Antiestrogens upregulate estrogen receptor β expression and inhibit adrenocortical H295R cell proliferation

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Abstract

The molecular mechanisms involved in adrenocortical tumorigenesis are still not completely understood. In this study, using the H295R cell line as a model system, we investigated the role of estrogens and estrogen receptor (ER) α and ERβ in the growth regulation of adrenocortical tumors. We demonstrated that H295R cells are able to convert androgens to estrogens by a constitutive expression of active cytochrome P450 aromatase protein and express ERβ to a greater extent than ERα. Moreover, physiological concentrations of 17β-estradiol (E_2) determined an increase of thymidine incorporation, suggesting the presence of an autocrine mechanism in maintaining H295R cell proliferation. Evaluating the response to ER antagonists like 4-hydroxytamoxifen (OHT) and ICI 182 780 (ICI), we observed an up-regulation of ERβ and a dose-dependent inhibition of H295R cell proliferation. Whereas ICI determined the growth arrest of H295R cells, OHT induced morphological changes that were characteristic of apoptosis. According to the above-mentioned observations, OHT but not ICI clearly induced a marked expression of FasL and the cleavage of both caspase-8 and caspase-3. Interestingly, the apoptotic effects of OHT in H295R cells may be consequent to the enhanced levels of ERβ which stimulate the expression of FasL interacting with activating protein (AP)-1 sites located within its promoter sequence. In conclusion, we have demonstrated that H295R cells are able to transform androgens to estrogens that activate an autocrine mechanism, mediated by their own receptors, and contribute to regulate the proliferation of these cells. Moreover, this study points towards a role for ERβ as an important mediator of the repressive effects exerted by antiestrogens on H295R cells; however, further studies are needed to clarify its role in the control of adrenocortical cell proliferation and on the potential benefits of antiestrogens for treatment of adrenocortical cancer.

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Introduction

Adrenocortical cancers are highly malignant and associated with a poor prognosis. The molecular mechanisms involved in adrenocortical tumorigenesis are still not completely understood. However, several studies have revealed an alteration of a wide variety of signaling pathways such as the mutation of the tumor-suppressor gene TP53 and *ras* gene family as well as the up-regulation of the insulin-like growth factor (IGF) II system (Logié *et al.* 1999, Barzon *et al.* 2001, Kirschner 2002).

The H295R adrenocortical carcinoma cell line (Rainey *et al.* 1994) was derived from H295 cells, which were established from a primary hormonally active adrenocortical carcinoma (Gazdar *et al.* 1990), and has been used as a model system to investigate the role of different signaling pathways in the growth regulation of adrenocortical tumors (Rossi *et al.* 1998, Logié *et al.* 1999, Bourcigaux *et al.* 2000). The IGF system has been well characterized (Weber *et al.* 2000): the IGF-II but not the IGF-I gene is strongly expressed in H295R cells as well as in human adrenocortical tumors and exhibits paternal isodisomy (loss of the maternal-derived allele and duplication of the IGF-II active paternal allele) or less frequently the loss of imprinting (Gicquel *et al.* 1997). IGF-II regulates the growth of adrenal cells by binding to the IGF receptor and inducing the activation of kinase systems as well as mitogen-activated protein kinases or protein kinase B/Akt (Kirschner 2002). Recently, in several tumoral cells the presence of a cross-talk has been reported between the IGF system and estrogens, which is able to activate the same pathway through the action of estrogen receptors (ERs; Hamelers & Steenbergh 2003).

It has been largely demonstrated that the effects of estrogens on target tissues are mediated by the ERα and ERβ, which act as transcription factors

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(Nilsson *et al.* 2001). In the human fetal adrenal gland the mRNA of ERβ was much more expressed than that of ERα and the ERβ protein was detected in the definitive zone of the adrenal cortex (Takeyama *et al.* 2001). The highly estrogenic environment during pregnancy has been reported to influence steroidogenesis of the primate fetal gland (Hirst *et al.* 1992, Albrecht *et al.* 1999) and it has been suggested that the effects of estrogens via ERβ may play an important role in modulating the development of both human and primate fetal adrenal glands (Albrecht *et al.* 1999, Takeyama *et al.* 2001). However, it remains to be elucidated whether estrogens can influence adrenocortical growth and function in the adult, and even tumorigenesis like that observed in other hormone-dependent tissues (Gao *et al.* 2002). A possible involvement of estrogens in adrenocortical tumor development is suggested by epidemiological and experimental studies. Adrenal tumors, and especially functioning tumors, are more frequently found in women than in men (Barzon *et al.* 2003). Moreover, adrenocortical cancers show a different distribution among genders, with functioning tumors, which represent about half of adrenocortical carcinomas, significantly more frequent in females, and non-functioning carcinomas more frequent in males. Interestingly, a case-control study demonstrated that use of estro-progestins was a risk-factor for the development of adrenocortical carcinomas (Hsing *et al.* 1996). Moreover, a recent study on H295R cells (Somjen *et al.* 2003) has shown that these cells are sensitive to low doses of estrogens in terms of proliferation and that they express mRNA of both ER isoforms.

In the present study, using the H295R cell as a model system, we investigated the involvement of estrogen and ERs in the growth regulation of adrenocortical carcinoma. We have demonstrated that H295R cells are able to transform androgens to estrogens that activate an autocrine mechanism, mediated by their own receptors, and contribute to the regulation of proliferation of these cells. Moreover, for the first time we have revealed that in H295R cells antiestrogens such as 4-hydroxytamoxifen (OHT) and ICI 182,780 (ICI) exert relevant growth-inhibitory effects through different pathways. For instance, OHT induces apoptosis in H295R cells, up-regulating the expression of FasL and determining the autocrine activation of caspases.

Materials and methods

Reagents

Forskolin (FSK), 17β-estradiol (E_2) and OHT were purchased from Sigma (St Louis, MO, USA), ICI was a gift from Astra-Zeneca (Italy) and Letrozole was a gift from Novartis Pharma AG (Basel, Switzerland). All these reagents were dissolved in dimethylsulfoxide (DMSO).

Cell cultures

H295R cells, a cell line established from a human adrenocortical carcinoma, was obtained from Dr W E Rainey (University of Texas Southwestern Medical Center, Dallas, TX, USA) and cultured in Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12; 1:1; Eurobio, Les Ulis, France) supplemented with 1% ITS Liquid Media Supplement (100 ×; Sigma), 10% calf serum and antibiotics (Eurobio), at 37 °C in an atmosphere of humidified air containing 5% CO_2. MCF-7 breast cancer cells were incubated in the same conditions but maintained in DMEM/F12 supplemented with 10% calf serum.

Plasmids and *in vitro* transcription/translation

The coding regions of ERα and ERβ were excised from their vector and subcloned into the pcDNA3·1 zeo(+) expression plasmid (Invitrogen, Carlsbad, CA, USA). 1 μg each plasmid was used for *in vitro* transcription/translation using T7 RNA polymerase in the rabbit reticulocyte system following manufacturer's protocol for the TNT kit (Promega, Madison, WI, USA) in a final volume of 50 μl.

Aromatase activity assay

The aromatase activity in subconfluent H295R cell culture medium was measured by tritiated water-release assay using 0·5 μM [1β-^{3}H(N)]androst-4-ene-3,17-dione (25·3 Ci/mmol; DuPont NEN, Boston, MA, USA) as a substrate (Lephart & Simpson 1991). Incubations were performed at 37 °C for 2 h under a 95%:5% air/CO_2 atmosphere. The results obtained were expressed as pmol/h and normalized to milligram of protein (pmol/h per mg protein).

Estradiol measurement

Subconfluent H295R cells, seeded in 12-well/plates at the concentration of 5×10^5 cells/well, were washed twice with PBS and grown in medium without serum. After 24 h, cells were washed again with PBS and grown in 0·5 ml/well fresh medium without serum for 48 and 72 h. The experiment was performed three times in sextuplicate. Total E_2 was measured in the supernatant of H295R cells by competitive immunoassays on ADVIA Centaur (Bayer Diagnostics, Tarrytown, NY, USA).

RNA isolation and reverse transcriptase (RT)-PCR

Total cellular RNA was extracted from H295R cells with Trizol reagent (Invitrogen, Life Technologies, San Giuliano Milanese, Italy) according to the protocol provided by the manufacturer. All the RNA was

DNase-treated using the DNA-free™ kit (Ambion, Austin, TX, USA), and purity and integrity of the RNA was checked spectroscopically and by gel electrophoresis before carrying out the analytical procedures. The evaluation of gene expression was performed by semiquantitative RT-PCR (Maggiolini *et al.* 1999). For cytochrome P450 aromatase (henceforth called P450 aromatase), ERβ and internal control gene 36B4 the primers were 5′-CTGGAAGAATGTATGGACTT-3′ (P450 aromatase forward), 5′-GATCATTTCCAGCAT GTTTT-3′ (P450 aromatase reverse), 5′-CAGCATTC CCAGCAATGTCAC-3′ (ERβ forward), 5′-GCAGAA GTGAGCATCCCTCTTTG-3′ (ERβ reverse), 5′-CTC AACATCTCCCCCTTCTC-3′ (36B4 forward) and 5′-CAAATCCCATATCCTCGTCC-3′ (36B4 reverse), to yield products of 660, 281 and 408 bp, respectively, with 30, 20 and 15 PCR cycles of 1 min at 95 °C for all genes, 1 min at 64 °C for P450 aromatase, 1 min at 58 °C for ERβ and 1 min at 57 °C for 36B4, followed by 1 min at 72 °C for all genes.

Western-blot analysis

Total cell protein extracts from H295R and MCF-7 cells were lysed in ice-cold Ripa buffer containing protease inhibitors (20 mM Tris, 150 mM NaCl, 1% Igepal, 0·5% sodium deoxycholate, 1 mM EDTA, 0·1% SDS, 1 mM PMSF, 0·15 units/ml aprotinin and 10 μM leupeptin). Nuclear extracts were prepared from H295R and MCF-7 as previously described (Andrews & Faller 1991). Briefly, cells plated onto 60 mm^2 dishes were scraped into 1·5 ml cold PBS. Cells were pelleted for 10 s and resuspended in 400 μl cold buffer A, containing protease inhibitors, 10 mM Hepes, pH 7·9, 1·5 mM $MgCl_2$, 10 mM KCl, 0·5 mM dithiothreitol, 0·2 mM PMSF, 10 μM leupeptin and 0·15 units/ml aprotinin, by flicking the tube. The cells were allowed to swell on ice for 10 min and then vortexed for 10 s. Samples were centrifuged for 10 s and the supernatant fraction discarded. The pellet was resuspended in 50 μl cold buffer C (20 mM Hepes, pH 7·9, 25% glycerol, 1·5 mM $MgCl_2$, 420 mM NaCl, 0·2 mM EDTA, 0·5 mM dithiothreitol, 0·2 mM PMSF, 10 μM leupeptin and 0·15 units/ml aprotinin) and incubated on ice for 20 min for high-salt extraction. Cellular debris was removed by centrifugation for 2 min at 4 °C and the supernatant fraction (containing DNA-binding proteins) was stored at −70 °C. The yield was determined by Bradford method (Bradford 1976). The proteins were separated on 11% SDS/polyacrylamide gel and then electroblotted onto a nitrocellulose membrane. The blots were incubated overnight at 4 °C with (1) mouse anti-(human P450 aromatase) antibody, raised against a conserved epitope within P450 aromatase (1:1000; Serotec, Oxford, UK), (2) anti-ERβ antibody against the C-terminal region of the ERβ (1:500; Serotec), (3) anti-ERα (F-10) antibody against the N-terminal region of the ERα (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), (4) anti-caspase-8 1C12 monoclonal antibody (1:1000; Cell Signaling Technology, Celbio, Milan, Italy), (5) anti-caspase-3 antibody (1:1000; Cell Signaling Technology), (6) anti-Fas (FL-335) antibody (1:1000; Santa Cruz Biotechnology) and (7) anti-FasL antibody (1:1000; DB Transduction Laboratories, Lexington, KY, USA). The antigen–antibody complexes were detected by incubation of the membranes at room temperature with goat anti-mouse IgG coupled to peroxidase, developed using the ECL Plus Western-blotting detection system (Amersham Biosciences, Cologno Monzese, Italy). As a loading control, membranes were stripped and reprobed with β-actin antiserum.

Cell-proliferation assay

A total of 1×10^6 cells were seeded onto six-well plates in complete medium, for proliferative analysis. After 3 days the medium was replaced with DMEM lacking Phenol Red as well as serum and ITS Liquid Media Supplement and treated with different concentrations of E_2, ICI 182 780 and OHT alone or in combination for 96 h. Control cells were treated with the same amount of vehicle alone (DMSO) that never exceeded the concentration of 0·01% (v/v).

[^{3}H]Thymidine incorporation was evaluated after a 24-h incubation period with 1 μCi [^{3}H]thymidine (PerkinElmer Life Sciences, Boston, MA, USA) per well. Cells were washed once with 10% trichloroacetic acid, twice with 5% trichloroacetic acid and lysed in 1 ml 0·1 M NaOH at 37 °C for 30 min. The total suspension was added to 10 ml optifluor fluid and was counted in a scintillation counter.

Staining for apoptosis detection

The apoptotic cells were stained using Hoechst 33342 staining (Sigma). H295R cells, plated on chamber slides, were fixed with 4% paraformaldehyde and stained with Hoechst 33342 dye to a final concentration of 5 μg/ml (150 μM) for 20 min at room temperature. Following washing with PBS, the cells were observed under an Olympus BX51 fluorescence microscope.

Chromatin immunoprecipitation (ChIP)

This assay was performed using the ChIP assay kit from Upstate Biotechnology (Lake Placid, NY, USA; http://www.nature.com/cgi-taf/DynaPage.taf?file=/onc/journal/v23/n45/full/1208014a.html-bib23#bib23) with minor modifications in the protocol. H295R cells were grown in 100 mm plates. Confluent cultures (90%) were treated for 24 h with 10 nM E_2, 10 μM OHT or 10 μM ICI. Control cells were treated with the same amount of

vehicle alone (DMSO) that never exceeded 0·01% (v/v). Following treatment DNA–protein complexes were cross-linked with 1% formaldehyde at 37 °C for 10 min. Next, cells were collected and resuspended in 400 µl SDS lysis buffer (Upstate Biotechnology) and left on ice for 10 min. Cells were sonicated four times for 10 s at 30% maximal power (Vibra Cell VCX 500; Sonics & Material, Inc. Newtown, CT, USA) and collected by centrifugation at 4 °C for 10 min at 11 000 ***g***. Of the supernatants 20 µl were kept as input (starting material, to normalize results) and 100 µl were diluted 1:10 in 900 µl ChIP dilution buffer (Upstate Biotechnology) and immunocleared with 80 µl sonicated salmon sperm DNA–Protein A agarose (Upstate Biotechnology) for 6 h at 4 °C. The precleared chromatin was immunoprecipitated overnight with 2 µg specific anti-ERβ antibody. The following day 60 µl salmon sperm DNA–Protein A agarose was added and precipitation was continued at 4 °C until the day after. After pelleting, precipitates were washed sequentially for 5 min with the following buffers: High Salt Immune Complex Wash Buffer, Low Salt Immune Complex Wash Buffer, LiCl Immune Complex Wash Buffer and then twice with TE buffer (all buffers are contained in the kit). The immune complexes were eluted with elution buffer (1% SDS and 0·1 M $NaHCO_3$). The eluates and the 20 µl input were reverse cross-linked by heating at 65 °C overnight and digested with proteinase K (0·5 mg/ml) at 45 °C for 1 h. DNA was recovered by phenol/chloroform extractions. A 2 µl aliquot of 10 mg/ml yeast tRNA was added to each sample and DNA was precipitated with ethanol for 1 h at −20 °C and then resuspended in 50 µl TE buffer. A 5 µl volume of each sample and 2 µl of input were used for PCR using the following FasL promoter primers (GenBank accession no. AF035584): sense, 5′-AAACTG AGGCAGGAGGATGT-3′, and antisense, 5′-TCG TAGTTAACTGCAGCCTC-3′. The PCR conditions were 1 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C for 30 cycles. The amplification products of 200 bp were analyzed on a 1% agarose gel and visualized by ethidium bromide staining. In control samples, nonimmune rabbit IgG was used instead of the specific antibody.

Statistical analysis

Statistical analysis was performed using one-way ANOVA. Data were analyzed using software from STATPAC (Minneapolis, MN, USA).

Results

Estradiol production and aromatase activity in H295R cells

We initially examined whether H295R cells, used as a model system, were able to synthesize estrogens. H295R cells were cultured for 48 and 72 h in serum-free medium; E_2 content was measured with a competitive immuno assay and revealed a time-dependent increase. E_2 medium content was 512 ± 36 pg/ml at 48 and 834 ± 67 pg/ml at 72 h.

We next investigated the expression and the activity of P450 aromatase in the absence or presence of the adenylate cyclase activator FSK. A 24-h treatment with FSK significantly increased aromatase mRNA expression (Fig. 1A and B) and protein levels (Fig. 1C and D). This behavior was reproduced with aromatase activity, which was $7{\cdot}43 \pm 0{\cdot}4$ pmol/h per mg protein in basal conditions and increased to $23{\cdot}28 \pm 2{\cdot}5$ pmol/h per mg protein after incubating cells for 24 h with FSK. The presence of the aromatase inhibitor Letrozole, used 1 h before and during the incubation with the tritiated aromatase substrate, inhibited basal and, in particular, FSK-induced aromatase activity to $1{\cdot}087 \pm 0{\cdot}1$ pmol/h per mg protein (Fig. 1E).

Expression of ERα and ERβ in H295R cells

Since the effects of estrogens on target tissues are mediated by the estrogen receptors (ERα and ERβ), we next investigated the expression of these factors in H295R cells. We subcloned the ERα and ERβ genes into the expression vectors pcDNA3·1 zeo(+) and used these vectors for *in vitro* transcription, and the synthetized proteins were utilized as a standard curve for ERα and ERβ in Western-blot analysis. Standard points were obtained using increasing amounts of the synthetized proteins (0·5, 1, 3 and 6 µl). Densitometric analysis, reference band intesities to the curve where the standard point with the lowest concentration was taken as 100%, indicated that in H295R cells ERβ is significantly higher than ERα. We also used MCF-7 human breast cancer cells, a known ERα-positive cell type, as a control, and found as expected a high expression of the ERα isoform, whereas ERβ was barely detectable (Fig. 2). Based on these results we focused our attention on the role of ERβ in mediating estrogen signaling in this adrenal cancer cell line.

E_2 enhances while antiestrogens and the aromatase inhibitor Letrozole inhibit the proliferation of H295R cells

We then aimed to investigate the effects of E_2 on the proliferation of H295R cells assayed using thymidine incorporation. Treatment with different concentrations of E_2 (0·1–1000 nM) for 96 h exhibited a slight but significative increase of thymidine incorporation (Fig. 3A), while shorter exposures (24, 48 and 72 h) did not determine significant effects on DNA synthesis (data not shown). To demonstrate the involvement of ERs, we also investigated the effects of antiestrogens on H295R

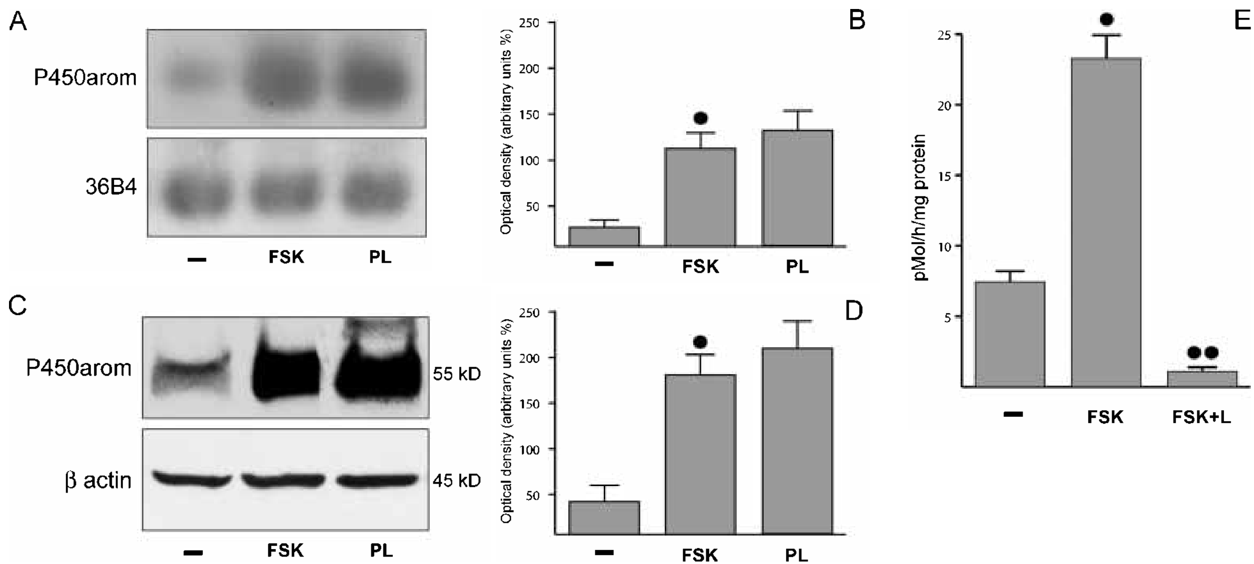

Figure 1 Effects of FSK on P450 aromatase expression and activity in cultured H295R cells. (A) P450 aromatase mRNA expression in H295R cells in the absence (−) or presence of FSK for 24 h was determined by semi-quantitative RT-PCR. Human placenta RNA (PL) was used as a positive control. 36B4 mRNA levels (lower panel) were also determined as a loading control. (B) Quantitative representation of data (means±S.E.M.) from three independent RT-PCR experiments after densitometry and correction for 36B4 expression. (C) Protein expression of P450 aromatase in H295R cells in the absence (−) or presence of FSK for 24 h. Whole-cell extracts (50 µg) were subjected to Western-blot analysis using anti-(human P450 aromatase) antibody (upper panel) and anti-actin antibody (lower panel) as a loading control. (D) Quantitative representation of data (means±S.E.M.) of three independent Western-blot experiments after densitometry and correction for β-actin expression. (E) Aromatase activity in H295R cells. The cells were cultured for 24 h in DMEM/F12 in the absence (−) or presence of FSK (25 µM), or FSK (25 µM) combined with Letrozole (4 µM; FSK+L). Aromatase activity was assessed using the modified tritiated water method. The results obtained were expressed as pmol [^{3}H]water released per h and were normalized for mg protein (pmol/h per mg protein). Values represent the means±S.E.M. from three different experiments, each performed with triplicate samples. ●, $P<0{\cdot}01$ compared with untreated cells (−); ●●, $P<0{\cdot}01$ compared with cells treated with FSK alone.

proliferation. Treatment with increasing amounts of ICI (0·1–10 µM) or OHT (0·1–10 µM) determined a dose-dependent inhibition of thymidine incorporation both under the basal conditions and in the presence of E_2 (10 nM) (Fig. 3B). To further confirm that H295R growth depends on estrogens we investigated the effect of aromatase inhibitor Letrozole on cell proliferation. As shown in Fig. 3C treatment with increasing amounts of Letrozole (1–10 µM) determined a dose-dependent inhibition of thymidine incorporation.

Antiestrogens modulate ERβ expression

The above results prompted us to evaluate whether ERβ expression can be modulated by E_2 and/or antiestrogens such as ICI and OHT. Performing a semiquantitative RT-PCR we observed that a 24-h exposure to ICI or OHT was clearly able to up-regulate the mRNA levels of ERβ, which were not modified by E_2 (10 nM) treatment (Fig. 4A and B). In fact, E_2 dose-response (0·1–1000 nM) or time-course (12, 24 and 48 h) experiments did not reveal any significant differences with respect to controls (data not shown). Besides, Western-blot analysis revealed an increase in ERβ protein levels after a 96-h exposure to antiestrogens (Fig. 4C and D), which paralleled the evaluation of cell proliferation.

Antiestrogens up-regulate FasL protein expression and induce activation of caspase pathway in H295R cells

Microscopic observation of H295R cells treated with 10 µM OHT and stained with Hoechst 33342 showed morphological changes characteristic of apoptosis after only 24 h (Fig. 5). These changes were not observed in cells treated with estradiol or ICI (data not shown).

These results led us to ascertain the involvement of the Fas/FasL pathway in the OHT-promoted apoptosis of H295R cells. Fas protein was expressed but not modulated by E_2, ICI or OHT in H295R cells (Fig. 6A and D), however the expression of FasL was up-regulated only by OHT (Fig. 6B and E). The autocrine mechanism of apoptosis in H295R cells by the Fas/FasL system was further supported by the expression of the active forms of caspase-8 (p43/45 and p28) and caspase-3 (p20 and p17) induced only by OHT treatment (Fig. 7).

ERβ binds the activating protein (AP)-1 site on the FasL promoter

Since for several ER-dependent genes transcription has been shown to be regulated through ER binding to the AP-1 complex, we used chromatin immunoprecipitation analysis to show ERβ binding to the AP-1 site on the FasL promoter in H295R cells. Samples were immunoprecipitated with the ERβ antibody and the FasL promoter region containing the AP-1 site was amplified with specific primers. The results obtained show that ERβ is able to interact with the protein complex bound to the AP-1 site in basal conditions and that this binding is maintained in treated cells (Fig. 8). Bands are specific as shown by the lack of amplification in samples immunoprecipitated with nonimmune rabbit IgG.

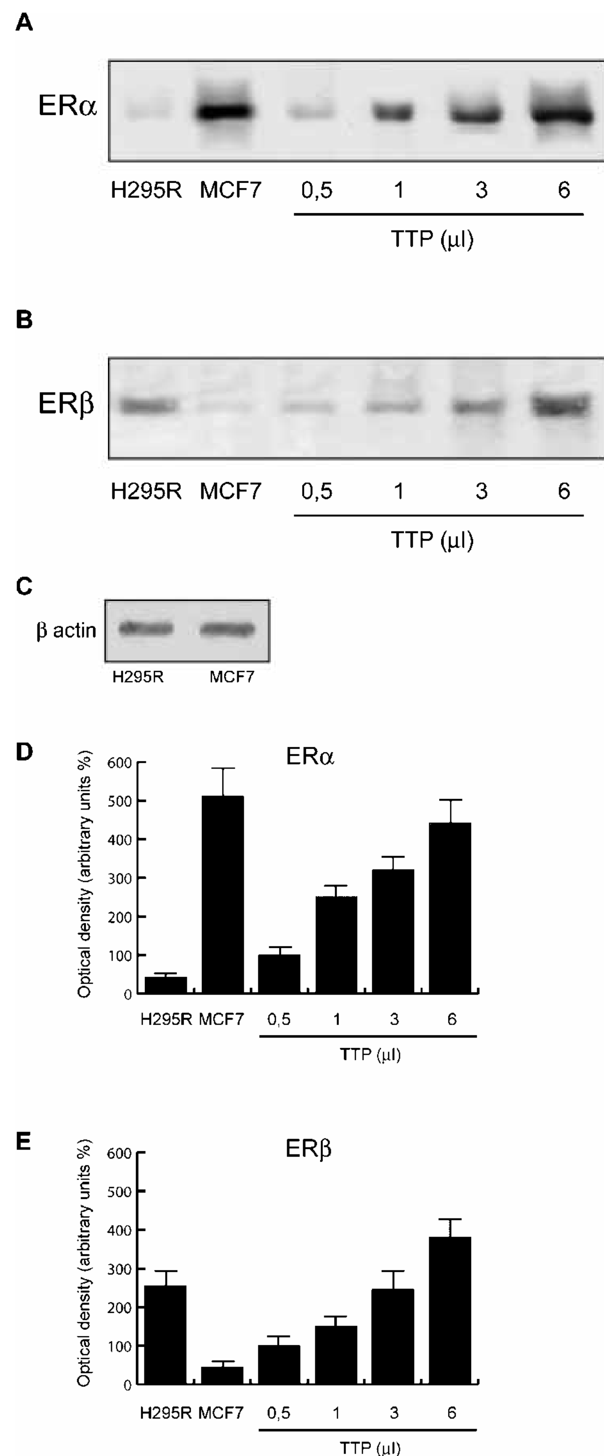

Figure 2 Expression of ERα and ERβ in H295R cells. Western-blot analysis was performed on total proteins from H295R or MCF-7 cells (50 µg) and on *in vitro*-transcribed and -translated protein (TTP) ERα and ERβ; (synthesized as described in the Materials and methods section) utilized as a standard curve at the indicated volumes. Anti-ERα (F10) (A), anti-ERβ (B) and anti-β actin (C) antibodies were used. These results are representative of those obtained in three independent experiments. (D and E) Quantitative represention of data (means±S.E.M.) from three independent Western-blot experiments performed after densitometry and correction for β-actin expression. For densitometric analysis, standard point with the lowest concentration was taken as 100% and band intesities were referred to the curve.

Discussion

In the present study we have shown that H295R adrenocortical carcinoma cells are able to convert androgens to estrogens, which, through a short autocrine loop, mediated by their own receptors, contribute to enhance H295R cell proliferation. Furthermore, both antiestrogens ICI and OHT up-regulate ERβ expression and dose-dependently inhibit basal and E_2-induced H295R cell proliferation by activating different pathways. In fact, whereas ICI determines the growth arrest of H295R cells, OHT treatment activates the Fas/FasL pathway which in turn induces apoptosis.

Androgens and estrogens determine various biological activities on mammalian tissues controlling cellular growth and differentiation through different signal transduction pathways. Previous studies have demonstrated that androgens inhibit H295R cell proliferation through the androgen receptor (Rossi *et al.* 1998), whereas the role of estrogens and ERs in both normal adult and malignant adrenocortical cells remains to be elucidated. Our results give a functional emphasis to recent studies which revealed that the H295R cell line is able to transform androgens into estrogens (Watanabe & Nakaijn 2004) and express ERα and ERβ mRNA (Somjen *et al.* 2003). We demonstrated that high levels of basal aromatase activity are present in H295R cells, allowing these cells to produce E_2. Moreover, H295R cells also express both ER protein isoforms, with a

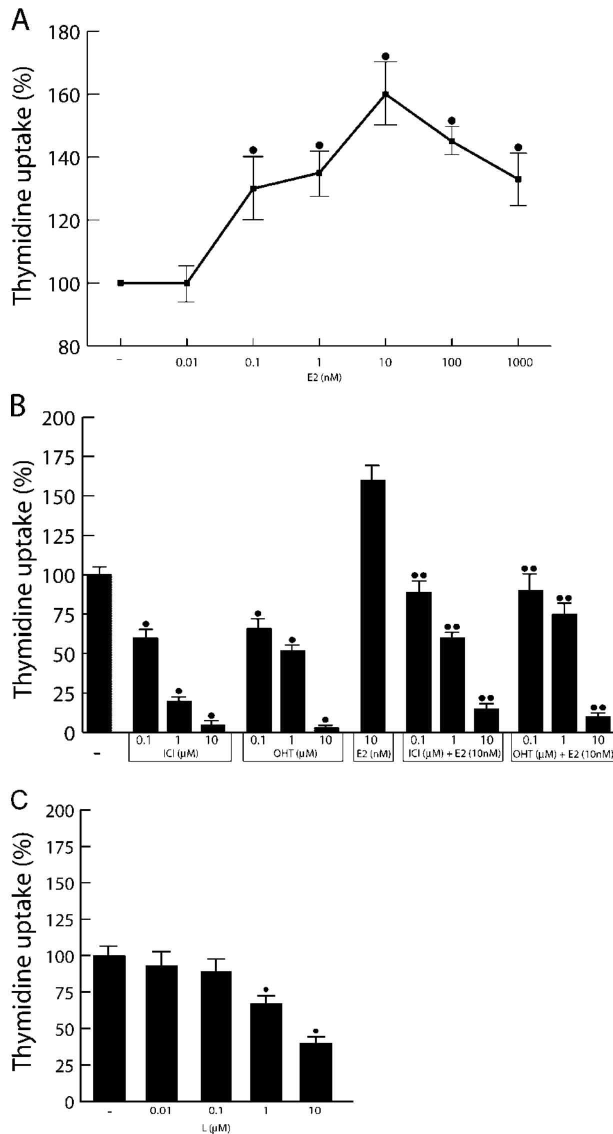

Figure 3 Proliferative analysis in H295R cells. Proliferation was evaluated by [^{3}H]thymidine incorporation analysis. (A) H295R cells were cultured for 96 h in DMEM in the absence or presence of E_2 at the indicated concentrations. (B) H295R cells were cultured for 96 h in DMEM in the absence or presence of E_2, ICI, OHT, E_2+ICI or E_2+OHT at the indicated concentrations. Control cells were treated with the same amount of vehicle alone (DMSO) that never exceeded 0·01% (v/v). (C) H295R cells were cultured for 96 h in DMEM in the absence or presence of Letrozole (L) at the indicated concentrations. Values expressed as percentages of untreated cells (100%); means ± S.E.M. from three independent experiments each performed with triplicate samples. ●, $P<0{\cdot}01$ compared with untreated cells (–); ●●, $P<0{\cdot}01$ compared with E_2-treated cells (E_2).

prevalence of ERβ, thus reproducing the same ER ratio present in the fetal adrenal gland (Takeyama *et al.* 2001). The exposure to a physiological concentration of E_2 determined a slight but significant increase of thymidine incorporation, confirming the results of a previous study (Somjen *et al.* 2003) and revealing the presence of an autocrine mechanism which could contribute to H295R cell proliferation. The observation that both antiestrogens and the aromatase inhibitor Letrozole determined a dose-dependent arrest of these proliferative effects supports this hypothesis. However, only OHT was responsible for the morphological changes associated with apoptosis as observed in previous studies performed on MCF-7 breast cancer cells using tamoxifen and its analogs (Bardon *et al.* 1987, Warri *et al.* 1993, Wilson *et al.* 1995). On the contrary, apoptotic events were shown to be induced also by ICI in ER-positive primary breast cancer cells (Ellis *et al.* 1997). Moreover a study performed on six malignant rhabdoid tumor cell lines revealed that OHT but not ICI induced apoptosis (Koshida *et al.* 2002). To explain these apparently controversial data we have to take into account that the pharmacological potency elicited by antiestrogens is dependent on the cellular context as well as on the interaction of these molecules with the two different ER isoforms. This hypothesis is supported by studies which have shown that ER agonists and antagonists are able to induce distinct conformations and biological activity of both ER isoforms (Van Den Bemd *et al.* 1999). A study using a fingerprint assay clearly indicates that OHT determines, for both ERα and ERβ, the exposure of unique peptide-binding surfaces that are not exposed in the presence of ICI (Paige *et al.* 1999). So, different ER–ligand complexes may be able to recruit different coactivator and corepressor proteins within the cell, and the overall biological response is determined by unique combinations of protein–protein interactions that occur in a given cell and promoter context (Jones *et al.* 1999). The present study contributes to the understanding of the biological activity of OHT and ICI, extending to adrenocortical cancer cells the different response to antiestrogens.

We ascertained that the antiestrogens OHT and ICI were able to induce the up-regulation of ERβ, whereas E_2 did not show any change in our experimental conditions. What is the molecular mechanism by which antiestrogens can enhance ERβ expression and what is the biological counterpart of this response? It is worth noting that Paech *et al.* (1997) reported an antiestrogen-dependent transcriptional activity of ERβ at AP-1 sites which were demonstrated to be located within the promoter region of ERβ gene (Li *et al.* 2000). In line with these findings, our data may provide new insight on ERβ autologous regulation by antiestrogens as displayed even in H295R cells. Moreover, could this up-regulation of ERβ mediate the above reported down-regulatory effects of ICI and OHT on H295R cell proliferation? This question opens an additional intriguing area of research that we are investigating currently.

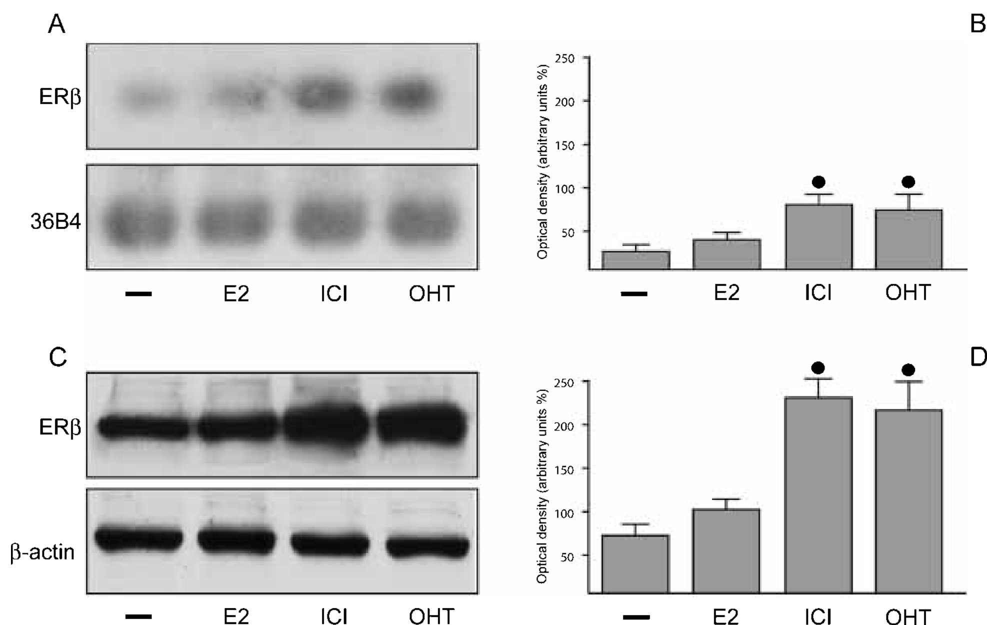

Figure 4 Effects of antiestrogens on ERβ expression in H295R cells. (A) ERβ mRNA expression in H295R cells in the absence (−) or presence of E_2 (10 nM), ICI (10 μM) or OHT (10μM) for 24 h was determined by semi-quantitative RT-PCR (upper panel). 36B4 mRNA levels (lower panel) were used as a loading control. (B) Quantitative representation of data (means±S.E.M.) from three independent RT-PCR experiments after densitometry and correction for 36B4 expression. (C) ERβ protein expression in H295R cells in the absence or presence of E_2 (10 nM), ICI (10 μM) or OHT (10 μM) for 96 h was determined by Western blotting (upper panel); β-actin was also used as a loading control (lower panel). (D) Quantitative representation of data (means±S.E.M.) of three independent Western-blot experiments after densitometry and correction for β-actin expression. ●, $P<0·01$ compared with untreated cells (−).

In H295R cells we also observed that ICI determined a dose-dependent inhibition of proliferation. This cytostatic effect could be explained by the inhibitory effects exerted by ICI on the IGF signaling pathway, which is activated strongly in H295R cells by the autocrine action of IGF-II through the type 1 IGF receptor (IGF-1R; Logié *et al.* 1999). In fact, in mammary tissue it has been demonstrated that the effect of estrogen on cell growth is mediated by the up-regulation of IGF-1R (Stewart *et al.* 1990), insulin receptor substrate (IRS)-1 and IRS-2 (Lee *et al.* 1999) expression and/or by the down-regulation of the inhibitory IGF-binding protein 3 (IGFBP-3; Huynh *et al.* 1996), while ICI reduced basal phosphorylation of IGF-1R, IRS-1, IRS-2, Akt-1 and the p85 subunit of phosphoinositide 3-kinase (Chan *et al.* 2001). These observations indicate that inhibition of cell growth by ICI may not only be attributable to competition between estrogens and ICI for ER but also to the interruption of the IGF signaling pathway. By doing so, ICI may also block a possible cross-talk between the ER and IGF-1R signaling pathways (Dupont *et al.* 2000). The molecular mechanism determining the cytostatic effect of ICI on H295R cells is currently under investigation, but preliminary results seem to confirm this hypothesis.

Interestingly, we demonstrated the involvement of the Fas/FasL system as a signal transduction pathway mediating the OHT-induced apoptosis. FasL is a membrane protein belonging to the tumor necrosis factor family (Suda *et al.* 1993), which is able to induce apoptosis by cross-linking with the Fas receptor (Takahashi *et al.* 1994). FasL is normally expressed on activated cells of the immune system and is used for killing cells infected with viruses or cancer cells expressing Fas (Suda *et al.* 1993). It has also been demonstrated that tumor cells can express FasL by which they kill Fas-positive immune cells evading the immune system (Walker *et al.* 1997, Nagarkatti 2000). Recently, it has been described that, in certain Fas-positive tumor cells, OHT induces apoptotic effects by up-regulating FasL (Nagarkatti & Davis 2003). Our study recalls these observations since we have shown that H295R cells are Fas-positive and that OHT is able to increase the expression of FasL which may activate pro-apoptotic events. This autocrine mechanism is further substantiated by the cleavage of caspase-8 and caspase-3 observed upon OHT treatment. Notably,

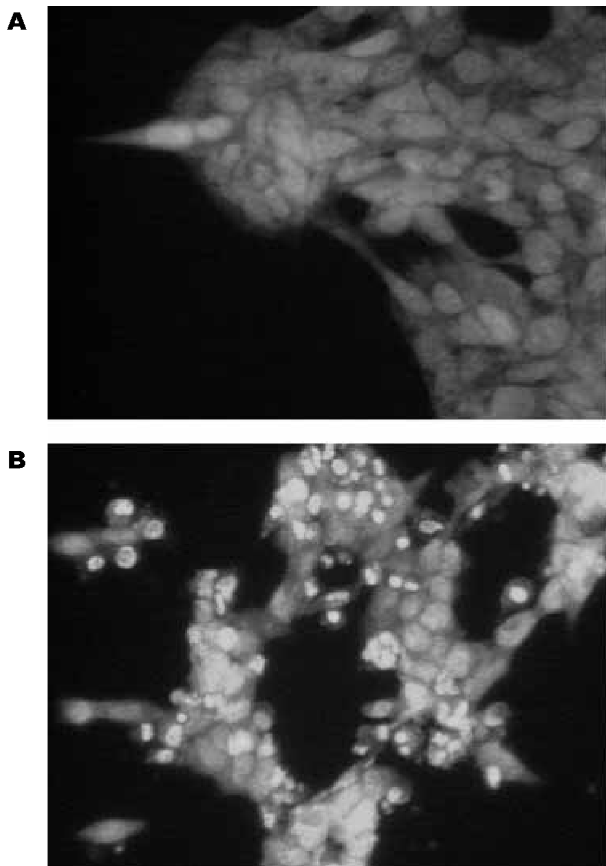

Figure 5 Induction of apoptosis in H295R cells by OHT. H295 cells were cultured for 24 h in the absence (A) or presence (B) of 10 μM OHT. Control cells were treated with the same amount of vehicle alone (DMSO) that never exceeded 0·01% (v/v). The cells were stained subsequently with Hoechst 33342. These results are representative of those obtained in three independent experiments.

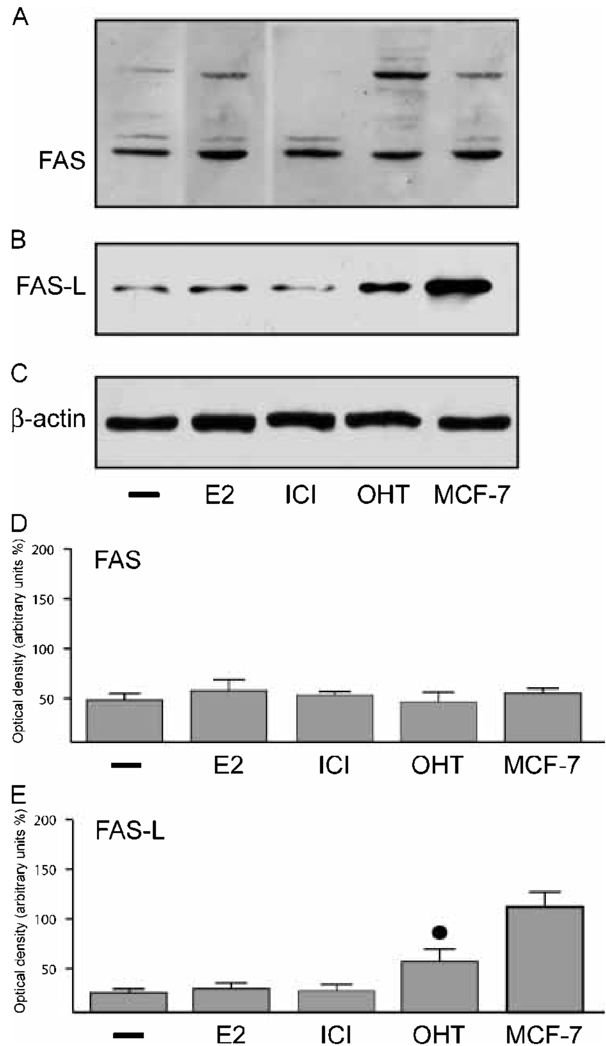

Figure 6 Expression of Fas and FasL in H295R cells. Western blots of Fas (A) and FasL (B) on H295R whole-cell extracts (50 μg) after 24 h of treatment with E_2 (10 nM), ICI (10 μM) or OHT (10 μM). (C) β-Actin was used as a loading control. (D) Quantitative representation of data (means±S.E.M.) from three independent Western-blot experiments using antibody anti-Fas after densitometry and correction for β-actin expression. (E) Quantitative representation of data (means±S.E.M.) from three independent Western-blot experiments using antibody anti-FasL after densitometry and correction for β-actin expression. MCF-7 whole cell extracts were used as positive controls. ●, $P<0·01$ compared with untreated cells (–).

these phenomena could be consequent to the interaction of the ERβ–OHT complex with the promoter of FasL, which contains a complete AP-1 sequence (Mor *et al.* 2000), while in our cellular context the ERβ–ICI complex seems to be unable to activate FasL transcription.

A previous study (Mor *et al.* 2000) reported that OHT inhibits the expression of FasL in MCF-7 and in T47D breast tumor cells through ERα. In contrast, other authors showed that OHT is able to up-regulate FasL in T47D cells (Nagarkatti & Davis 2003), depending on the concentrations of treatments and culture conditions. A role for ERβ in the regulation of FasL has been hypothesized by a study on neuronal cells demonstrating that ERβ mediates apoptosis induction in cells expressing Fas/FasL proteins, while ERα has antiapoptotic and neuroprotective effects (Nilsen *et al.* 2000). Mechanisms controlling the activation of ER-dependent genes through AP-1 sites are not completely clear. ERβ shows a unique capacity to enhance AP-1 activity in response to selective antiestrogens (Paech *et al.* 1997, Weatherman & Scanlan 2001); this is due to ERβ interactions with corepressors, as such interactions are inhibited by antiestrogens and increased by agonists (Webb *et al.* 2003, Uht *et al.* 2004). Our data show that ERβ is able to interact with the proteins bound to the

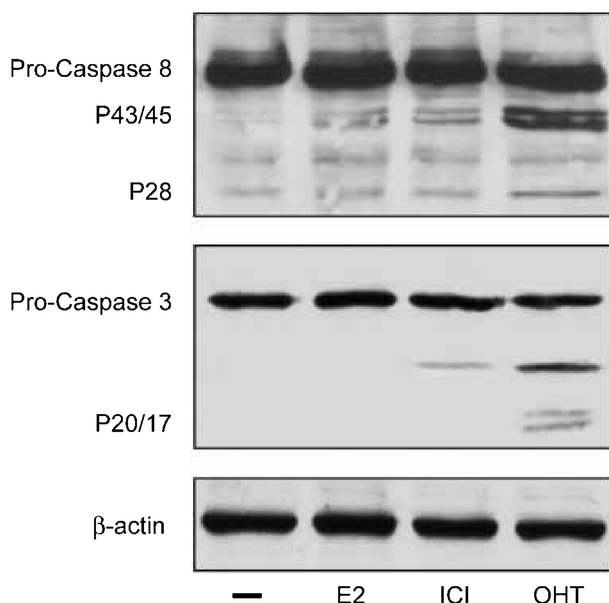

Figure 7 OHT induces caspase-8 and caspase-3 activation in H295R cells. The effect of E_2 (10 nM), ICI (10 μM) or OHT (10 μM) on caspase-8 and caspase-3 activation was evaluated by Western-blot analysis. Active forms of caspase-8 (p43/45 and p28; upper panel) and caspase-3 (p20 and p17; middle panel) were revealed only after OHT treatment for 24 h; β-actin was used as a loading control (lower panel). These results are representative of those obtained in three independent experiments.

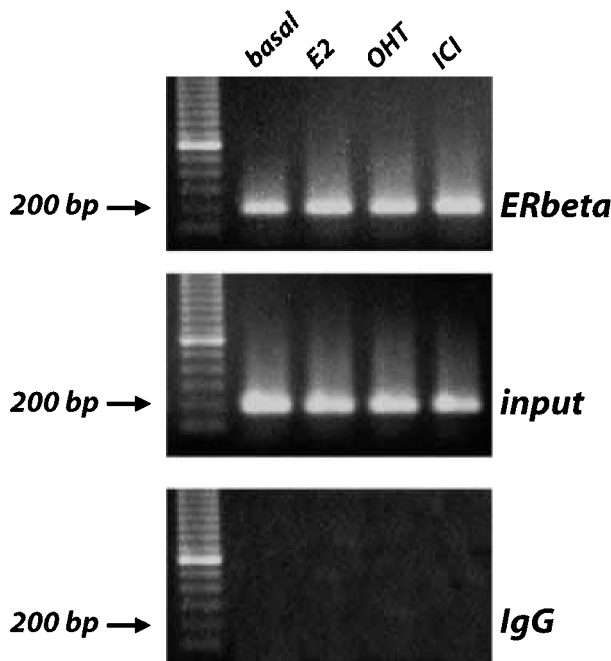

Figure 8 ERβ is recruited to the FasL promoter. H295R cells were incubated for 24 h with E2 (10 nM), OHT (10 μM) or ICI (10 μM). Control cells were treated with the same amount of vehicle alone (DMSO) that never exceeded 0·01% (v/v). *In vivo* binding of ERβ to the FasL promoter was examined using ChIP assay. Immunoprecipitated (ERβ, IgG) and total (10% input) DNA were subject to PCR using specific primers. These results are representative of those obtained in three independent experiments.

AP-1 complex on human FasL promoter and confirm that transcriptional activity does not depend only on ERβ binding to the promoter but instead depends on ERβ's ability to recruit specific cofactors. In our cellular context, the interaction between ERβ and OHT may be able to recruit corepressors bound to the AP-1 complex determining the activation of FasL expression that in turn promotes apoptosis. Nevertheless we cannot exclude that high doses of OHT are also able to induce apoptosis involving other factors such as p53 or c-Myc in a direct manner (Mandlekar & Kong 2001). Whatever the mechanism involved, what clearly emerges from this study is that the H295R adrenocortical cancer cell line exhibits estrogen-sensitive proliferation which can be inhibited by exposure to antiestrogens ICI and OHT or the aromatase inhibitor Letrozole. The present findings and our preliminary observations (Barzon *et al.*, unpublished observations), which clearly reveal (by real-time PCR and western-blot analysis) the expression of ERβ in human adrenocortical carcinoma tissues, open new perspectives on the potential therapeutic benefits of antiestrogens as pharmacological agents in antagonizing adrenocortical carcinoma cell growth and progression. However, further studies are needed to clarify the role for ERβ as a possible mediator of adrenocortical cell proliferation.

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