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Estrogenic *in vitro* assay on mouse embryonic Leydig cells

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ABSTRACT We and others have reported that mouse embryonic testes contain a subpopulation of somatic cells expressing estrogen receptor α (ERα). In order to provide evidence for a possible direct estrogen effect on mammalian testes from the early stage of their differentiation, here we devised a method for the *in vitro* culture of the ERα-expressing cells from 12.5 days post coitum mouse testes and their transfection with plasmids containing the classical estrogen responsive element (ERE) or the alternative estrogen AP-1 responsive element upstream of the luciferase reporter gene (ERE-Luc and AP-1-Luc). StAR immunopositivity of the most part of the ERα+ cells grown in culture and subjected to the estrogenic assay, allowed their identification as embryonic Leydig cells. Maximum induction of the ERE-Luc activity was achieved with 10 nM 17-β estradiol (E2), from 1.7 to 3-fold in such cells and from 2.3 to 5.7-fold in MCF-7 cells used for comparison; the anti-estrogen ICI 182.780 abolished such effects. AP-1-Luc was less sensitive to E2 in both cell types (10 nM E2, 1.2 to 2.7-fold increase in embryonic Leydig cells; about 3-fold in MCF-7 cells) and the effect was not ICI-dependent. Eventually, we stimulated the transfected cells with various xenoestrogens such as lindane, bisphenol A or mono-(2-ethylhexyl) pthalate and with the phytoestrogen zeralenone obtaining evidence for ERE-Luc, but not AP-1-Luc stimulation in embryonic Leydig cells. These results represent evidence of functional ERα-dependent genomic pathways in embryonic Leydig cells and describe an *in vitro* assay suitable for evaluating the activity of putative estrogenic compounds on such cells.

KEY WORDS: *17-β-estradiol, lindane, ZEA, MEHP, BPA, Leydig cells, ERE, AP-1*

Introduction

Estrogens are key regulators of growth, differentiation and function in a broad range of target tissues, including the male and female reproductive tracts, mammary gland, bone, brain and the cardiovascular system. The biological effects of estrogens are mediated through estrogen receptor a (ERα) and estrogen receptor β (ERβ), which belong to a large superfamily of nuclear receptors that act as ligand-activated transcription factors (for a review Mangelsdorf *et al.*, 1995). The classical mechanism of activation of ERs depends on ligand binding to the receptors, after which the receptors dimerize and bind to estrogen response elements (EREs) located in the promoters of estrogen-responsive genes (McKenna *et al.*, 1999; Klein-Hitpass *et al.*, 1989). ERs may also regulate gene expression in the absence of DNA-binding by modulating the activities of other transcription factors via protein-protein interactions on DNA. This mechanism is referred to as cross-talk and is common for several nuclear receptors. For example, ligand-bound ERs upregulate and downregulate transcription from genes that contain activator protein (AP)-1 sites, binding sites for the c-Jun/c-Fos complex, in a manner that depends on the type of cells and the subtype of ERs.

In 1993, Sharpe and Skakkebaek formulated the hypothesis that the increased concentration in environment and in food of human made compounds that mimic the action of estrogens termed xenoestrogens, was responsible for increased disorder of the male reproductive function termed Testicular Dysgenesis Syndrome (TDS), including decrease of sperm count, increase in the incidence of testicular cancer and hypospadias and cryptorchidism (Sharpe and Skakkebaek, 1993). They then argued that the exposure to xenoestrogens during fetal and neonatal

Abbreviations used in this paper: AP-1, activator protein-1; BPA, bisphenol A; DES, diethylstilbestrol; E2, 17-β-estradiol; EGFP, enhanced green fluorecence protein; ERα, estrogen receptor α; ERE, estrogen dependent element; Luc, luciferase; MEHP, mono-(2-ethylhexyl) pthalate; MIS, mullerian inhibitory substance; SF-1, steroidogenic factor-1; StAR, steroidogenic acute regulatory; TDS, testicular dysgenesis syndrome; ZEA, zeralenone.

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period was the origin of this syndrome (Skakkebaek *et al.*, 2001). A prerequisite of such hypothesis is that reproductive tissues and gonads express functional ERs from early stages of development. Several studies actually reported that ERs are expressed in reproductive tissues and gonads from early stages of embryo development in mammals, including humans (Saunders *et al.*, 1998, Jefferson *et al.*, 2000, Vicini *et al.*, 2006). In particular, we and others have reported that mouse embryonic testes from 12.5 dpc onward contain a subpopulation of somatic cells, identifiable mostly as Leydig cells, expressing ERα (Greco *et al.*, 1992; Nielsen *et al.*, 2000; Moe-Beherens *et al.*, 2003). This marks testes as a possible target for estrogens and estrogenic compounds from early stages of development.

Results are actually accumulating about ERα-dependent estrogen and estrogenic effects on endocrine functions of fetal Leydig cell (Delbes *et al.*, 2005; Cederroth *et al.*, 2007). No simple assays exist, however, to evidence estrogenic activity at genomic level of compounds on ERα expressing cells within the embryonic testes. Aim of this work was to verify the presence of functional ERα in such cells, defined by us embryonic Leydig cells, using an ERE-and AP-1-Luc assay that could be used to quantify the genomic activity of estrogens and estrogenic compounds on such cells.

Results and Discussion

RT-PCR analyses carried out to verify the expression of ER transcripts on testis SCs after 3 days of culture and MCF-7 cells used as control, confirmed the expression of ERα and not of ERβ in either cell types (Fig. 1).

Using IF with antibodies specific for the three main somatic cell types of the testis, namely anti-MIS for Sertoli cells, anti-Desmin for myoid cells and anti-StAR for Leydig cells, we next estimated that under our culture conditions the percentages of cells positive for these markers after three days of culture were (mean ± standard error) 12 ± 2.7% for MIS, 20 ± 1.8% for Desmin and 50 ± 6.5 % for StAR (Fig. 2). On the basis of such expression, we consider these cells as Sertoli, myoid cell precursors and embryonic Leydig cells, respectively. Moreover, while virtually all StAR+ cells were ERα+ (Fig. 2D), only about 10% of the ERα+cells were StAR-. Overall these observations indicated that around 60% of the cells to be subjected to the ERE- and AP-1-Luc assay expressed ERα and that the large majority of such cells being StAR+ could be identified as embryonic Leydig cells. Since in preliminary experiments, using pEGFP-C1 construct, the efficiency of transfection of the embryonic Leydig StAR+ ERα+ cells was around 30% and the number of other types of transfected cells was less that 5% (not shown), we attribute to the embryonic Leydig cells the results of the estrogenic assays reported below. In order to verify the functionality of ERα in such cells, we next transfected cultured testis SCs with ERE- and AP-1-Luc plasmids and then after 24 hr of starvation in serum-free medium, stimulated them with 1-100 nM E2 for further 24 h. The results of the ERE-Luc assay show maximal response of 2.8 ± 0.4-fold stimulation at 10 nM E2 relative to vehicle-treated controls (Fig. 3A). Similar results were obtained when the assay were carried out on MCF-7 cells (Fig. 3B). In accord to previous results (Balaguer *et al.*, 2001), however, in these cells the maximal effect achieved with 10 nM E2 reached a significant higher value of 5.7 ± 0.7-fold stimulation. In both cell types, 10 nM E2 stimulation was abolished by the presence of 10 μM ICI. The AP-1-Luc assay carried out on fractions enriched in embryonic Leydig cells and MCF-7 cells showed that in both cell types AP-1 site was less responsive to the hormone. In fact, we observed only 1.76 ± 0.23-fold and 3 ± 0.15-fold stimulation by 10 nM E2 in fractions enriched in embryonic Leydig cells and MCF-7 cells, respectively. Interestingly, ICI failed to inhibit this effect (Fig. 4). The low activity of the AP-1-Luc construct reported here is in line with the notion that ERα is a weaker activator of AP-1 in comparison to ERβ, while the lack of the ICI inhibitory effect can be explained by the fact that ERs can enhance AP-1 activity through two different mechanisms (for a review, see Kushner *et al.*, 2000). It is actually well established that ER action at EREs is mediated by transactivation functions. ERα contains two separate transactivation functions, AF-1 and AF-2, which synergize strongly to give the overall level of estrogen response. AF-2 consists of a small hydrophobic patch on the surface of the estrogen-liganded ligand binding domain (LBD). ERs can enhance AP-1 activity in a manner that requires ER transactivation functions, but not the ER-DNA binding domain (DBD) or with a mechanism that is independent of ER activation functions, but does require the ER-DBD. While the former is activated by estrogens, the latter is activated by antiestrogens, especially those with high antiestrogenic potential like ICI (for a review, see Kushner *et al.*, 2000).

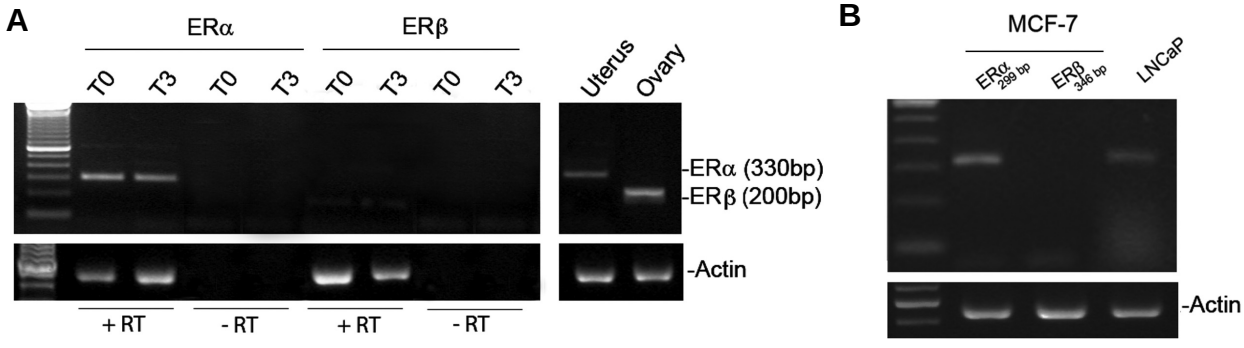

Fig. 1. Expression of ERα and ERβ transcripts in cultured testis somatic cells (SCs) and MCF-7 cells. *RT-PCR analyses on SCs obtained from 12.5 dpc testes and cultured for 0 and 3 days* **(A)** *and on MCF-7 cells* **(B)**. *Both cell types show the presence of ERα but not of ERβ. Uterus and ovary were used as positive control for mERα and mERβ, respectively; LNCaP cells was used as control for hERβ. RT, retrotrascriptase.*

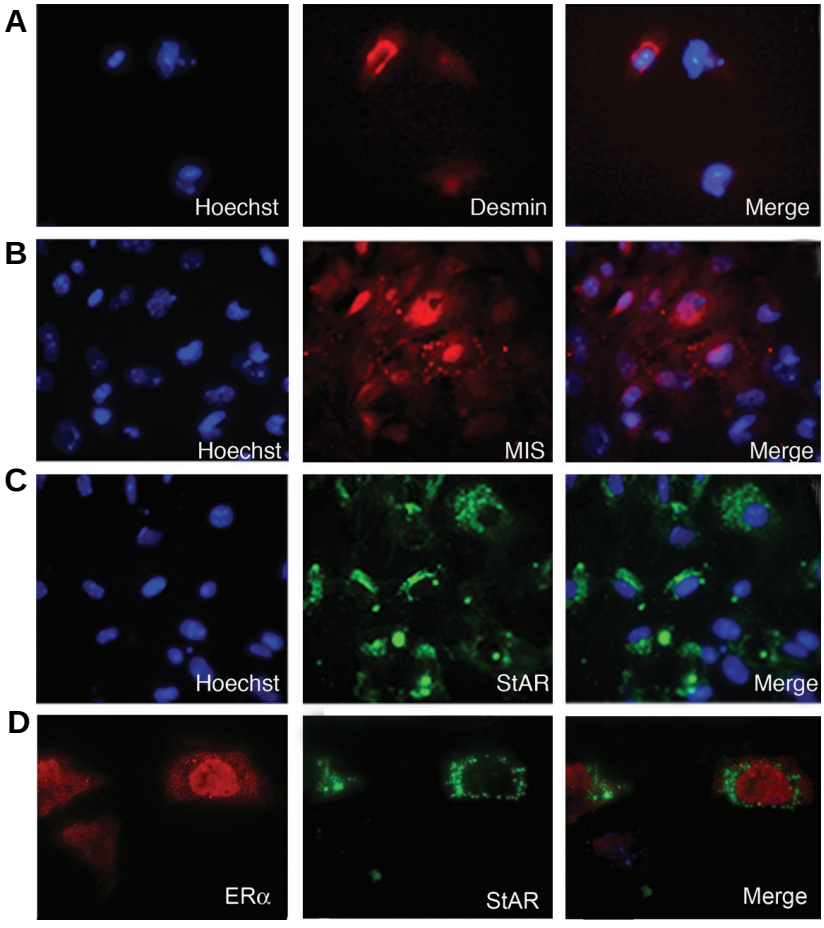

Fig. 2. Immunofluorescence on testis somatic cells (SCs) after 3 days of culture. *Representative fields of SCs stained as follows: Desmin* **(A)** *for myoid cells, MIS* **(B)** *for Sertoli cells and StAR* **(C)** *for Leydig cells;* **(D)** *double immunolocalization of ERα+ and StAR+. Original magnification 40x.*

Finally, to further validate our assay, we stimulated cultured fractions enriched in embryonic Leydig cells and MCF-7 cells with lindane, the γ isomer of hexacyclohexane (γ-HCH), bisphenol A (BPA, 2,2-bis (4hydroxyphen) propane), mono-(2-ethylhexyl) pthalate (MEHP) and zeralenone (ZEA), considered compounds with estrogenic activity (Traina *et al.*, 2003; Washington *et al.*, 2001; Okubo *et al.*, 2003; Shier *et al.*, 2001, and references herein). The results reported in Fig. 5, show that at concentrations able to elicit estrogen-dependent effect in MCF-7 cells in our assay and in literature (Balaguer *et al.*, 2001, Martin *et al.*, 1978, Ricupito *et al.*, 2009, Steinmetz *et al.*, 1996), these compounds stimulated ERE-Luc activity in fractions enriched in embryonic Leydig cells in a ICI-dependent manner at similar (3.1 ± 0.5 fold lindane and 3.33 ± 0.2-fold BPA) or higher (4.6 ± 0.1 fold ZEA) level than E2 (2.8 ± 0.4 fold). On the other hand, although 10μM MEHP appeared no estrogenic on MCF-7 (Fig. 5A) (see also Okubo *et al.*, 2003), it caused 4.0 ± 0.5-fold increase of ERE-Luc activity in fractions enriched in embryonic Leydig cells in our assay. Not consistent inhibitory effect of ICI on the MEHP effect (Fig. 5A) suggests complex not canonical ERα-dependent action of this compound on such cells. Interestingly, in the mouse in utero phthalate exposure altered development of fetal seminiferous cord and caused gonocyte multinucleation without modifying testosterone level (Gaido *et al.*, 2007). Finally, all these compounds failed to significantly stimulate AP-1-Luc activity in fractions enriched in embryonic Leydig cells. With the exception of ZEA, they were, however, able to stimulate such activity in ICI-independent manner in MCF-7 at levels comparable to those elicited by E2 (Fig. 4 and Fig. 5B). On the whole the comparison of estrogen responsiveness both in terms of ERE and AP-1-Luc activity allows to evidence significant less sensitivity in embryonic Leydig cells, than in MCF-7. This can be due to either higher levels of ERα or more efficient ERα signalling or both in these latter (Steinmetz *et al.*, 1996; Balaguer *et al.*, 2001).

Besides the above reported study on pthalates carried out by Gaido *et al.* (2007), several studies have demonstrated various effects of the prenatal exposure to estrogens and estrogenic compounds including environmental xenoestrogens, on the development of the rodent fetal testis (for a review, see Delbès *et al.*, 2006; 2007). For example in the rat, in utero exposure from 11.5 to 15.5 *dpc* to E2 and various xenoestrogens altered expression of steroidogenic factor-1 (SF-1) in the fetal testis (Majdic *et al.*, 1997) while BPA exposure on day 6-21 of gestation led to increased number of Sertoli cell. In culture, E2 and diethylstilbestrol (DES) caused various alteration in rat Leydig cells, Sertoli cells and gonocytes (Lassurguère *et al.*, 2003). In the mouse, DES inhibited the expression of StAR protein in fetal testis (Guyot *et al.*, 2004). Moreover using ERα knockout mice, it has been reported that endogenous estrogens inhibit fetal Leydig cell development (Delbès *et al.*, 2005) and that ERα is a major contributor to estrogen-mediated testis dysgenesis and cryptorchidism (Cederroth *et al.*, 2007). In spite of these

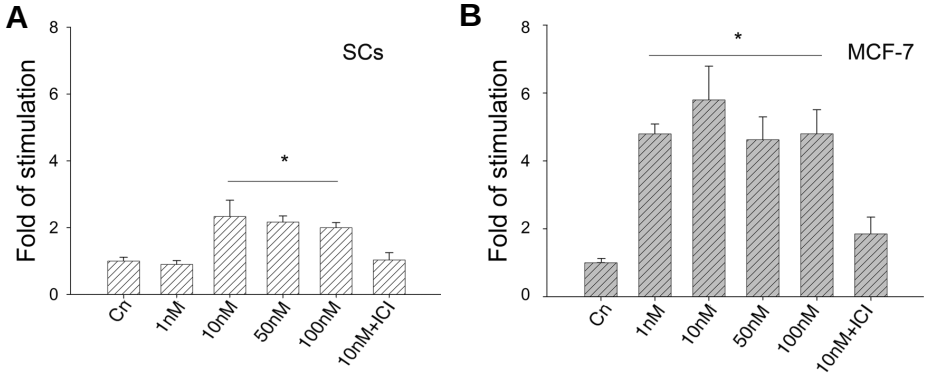

Fig. 3. ERE-Luc activity in testis SCs and MCF-7 cells stimulated by increasing concentrations of 17-β-estradiol (E2). (A) *SCs (fraction enriched in embryonic Leydig cells),* **(B)** *MCF-7 cells. Data represent the mean ± SE of at least three separate experiments. *, p < 0.05 vs Control (Cn).*

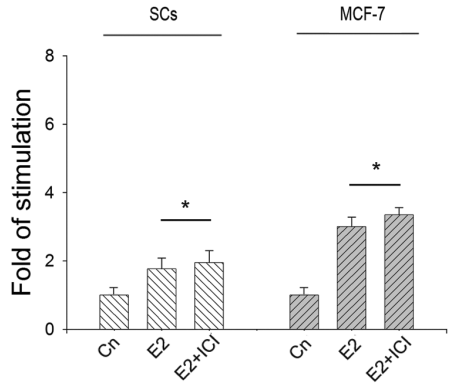

Fig. 4. AP-1-Luc activity in testis somatic cells (SCs; fraction enriched in embryonic Leydig cells) and MCF-7 cells stimulated by 10 nM 17-β-estradiol (E2). *Data represent the mean ± SE of at least three separate experiments. *, p<0.05 vs Control (Cn).*

results, while the estrogen responsiveness of MCF-7 cell in term of proliferation and apoptosis or gene expression is well documented (Detre *et al.*, 1999; Truchet *et al.*, 2000; Inoue *et al.*, 2002), little is known about the estrogen and xenoestrogen ERα-dependent effects on the embryonic Leydig cells. In this regard the results presented here are particular relevant since they constitute a clear evidence about the existence of functional ERα-mediated genomic pathways in embryonic Leydig cells. Moreover, the methods described here represent simple *in vitro* assays suitable for a rapid screening of estrogenic action of compounds on mammalian embryonic testes. These can be used as basis for studies aimed to identify effects of estrogens and xenoestrogens on biological functions and gene expression in Leydig cells at the very early stage of their differentiation within the mammalian testes.

Materials and Methods

Isolation and culture of somatic cells from 12.5 dpc mouse testes and MCF-7 cell culture

All experiments were carried out in compliance with the ethical provisions enforced by the European Union and authorized by the National Committee of the Italian Ministry of Health. CD-1 female mice (Charles River, Italy) were mated with CD-1 male mice and the detection of a vaginal plug the morning following mating was designated 0.5 *day post coitum* (*dpc*). Somatic cells (SCs) were obtained from the 12.5 dpc testes of CD-1 mice embryos following the method described in Pesce *et al.*, 1995. About $5x10^4$ testis SCs in 500 µl of culture medium were seeded in each well of a 24-well Falcon dish. The culture medium consisted of phenol red-free DMEM with high glucose (GibcoBRL/Invitrogen, Italy) containing non essential amino acids, 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine, 0.25 mM pyruvate, 75 mg/L penicillin-G, 50 mg/L streptomycin, 0.5 mg/ml N-acetyl-L-cysteine (NAC) and 5% Horse Serum (HS) and 2.5% Fetal Calf Serum (FCS) (GibcoBRL/Invitrogen (Italy). Culture was carried out in a humidified incubator at 37°C in 5% CO_2 air. The MCF-7 human breast cancer cell line was purchased from ATCC (American Type Culture Collection, USA) and cultured in DMEM w/o phenol red, 5 % fetal bovine serum (FBS), L-glutamine and antibiotics (GibcoBRL/Invitrogen, Italy).

Immunofluorescence (IF)

At the indicated time, cells were fixed in 4% paraformaldehyde, extensively washed and then permeabilized with Triton 0.2% for 5 min before incubation with the primary antibodies. Immunopositivity for Steroidogenic Acute Regulatory (StAR), Mullerian Inhibitory Substance (MIS) and Desmin proteins was used as Leydig, Sertoli and myoid cell markers, respectively. Cells were incubated over night at 4 °C with anti-StAR (1:250) (Santa Cruz, USA, cat. No. sc25806), anti-MIS (1:50) (Santa Cruz, USA, cat. No. sc-6886), and with anti-Desmin (1:20) (Amersham, Italy). All antibodies were diluted in PBS with 1% BSA. Cells were washed and transferred in secondary antibodies, anti-rabbit (StAR), anti-goat (MIS) and anti-mouse (Desmin) IgGs were added (1:500) for 1 h. Negative controls, omitting the primary antibodies, were included. IF for ERα detection was performed using anti-ERα antibody H222 (a kind gift by Prof. GL Green, University of California, USA) following the protocol for permeabilized cells described in Norfleet *et al.* (1999). For double staining, after fixation and permeabilization according to the ERα detection protocol and StAR immunolocalization as reported above, SCs were incubated with the anti-ERα for 2h and then with anti-rat IgGs made in rabbit for 30 min at room temperature.

RT-PCR for ERα and ERβ

Total RNA was extracted from cultured testis SCs and from MCF-7 cells with RNeasy minikit (Qiagen, Italy) in accordance to the manufacturer's instructions. First-strand cDNA synthesis was performed as follows: 200 ng total RNA was reverse transcribed by 50U of Superscript™II (Invitrogen, Italy) using 50 ng random hexamers, in the presence of 0.5 µM deoxynucleotide triphosphates in a final volume of 20 µl. DNA contamina-

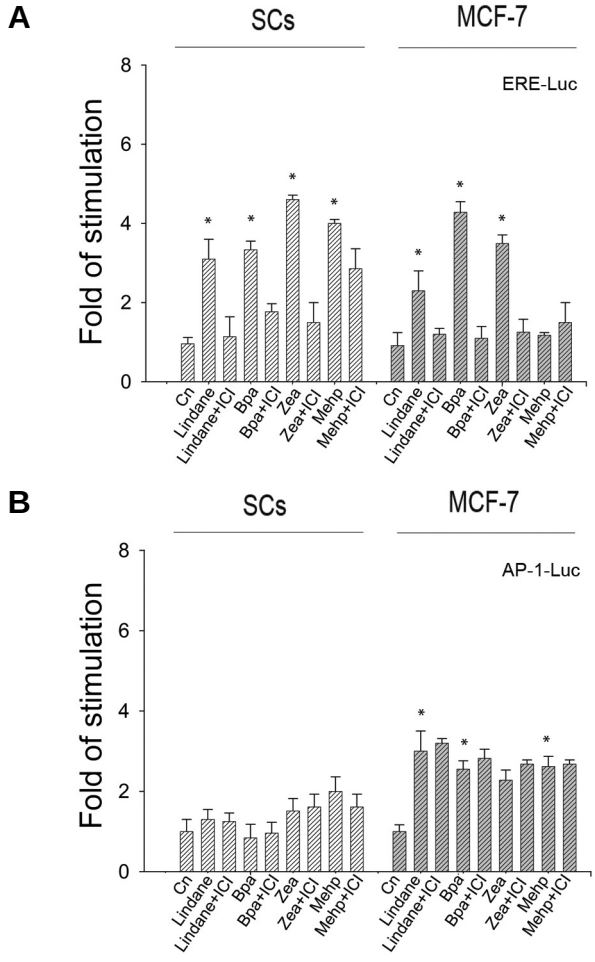

Fig. 5. ERE-Luc and AP-1-Luc activity in testis somatic cells (SCs; fraction enriched in embryonic Leydig cells) and MCF-7 cells stimulated by 10 µM lindane, 25 µM BPA, 10 µM ZEA or 10 µM MEHP. (A) *ERE-Luc,* **(B)** *AP-1-Luc. *, p < 0.05 vs Control (Cn).*

tion or PCR carry over controls were performed omitting reverse transcriptase during reverse transcription. The reaction mixture was incubated for 1 h at 42°C, then heat denatured for 15 min at 75°C 3 µl of the obtained cDNA was used to amplify ERα, ERβ. Primer sequences were:

mERα: 5'-ACCATTGACAAGAACCGGAG-3'
5'-ATAGATCATGGGCGGTTCAG-3'
mERβ: 5'-TCTGCATAGAGAAGCGATGA-3
5'-GGCATTCTACAGTCCTGCTG-3'
hERα 5'-AGACATGAGAGCTGCCAACC-3
5'-GCCAGGCACATTCTAGAAGG-3
hERβ 5'-TCACATCTGTATGCGGAACC-3'
5'-CGTACCACTTCCGAAGTCGG-3'

Reactions were performed using the following reagents: 0.5 µM of primers, Taq polymerase (2U/tube) in a final volume of 20 µl. The amplification consisted of: 35 cycles at 95°C for 1 min, 58°C for 1 min, 72°C for 1 min for mERα; 35 cycles 95°C for 1min, 62°C for 1 min, 72°C for 2 min for mERβ; 35 cycles 95°C for 1min, 58°C for 30 sec, 72°C for 30 sec for hERα/ hERβ. Adult uterus and ovary served as positive controls for mERα and mERβ respectively, while human prostate carcinoma cell line (LNCaP) as a positive control for hERβ.

ERE-and AP-1-Luc assay

After 24 h of culture, testis SCs in each well of a 24-well Falcon dish were transfected with 1 µg of pEGFP-C1 construct (Clonetech) or an ERE- or AP-1 dependent luciferase reporter gene (3XERE-TATA-Luc, provided by Prof. DP McDonnell, Duke University, North Caroline, USA; AP-1-Luc, Stratagene) and Renilla luciferase reporter gene (10 ng) as an internal transfection control using the TransFastTM Transfection Reagent (Promega, Italy) according to the manufacturer's instructions. After 24h of culture, transfected cells were extensively washed and starved for 24h in the same culture medium as above but without serum. Eventually, cells were incubated in the presence of 17-β-estradiol (E2) lindane (γ-HCH), bisphenol A (BPA), zeralenone (ZEA) (Sigma, Italy) or mono-(2-ethylhexyl) pthalate (MEHP) (TCI, Japan) at the indicated concentrations with or without 10 µM of the antiestrogen ICI 182.780 (ICI) (Tocris, UK) for additional 24 h. Stock solutions of compounds were prepared in ethanol and freshly diluted at the final concentrations with the culture medium. Control cultures were treated with vehicle alone (1: 1000 v/v ethanol). At the end of the incubation, cells in each well were harvested and lysed in 250 µl passive lysis buffer (PLB) (Dual-Luciferase Reporter Assay System, Promega, Italy) for 15 min at room temperature. Cell lysates were cleared for 30 sec by centrifugation at top speed in a refrigerated microcentrifuge and transferred to a fresh tube prior to reporter enzyme analysis. Ten microliters of cell lysates from each well were mixed with 100 µl of Luciferase Assay Reagent II (LAR II) (Promega, Italy), and the ERE-firefly luciferase activity was determined using a biocounter luminometer. For the assessment of the Renilla luciferase activity, 100 µl of Stop & GloR Reagent were added to the same sample.

Statistics

All experiments were replicated at least three times. The means were tested for homogeneity of variance and analyzed by ANOVA. The level of significance was set at P= 0.05% and P= 0.01%.

Acknowledgements

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