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Research Article

Hypoxia and estrogen receptor profile influence the responsiveness of human breast cancer cells to estradiol and antiestrogens

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Abstract. Angiogenesis activation mediated by vascular endothelial growth factor (VEGF) is one of the factors that can cause antiestrogen treatment failure in estrogen receptor (ER)-positive breast cancer patients. Since VEGF synthesis is modulated not only by hypoxia but also by steroid hormones, we investigated the relationship between hypoxic and estrogenic/antiestrogenic stimuli in two human breast cancer cell lines expressing both ERα and ERβ (MCF7) or only ERβ (MDA-MB231). In both cell lines, the VEGF level was significantly influenced by hypoxic conditions and in antiestrogen-responsive MCF7 cells, this effect was not counteracted by tamoxifen or ICI 182,780, thus providing an experimental explanation for the resistance to endocrine treatment observed in patients with ER-positive tumors. In MDA-MB231 cells, estradiol significantly reduced the VEGF level, suggesting that through the ERβ isoform it may function as a negative modulator of VEGF synthesis under hypoxia, and providing evidence for a complex interplay of the estrogen-dependent and hypoxia-dependent pathways.

Key words. VEGF; HIF-1α; estradiol; tamoxifen; breast cancer cell line.

Although hormonal therapy is effective for the treatment of most patients with estrogen receptor (ER)-positive breast cancer, resistance to hormonal agents is frequent [1]. Among the hypotheses proposed to explain treatment failure is the overexpression and/or dysregulation of angiogenic factors such as vascular endothelial growth factor (VEGF), which may cause resistance to hormonal agents in ER-positive human breast cancer [2]. In accordance with this finding, we observed in a recent translational study [3] that in postmenopausal women with resectable ER-positive breast cancer submitted to surgery and adjuvant tamoxifen (TAM), the risk of 6-year relapse increased with increasing values of intratumoral VEGF. This finding was primarily evident in patients whose tumors had a low/intermediate ER content, probably indicating that TAM failed to inhibit tumor progression activated or sustained by high VEGF levels.

An increasing body of experimental evidence indicates that VEGF synthesis is activated by several stimuli, the most important of which is the decrease in oxygen supply (hypoxia) that is a common feature of solid tumors, including breast cancer [4]. Through a transcription factor named hypoxia-inducible factor (HIF)-1 and a specific consensus sequence on the promoter region of some HIF-1-dependent genes including the VEGF gene, hypoxia triggers a cascade of molecular pathways aimed at activating essential early (i.e., glycolytic enzyme synthesis) and late (angiogenesis activation) responses, the

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latter having important implications for tumor progression [5].

However, in breast tissue, VEGF synthesis is also physiologically modulated by steroid hormones, in particular estradiol (E_2). In fact, although the VEGF gene promoter lacks a perfect palindromic estrogen-response element, a functional response element for the ER has been identified in the 5′-flanking region of the gene [6].

In view of the importance of these two different pathways in inducing VEGF synthesis and contributing to malignant progression and acquired resistance to chemical and physical agents, we investigated the relationship between hypoxic and estrogenic stimuli on VEGF synthesis in the presence of TAM, the conventional antiestrogenic agent known to exert both agonist and antagonist activities, or in the presence of the pure antiestrogen ICI 182,780 (ICI). Moreover, since, in addition to the conventional ER (renamed as ERα), a new isoform (ERβ) characterized by a different response to estrogen and antiestrogens has been identified [7, 8], we investigated whether a different ER profile may influence this relationship. For this purpose, we chose two human breast cancer cell lines characterized by different ER isoform profiles: MCF7 cells, which express both ERα and ERβ, and MDA-MB231 cells, which express only ERβ.

Materials and methods

Hormones and chemicals

E_2 and 4-OH-tamoxifen (OH-TAM) were purchased from Sigma (St. Louis, Mo.). ICI 182,780 was a gift from Dr A. E. Wakeling (AstraZeneca, Macclesfield, U. K.).

Since cobalt mimics hypoxia and causes HIF-1α accumulation by occupying the von Hippel-Lindau domain of the HIF-1α protein and thereby preventing protein degradation and inducing expression of the downstream hypoxia-inducible genes [9], we induced a 'hypoxia-like' stimulus by treating the cells with 100 µM cobalt chloride ($CoCl_2$) (Sigma).

Cell culture and experimental conditions

MCF7 and MDA-MB231 breast cancer cells (American Type Culture Collection, Rockville, Md.) were routinely maintained in DMEM/F12 without phenol red (Life Technologies, Grand Island, N. Y.) supplemented with 5% fetal bovine serum (FBS) at 37 °C and 5% CO_2.

For in vitro experiments, the cells (4×10^6) were plated in 150-cm^2 tissue culture flasks (Corning, Corning, N. Y.) in 5% dextran-charcoal-stripped FBS (DCC-FBS) to avoid uncontrolled endogenous estrogenic activity. Cells were allowed to attach for 24 h, following which the seeding medium was removed and replaced by experimental medium. Cells were kept for 24 h in medium supplemented with 10 nM E_2, 0.1 µM OH-TAM, and 0.1 µM ICI, separately or combined (10 nM E_2 plus 0.1 µM OH-TAM or 0.1 µM ICI). The vehicle for all hormone and antihormone treatments was ethanol (final concentration <0.1% v/v). Experiments were performed in triplicate. To investigate the effect of estrogen and antiestrogens under hypoxic conditions, the above described experiments were carried out in the presence of 100 µM $CoCl_2$.

Western blotting for ERβ analysis

ERβ protein expression was evaluated by Western blotting using a non-commercial rabbit polyclonal antibody (MYEB) developed and kindly provided by Dr M. Younes, and generated against a 12-amino-acid synthetic peptide corresponding to the NH_2 terminus of human ERβ and unable to discriminate among the ERβ isoforms identified to date [10]. Anti-actin mouse monoclonal antibody (Sigma) was used to detect the internal control. Immune complexes were detected using the ECL chemoluminescent system and bands were identified by a scanner, quantified by TotalLab software, and normalized according to the internal control. Before using actin bands to normalize the results, we verified the presence of a linear range of protein concentrations (i.e., a proportional increase in the actin signal by increasing the amount of starting protein concentration) [11].

Human VEGF immunoassay

VEGF levels were measured in cell culture medium and in cell extracts using a quantitative immunoassay kit (Quantikine kit from R&D Diagnostics, Minneapolis, Minn.) and intracellular and secreted VEGF levels were globally expressed as pg/million cells.

HIF-1 activity assay

Since the 'hypoxia-like' effect exerted by $CoCl_2$ is obtained by stabilizing HIF-1α protein under normoxic conditions, we decided to evaluate the biologic activity of the protein using a recently commercialized HIF-1 transcription factor assay kit (TransAM; Active Motif Europe, Rixensart, Belgium), an ELISA-based kit able to detect and quantify only the HIF-1α subunit (the inducible part of the heterodimer HIF-1). Briefly, the kit consists of a 96-well plate coated with an oligonucleotide containing the hypoxia response element (HRE). HIF-1 contained in nuclear extracts binds specifically to this oligonucleotide. By using an antibody directed against HIF-1α, the HIF-1α amount bound to the oligonucleotide is detected. Addition of a secondary antibody conjugated to horseradish peroxidase provides a sensitive colorimetric readout easily quantified by spectrophotometry according to the manufacturer's instructions. After the colorimetric reaction, samples were measured at 450 nm with a reference wavelength of 655 nm. HIF-1 activation was expressed as optical density (OD_{450}). $CoCl_2$-treated COS-7 cells were used as positive control.

Statistical analysis

The effect of individual drugs on ERβ, VEGF expression or HIF-1α activity was statistically assessed by Student's t test and all p values were two-sided. Interaction between hypoxia and estrogenic/antiestrogenic compounds on ERβ was determined by the Drewinko test [12] modified by Bhuyan et al. [13]. Briefly, to describe the kind of interaction between agents, we divided the effect observed in the cells treated with both agents (hypoxia and E_2 or an antiestrogen) simultaneously (O) by the calculated expected value (E). A positive interaction or synergy (i.e., an increase in ERβ or VEGF protein expression or HIF-1α activity) was assumed when the O/E ratio was >2, whereas an O/E ratio <0.5 would indicate a negative interaction or antagonism (i.e., decrease in ERβ or VEGF protein expression or HIF-1α activity).

Results and discussion

E_2 and hypoxia upregulate ERβ expression in MCF7 but not in MDA-MB231 cells

Figure 1 shows the basal expression of ERβ in the two cell lines and the effect of 24-h treatment with estrogen and antiestrogens in the absence or presence of 100 µM $CoCl_2$. MDA-MB231 cells showed a significantly higher normoxic basal ERβ protein level than MCF7 cells ($p < 0.01$), which was unaffected by $CoCl_2$ treatment alone or in combination with E_2 or an antiestrogen, suggesting that in this cell line, hypoxic conditions did not influence ERβ expression. By contrast, in MCF7 cells, the ERβ protein level was slightly but significantly increased by the presence of E_2 (+83%, $p < 0.05$), OH-TAM (+100%, $p < 0.05$), ICI (+88%, $p < 0.05$) and their

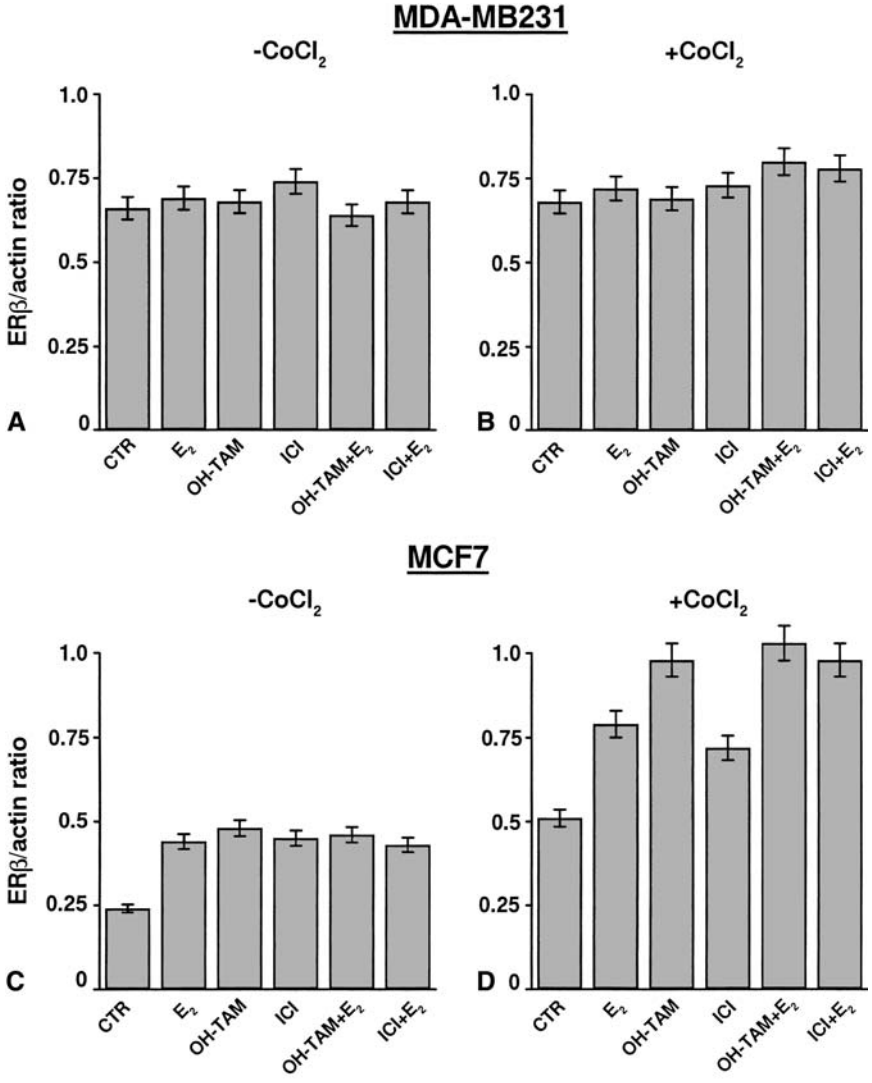

Figure 1. Effects of E_2, OH-TAM and ICI, alone or in combination, on ERβ expression in the MDA-MB231 and MCF7 cell lines. Cells were treated for 24 h with E_2 (10 nM), OH-TAM (0.1 µM), ICI (0.1 µM), E_2 plus OH-TAM or E_2 plus ICI, in the absence (*A, C*) or presence (*B, D*) of 100 µM $CoCl_2$, which induced a 'hypoxic-like' effect. ERβ levels were evaluated by Western blotting; actin was used as internal control and the results were expressed as the ERβ/actin ratio. Results represent the mean ± SE of three experiments.

combination (E_2 plus OH-TAM or ICI: +92%, $p < 0.05$ and +79%, $p < 0.05$, respectively) already under normoxic conditions. The increase in ERβ protein level observed in MCF7 cells is not surprising because E_2 [14] and other estrogenic compounds [15] have been demonstrated to increase ERβ mRNA expression also in T47D, another phenotypically ER-positive breast cancer cell line but only in the presence of detectable ERα protein levels [15]. Our finding that both antiestrogens induced ERβ expression, which is in disagreement with those reported by other authors [14], could be explained by a non-classical receptor-mediated mechanism of action, which does not necessarily require DNA-protein interaction between the receptor and a promoter element but, rather, protein-protein interactions between the receptor and other transcription factors, including AP1 or Sp1. In particular, physical and functional interactions between both ERα and ERβ with Sp1 have been reported by Saville et al. [16], who demonstrated that the effect of this interaction on gene transcription is ligand, promoter and ER subtype dependent. In fact, in experiments based on reporter gene activity in MCF7 cells (expressing ERα and a low level of ERβ), in the presence of the ERα/Sp1 complex, E_2, TAM and ICI upregulated reporter gene activity, while the ERβ/Sp1 complex did not. In addition, when ERα-negative MDA-MB231 cells were transfected with ERα/Sp1, TAM and ICI were both agonists, while they were ineffective on ERβ/Sp1 reporter gene activation. Therefore, since, as demonstrated by Li et al. [17], the ERβ gene promoter contains two Sp1 sites, such a different regulation by E_2 and antiestrogens as a function of the ER subtype is not surprising, even though we have no direct evidence in our cellular model. The hypothesis of a non-classical receptor-mediated mechanism is corroborated by the observation that $CoCl_2$ also upregulated the ERβ protein level (+113%, $p < 0.05$). A sequence alignment check by BLAST analysis (access number: AF191544) revealed that in addition to consensus sequences for other transcription factors, the human ERβ promoter region contains at least one specific consensus sequence for the HRE. Since $CoCl_2$ induces hypoxia-like effects by preventing HIF-1α degradation and causes the expression of the downstream hypoxia-inducible genes, the observed ERβ overexpression does not appear so strange. In light of the above-mentioned mechanisms, the effect on ERβ expression observed under hypoxia conditions in the presence of E_2 (O/E ratio: 3.59), OH-TAM (O/E ratio: 4.08), ICI (O/E: 3.13) or their combination (O/E: 4.48 and 4.45 for E_2 plus OH-TAM or ICI, respectively) is not surprising, since hypoxia and estrogen/antiestrogens should exert both a stimulatory effect on gene transcription even through completely different pathways.

In MCF7 cells, ICI counteracts E_2-induced VEGF synthesis but only in normoxia, whereas in MDA-MB231 cells, E_2 reduces the VEGF concentration both in normoxic and hypoxic conditions

Figure 2 shows the effect of 24-h treatment with estrogen and/or antiestrogens in the absence or presence of 100 µM $CoCl_2$ on total (intracellular plus secreted) VEGF protein in the two cell lines. MDA-MB231 cells showed an approximately tenfold higher (4603 vs 330 pg/million cells, $p < 0.001$) basal total VEGF concentration than MCF7 cells, whereas in both cell lines, the intracellular fraction of VEGF represented less than 1% of the total. The observation that, in addition to a high VEGF concentration, MDA-MB231 cells expressed a high ERβ protein level is in agreement with findings on a series of clinical specimens in which high VEGF expression was more likely to be associated with an ERα-negative phenotype but correlated positively also with high ERβ levels [18].

Like Buteau-Lozano [19], in MCF7 cells under normoxic conditions we observed a statistically significant increase in VEGF protein concentration after 24-h treatment with E_2 alone (+88%, $p < 0.05$) or in combination with OH-TAM (+65%, $p = 0.06$), but not in combination with ICI, supporting the hypothesis of ERα-mediated VEGF synthesis that was blocked by the presence of the pure antiestrogen. A totally different situation, in disagreement with the Buteau-Lozano results, was observed in MDA-MB231 cells (expressing only ERβ) in which E_2, alone or in combination with OH-TAM or ICI, significantly reduced the VEGF protein concentration (–50%, $p < 0.05$; –20%, $p = 0.09$; –52%, $p < 0.05$, respectively), supporting once again the hypothesis of a non-classical receptor-mediated mechanism with a different transcriptional regulation of VEGF synthesis by ERα and ERβ depending on the nature of the ER subtype.

In the presence of $CoCl_2$, also, the two cell lines showed a different responsiveness to E_2 and antiestrogens. In fact, in MCF7 cells, a statistically significant increase of about two- to threefold with respect to the normoxic basal value was observed in the total VEGF concentration regardless of the treatment conditions, suggesting the inability of antiestrogens to counteract the $CoCl_2$-induced VEGF overexpression, and providing a possible experimental explanation for the TAM failure we observed in patients with ER-positive tumors [3].

Conversely, in MDA-MB231 cells, which are characterized by a very high basal VEGF concentration, $CoCl_2$ treatment further increased the VEGF concentration (+56%, $p < 0.05$) but, as observed under normoxic conditions, E_2 alone (–58%, $p < 0.05$) or combined with an antiestrogen was able to reduce the VEGF concentration to normoxic basal levels and below (E_2 plus OH-TAM: –36%, $p = 0.07$; E_2 plus ICI: –41%, $p = 0.06$). This would suggest that, in addition to the different transcriptional regulation of VEGF synthesis by ERβ, E_2 could reduce hypoxia-induced bind-

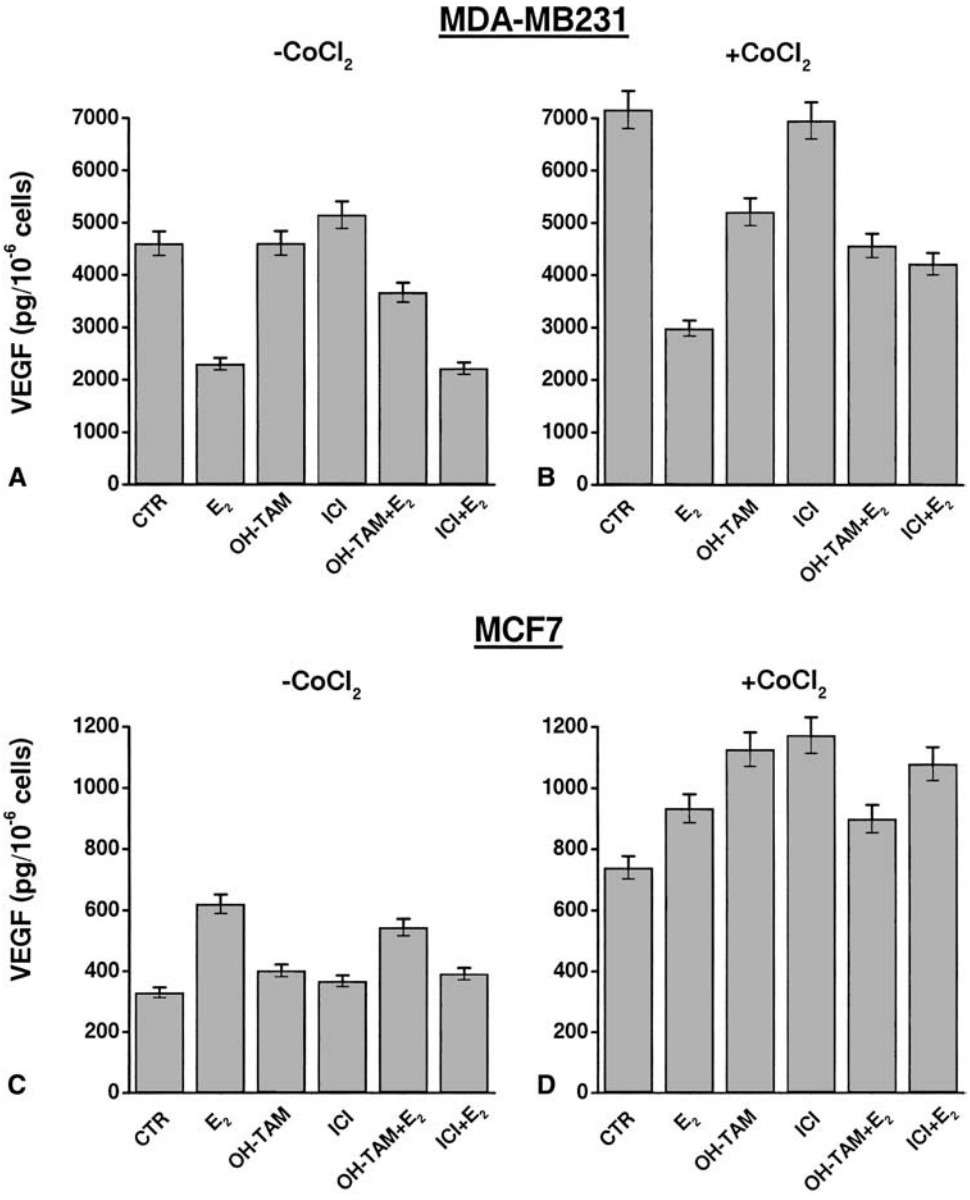

Figure 2. Effects of E_2, OH-TAM and ICI, alone or in combination, on total (intracellular and secreted) VEGF concentration in the MDA-MB231 and MCF7 cell lines. Cells were treated for 24 h with E_2 (10 nM), OH-TAM (0.1 µM), ICI (0.1 µM), E_2 plus OH-TAM or E_2 plus ICI, in the absence (*A, C*) or presence (*B, D*) of 100 µM $CoCl_2$, which induced a 'hypoxic-like' effect. The VEGF concentration was determined by immunometric assay and expressed as pg/million cells. Results represent the mean ± SE of three experiments.

ing of HIF-1α to the VEGF promoter site as described by Miyamoto et al. [20], who demonstrated that under hypoxic conditions, estrogen may function as a negative modulator of VEGF production by inhibiting HIF-1 binding and thereby VEGF transactivation.

E_2 partially reduces the hypoxia-induced increase in HIF-1α activity in MDA-MB231 cells

The finding concerning the effect of E_2 alone or in combination with an antiestrogen on HIF-1α activity confirmed the strong relationship between HIF-1α and VEGF and the inhibitory effect of E_2 on hypoxia-induced VEGF synthesis observed in MDA-MB231 cells. In fact, as shown in figure 3, under normoxic conditions, both cell lines had a very low basal HIF-1α activity, not influenced by any treatment, whereas in the presence of $CoCl_2$, as expected, HIF-1α activity was substantially increased in both cell lines: more than 40-fold in MCF7 cells and about 20-fold in MDA-MB231 cells. However, while this increase was unaffected by any hormonal treatment in MCF7 cells, except for a non-statistically significant reduction of about 30% ($p = 0.08$) in HIF-1α activ-

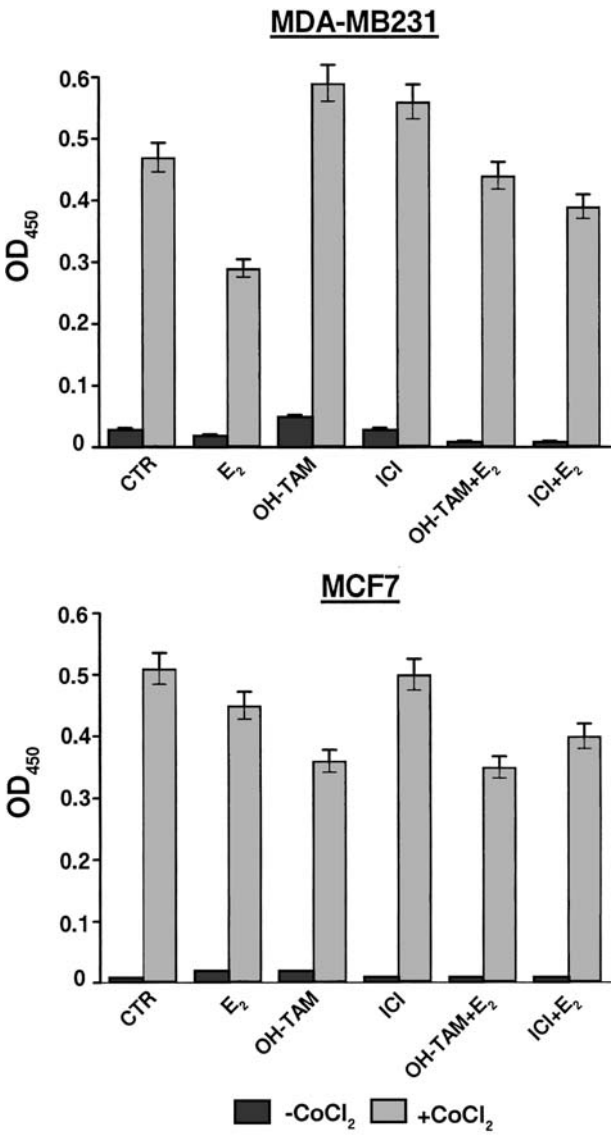

Figure 3. Effects of E_2, OH-TAM and ICI, alone or in combination, on HIF-1α activity in the MDA-MB231 and MCF7 cell lines. Cells were treated for 24 h with E_2 (10 nM), OH-TAM (0.1 µM), ICI (0.1 µM), E_2 plus OH-TAM or E_2 plus ICI, in the absence or presence of 100 µM $CoCl_2$, which induced a 'hypoxic-like' effect. HIF-1α activity was measured by an ELISA-based kit able to detect and quantify only the HIF-1α subunit and expressed as OD_{450}. The results represent the mean ± SE of three experiments.

ity following TAM or TAM plus E_2, in MDA-MB231 cells, the presence of E_2 reduced HIF-1α activity by about 40% ($p = 0.05$). Interestingly, the decrease in HIF-1α activity induced by E_2 was consistent with that observed for the modulation in VEGF synthesis.

In conclusion, our results showed that in both cell lines, the production of VEGF was considerably influenced by hypoxic conditions and that in the antiestrogen-responsive MCF7 cell line, this effect was not counteracted by the presence of TAM or ICI, providing an experimental explanation for the TAM failure observed in some patients with ER-positive tumors [3]. In addition, the finding that in MDA-MB231 cells, which express only the ERβ subtype, E_2 was able to significantly reduce the VEGF level under both normoxic and hypoxic conditions suggests that, through this isoform (which has been described to exert a protective effect in TAM-treated patients [10]), E_2 may function as a negative modulator of VEGF synthesis especially in hypoxia. In fact, E_2 also appeared to be able to counteract hypoxia-induced VEGF by reducing HIF-1α activity, thus providing evidence for a complex interplay of the two pathways (estrogen dependent and hypoxia dependent) involved in VEGF synthesis and angiogenesis activation.

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