Abstract. CCAAT/enhancer binding protein-α (C/EBPα) is involved in the control of cell differentiation and proliferation. It has been previously shown that hypoxia (H) down-regulates C/EBPα in breast cancer cells; here the effect of estrogen (E2) during H on C/EBPα in T-47D cell line was examined. By quantitative RT-PCR the C/EBPα mRNA stability was analyzed at 21% O2 and 1% O2 under E2. The H-E2 combination but not E2 alone reduced the half-life of the C/EBPα mRNA. C/EBPα promoter activity studies covering -576 bp do not show any effect of E2; a significant decrease of C/EBPα transcriptional activity was found in the C/EBPα promoter between -576/416 bp as previously observed when cells were treated with hypoxia alone. By immunocytochemistry, H-E2 combination alters the cellular distribution of C/EBPα in T-47D cells and locates this protein mainly at the cytosol. Therefore, the observed down-regulation of C/EBPα by the H-E2 combination in T-47D cells is mainly due to the hypoxia effect.

CCAAT/enhancer binding protein alpha (C/EBPα) is a basic leucine zipper transcription factor that inhibits cell cycle progression and regulates differentiation in different cell types (1). Greatly diminished expression of C/EBPα occurs in numerous types of human epithelial cancer including lung, liver, endometrial, skin and breast (2-5), suggesting a possible tumor suppressor gene function for C/EBPα (6, 7). As both estrogen and hypoxia have profound effects on cancer cell growth, the aim was to understand the cross-talk between hypoxia and estrogen on C/EBPα regulation.

The estrogen receptor (ER) is a member of a superfamily of nuclear receptors (NR3A) that have common structural and functional domains. Estrogen and its receptors ERα and ERβ play a major role in growth stimulation of a large percentage of breast cancers and approximately two-thirds of breast cancers express these functional receptors. Following E2 stimulation, the liganded ERα binds to its responsive element (ERE) and modulates the transcription of E2 target genes. These transcriptional effects of ER probably account for its well-documented implication in the development of hormone-dependent cancers (8). Anti-estrogen (anti-E2) therapy with E2 receptor antagonists has a significant benefit in women with hormone-dependent breast cancer (9).

Hypoxia, a common consequence of solid tumor growth at many sites, acts to promote tumor development (10) and is able to profoundly alter the expression of a wide range of genes (11). It was initially shown, by microarray analysis, that hypoxia and estrogen down-regulate C/EBPα mRNA in T-47D breast cancer cells (12). Following this, the mechanism by which this down-regulation by hypoxia takes place was detailed (13). The effect of estrogen during hypoxia on C/EBPα in the hormone-responsive T-47D human breast cancer cells is examined in this paper.

Using quantitative real-time reverse transcriptase PCR (qRT-PCR), Western blots, C/EBPα promoter activity studies and immunocytochemistry, it is shown that the E2 does not affect the down-regulation of C/EBPα under hypoxia and that this phenomenon was mostly hypoxia-dependent.

Materials and Methods

Cell lines and cell culture. The estrogen receptor-expressing human breast cancer cell line T-47D (ATCC® HTB-133) was cultured as previously described (13). Briefly, it was maintained at 37°C, 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Cergy-Pontoise, France) supplemented with 10% fetal bovine serum (FBS), 0.1 mM insulin (Sigma-Aldrich, Saint Quentin Fallavier, France), 0.5 μg/ml fungizone (Squibb, Princeton, NJ, USA), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Twenty-four h before exposure to hypoxic conditions, the medium was replaced with medium without phenol red and steroids (achieved by charcoal-filtering of fetal bovine serum). This medium was used for all experiments.
Estrogen and hypoxia treatment. Hypoxia treatment was performed by placing the cell culture dishes in a RSBiotech MiniGalaxy A O2 control incubator kept at 1% O2, 5% CO2, 94% N2, 37°C and constant humidity. When cells were exposed to the combination of E2 and hypoxia, E2 was added at the final concentration of 10–8 M immediately before placing the cells in the hypoxia incubator for 24 h.

Transcriptional inhibition. Transcription inhibitor 5,6-dichloro-benzimidazole 1-β-D-ribofuranoside (DRB; Sigma) was used as previously described (13). The cells were then exposed to E2 (10–8 M) and/or hypoxia (1% O2) for 1, 2 or 4 h. At these time points, the cells were harvested for PCR analysis of C/EBPα mRNA levels.

Quantitative real-time reverse transcription PCR. Total RNA was extracted from the T-47D cells, first-strand cDNA was generated and real-time PCR was performed. All PCRs were performed in duplicate. The sequences of C/EBPα primers were designed with Oligo Analyzer 1.0.2 (Kuopio University, Finland) based on human C/EBPα mRNA sequence (13). mRNA C/EBPα expression was determined by the 2−ΔΔCt method and normalized to RPL13A levels. The results are reported as relative fold increase or decrease compared with cells cultured under normoxia in the absence of estrogen.

Plasmids and transient transfection. A firefly luciferase reporter plasmid containing various regions of the C/EBPα promoter (C/EBPαuc-576, C/EBPαuc-416, C/EBPαuc-119 and C/EBPαuc-76) and the control plasmid without the C/EBPα promoter (pXp1-luc) (14) were kindly provided by G. J. Darlington (Baylor College of Medicine, Houston, Texas) and used to carry out transient transfections of plasmids using the Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer’s instructions and as described previously (13). After 16 h the cells were exposed to 10–8 M E2 and/or hypoxia (1% O2) for 24 h. After harvesting, luciferase activity was assessed, transfections were performed in duplicate and the activity of the firefly luciferase was normalized to the protein levels of the samples (assessed by BioRad detergent-compatible (DC) protein assay).

Immunocytochemistry studies. T-47D cells were seeded at a density of 4x10^4 cells in 1 mL of experimental medium in a 4-well Lab-Tek II chamber slide. The same protocol previously described was used for this study (13). The C/EBPα goat polyclonal antibody (sc-9315; Santa Cruz Biotechnology, Santa Cruz, California USA; 1:250 dilution in 1% Donkey serum-PBS) and donkey anti-goat IgG Alexa 488 (1:600 dihydro in PBS; Molecular Probes, Invitrogen) antibodies were used. Fluorescence was assessed by epifluorescence microscopy (Epifluorescence Nikon Eclipse TE-2000E).

Statistical analysis. Unless otherwise specified, means of treatment groups were compared with one-way analysis of variance (ANOVA). When the ANOVA showed that there were significant differences between the groups, Dunnett’s test or Bonferroni’s test was used to identify the sources of these differences. A p-value of ≤0.05 was considered statistically significant.

Results

Estrogen and H-E2 reduced C/EBPα expression in breast cancer cells. By qRT-PCR it was found that H, E2 and H-E2 combination significantly reduced C/EBPα mRNA levels in T-47D breast cancer cells (Table I A). The reductions in C/EBPα mRNA were followed by a reduction in C/EBPα protein levels (data previously shown) (12).

Estrogen did not influence the effect of hypoxia on C/EBPα mRNA stability. Half-lives of C/EBPα mRNA were estimated using exponential regression. Both hypoxia and H-E2 combination significantly reduced the half-life of the C/EBPα mRNA (p<0.05); E2 alone did not influence the mRNA stability (Table I B). Therefore, the reduction seen in C/EBPα protein when cells are submitted to hypoxia with or without E2 is preceded by a reduction in C/EBPα mRNA stability.

Estrogen did not influence the effect of hypoxia on the transcriptional activity of the C/EBPα promoter. The effect of estrogen on the down-regulation by hypoxia of C/EBPα transcription was investigated by transfecting T-47D cells with a reporter plasmid coupled to different fragments of the C/EBPα promoter (Figure 1A). Cells transfected with C/EBPαuc-576 [-576, +1] showed a ~30% reduction in
luciferase reporter activity under hypoxia in the presence or absence of E2 compared to normoxic controls (p<0.001); however, there was no significant effect of E2 alone (Figure 1B). In cells transfected with smaller promoter fragments C/EBP-416 [-416, +1] and C/EBP-119 [-119, +1], luciferase activity was not influenced by E2 either in normoxia or in hypoxia.

Estrogen did not modify the cytoplasmic localisation induced by hypoxia of C/EBPα. It was observed that the localization of C/EBPα was highly regulated by oxygen (13). Immunocytochemical analysis showed that C/EBPα was localized in the nucleus at 21% O2, but was mostly cytoplasmic under 1% O2. Herein, the effect of E2 on these modifications was investigated. As seen in Figure 2, E2 did not

Figure 1. Hypoxia but not E2 reduces luciferase activity in a C/EBPα luciferase reporter assay. A, C/EBPα promoter activity was assessed in T-47D cells transfected with progressive deletions of the C/EBPα promoter fragments coupled to firefly luciferase. B, The plasmids were transfected into T-47D cells and luciferase activity was assessed under normoxia (21% O2) or hypoxia (1% O2) with or without estrogen (E2). The results represent the mean of 3 independent experiments ± standard deviation (SD). *Significantly reduced compared to cells cultured under normoxia (p<0.05).

Figure 2. HE2 combination modifies the subcellular localization of C/EBPα. AT-47D cells were incubated for 24 h under 21% O2 + E2 (a) or 1% O2 + E2 (b), fixed and stained for C/EBPα with anti-C/EBPα antibodies (sc-9315)/Alexa-488-conjugated secondary antibody. Cell nuclei were stained with DAPI, and fluorescence was assessed by epifluorescence microscopy (Epifluorescence Nikon Eclipse TE-2000E) analysis. The images are representative of three independent experiments. Magnification: ×100 for 21% O2 + E2; ×40 for 1% O2 + E2.
affect the localisation of C/EBPα either in normoxia or in hypoxia. C/EBPα kept its nuclear localisation under normoxic+E2 conditions and became mostly cytoplasmic under hypoxic+E2 conditions.

**Discussion**

Breast cancer growth is regulated by coordinated actions of the estrogen receptor (ER) and various growth factor receptor signaling pathways, which could explain the reasons behind endocrine therapy action and resistance (15). Combining endocrine and non-endocrine therapies that block different signaling pathways is therefore becoming very interesting (16).

It has been previously shown that hypoxia by itself down-regulates C/EBPα expression in breast cancer cells by several mechanisms, including transcriptional and post-transcriptional effects (13). Herein, the possibility of E2 interfering with these processes was investigated. In the present study, it has been shown that E2 and the hypoxia-E2 combination reduced mRNA and protein levels of C/EBPα in T-47D cells. Therefore, the mechanisms responsible for the down-regulation of the C/EBPα gene expression by E2 in normoxia and in hypoxia were examined. Estrogen did not modify the C/EBPα mRNA stability at 21% O2 but decreased C/EBPα mRNA stability at 1% O2. As the same reduction of stability was previously observed when the cells were treated by hypoxia in absence of E2 (13), this could only be due to hypoxia.

To investigate whether estrogen regulated C/EBPα at the transcriptional level in normoxia and hypoxia, T-47D cells were transfected with different regions of the C/EBPα promoter coupled to a luciferase reporter gene. Estrogen did not alter luciferase reporter activity of any of the C/EBPα promoter constructs, although a significant decrease of C/EBPα transcriptional activity was found in the [-576, +1] region as previously observed when cells were treated with hypoxia alone. In fact, scanning the C/EBPα promoter up to −7,000 pb by Genomatrix software did not reveal any EREs in the screened region. This suggests that the reduction in C/EBPα mRNA levels in hypoxia is not E2 dependent in T-47D cells.

Hypoxia combined with estrogen altered the cellular distribution of C/EBPα in T47D cells and located this protein mainly at the cytosol. The same phenomenon was observed when cells were treated with hypoxia alone (13). Both the reduction in C/EBPα protein and its main localization in the cytosol could act in concert to reduce the functional activity of this protein in the nucleus.

In conclusion, the findings of the present study demonstrate that hypoxia leads to a down-regulation of C/EBPα in breast cancer cells by several mechanisms and that estrogen cannot counteract the predominant effect of hypoxia. The down-regulation of C/EBPα by the hypoxia estrogen association could involve protein-protein interactions between the ERα and HIF-1α as previously described (17).

Hypoxia is commonly found in solid tumors and up-regulates several target genes involved in angiogenesis, anaerobic energy metabolism, cell survival, cell invasion and drug resistance, thereby contributing to the progression of a more malignant phenotype and to increased resistance to radio- and chemotherapy (18). These results suggest that in ER-dependent breast carcinoma with hypoxic regions, hypoxia is predominant over E2, therefore this could interfere with the response to hormone therapy. This justifies further examination of C/EBPα as a possible therapeutic target in breast cancer with large hypoxic regions.

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**References**


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