhibition *via* iXRE (like increase in ER degradation, altered E2 synthesis/metabolism or squelching of shared co-activators) cannot be ruled out. Further research would confirm or reject these hypotheses and establish the exact mechanism underlying the inhibition of ER-driven transcription described herein for the chicken vitellogenin gene.

In conclusion, in the present study we demonstrate that β-naphthoflavone disrupts estradiol-induced chicken vitellogenin promoter activity, most probably through a unidirectional inhibiting crosstalk between the AHR and the ER signaling. This constitutes the first report of the AHR–ER inhibitory crosstalk in the chicken. In addition, we present a useful and convenient model for studying the potential antiestrogenic or estrogenic effects of AHR ligands utilizing the LMH cell line and a reporter construct bearing a vast region of the chicken vitellogenin II gene promoter. Our demonstration of the modulatory effect of a CYP1A-inducing compound on vitellogenin transcription in chicken liver-derived cells takes on added significance when one considers the potential exposure of birds, particularly poultry, to environmental toxic compounds.

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