Hypoxia Down-regulates CCAAT/Enhancer Binding Protein-α Expression in Breast Cancer Cells

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Abstract

The transcription factor CCAAT/enhancer binding protein-α (C/EBPα) is involved in the control of cell differentiation and proliferation, and has been suggested to act as a tumor suppressor in several cancers. By using microarray analysis, we have previously shown that hypoxia and estrogen down-regulate C/EBPα mRNA in T-47D breast cancer cells. Here, we have examined the mechanism by which the down-regulation by hypoxia takes place. Using the specific RNA polymerase II inhibitor 5,6-dichlorobenzimidazole-1-β-D-ribofuranoside, the mRNA stability was analyzed under normoxia or hypoxia by quantitative reverse transcription-PCR. Hypoxia reduced the half-life of C/EBPα mRNA by ~30%. *C/EBPα* gene promoter studies indicated that hypoxia also repressed the transcription of the gene and identified a hypoxia-responsive element (−522; −527 bp), which binds to hypoxia-inducible factor (HIF)-1α, as essential for down-regulation of C/EBPα transcription in hypoxia. Immunocytochemical analysis showed that C/EBPα was localized in the nucleus at 21% O_2, but was mostly cytoplasmic under 1% O_2. Knockdown of HIF-1α by RNAi restored C/EBPα to normal levels under hypoxic conditions. Immunohistochemical studies of 10 tumor samples did not show any colocalization of C/EBPα and glucose transporter 1 (used as a marker for hypoxia). Taken together, these results show that hypoxia down-regulates C/EBPα expression in breast cancer cells by several mechanisms, including transcriptional and posttranscriptional effects. The down-regulation of C/EBPα in hypoxia is mediated by HIF-1. [Cancer Res 2008;68(7):2158–65]

Introduction

The CCAAT/enhancer binding protein (C/EBP) transcription factors belong to a six-member family (α, β, γ, δ, ε, and ζ) of leucine-zipper DNA-binding proteins, which are involved in cell cycle regulation and differentiation in various cell types. C/EBPα, the founding member of the C/EBP family, regulates the transcription of several target genes in the mammary gland and in some other tissues such as adipose tissue, liver, and peripheral blood mononuclear cells (1). C/EBPα induces cell differentiation and slows down proliferation by stabilization of p21/WAF1, resulting in inhibition of cyclin-dependent kinase activity and E2F-mediated transcription (2–6), and it has been shown that C/EBPα expression is down-regulated in breast cancers (7, 8). Reduced C/EBPα expression or *C/EBPα* gene mutations have also been associated with cancer of the lung, breast, and skin and myeloid leukemias (7, 9–11), and C/EBPα has been shown to function as a tumor suppressor (12, 13).

We have previously shown that both hypoxia and estrogen down-regulate C/EBPα mRNA levels in T-47D breast cancer cells using DNA microarrays (14). Both estrogen and hypoxia have profound effects on cancer cell growth. Hypoxia is commonly found in solid tumors and is able to profoundly alter the expression of a wide range of genes, leading to a dedifferentiated, more aggressive phenotype as well as therapy resistance (15, 16). The cellular response to hypoxia is mainly mediated by hypoxia-inducible factor 1 (HIF-1), a heterodimer composed of HIF-1α and HIF-1β (also known as aryl hydrocarbon receptor nuclear translocator; refs. 17, 18). Whereas HIF-1β is constitutively expressed, HIF-1α is tightly regulated by the oxygen concentration and determines the activity of HIF-1. HIF-1α is overexpressed in biopsies of several human cancers including breast cancer (19). Whereas the effects of hypoxia on angiogenesis and cellular metabolism have been well characterized, the mechanism through which it alters cellular differentiation and cell cycle control is poorly understood. We hypothesize that one possible mechanism is the down-regulation of critical transcription factors such as C/EBPα.

The aim of the present study was to examine the mechanism(s) through which the hypoxia-induced down-regulation of C/EBPα takes place. Using quantitative reverse transcription-PCR (RT-PCR), electrophoretic mobility shift assay (EMSA), Western blots, C/EBPα promoter activity studies, and small interfering RNA (siRNA) knockdown of HIF-1α, we show that hypoxia down-regulates C/EBPα in T-47D breast cancer cells through multiple mechanisms. Most notably, down-regulation of C/EBPα under hypoxia was found to occur by transcriptional repression mediated by HIF-1α.

Materials and Methods

Cell lines and cell culture. The estrogen receptor–expressing human breast cancer cell lines T-47D (ATCC HTB-133) and MCF-7 (ATCC HTB-22) were maintained at 37°C, 5% CO_2 in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 0.1 μmol/L insulin (Sigma-Aldrich), 0.5 μg/mL fungizone (Squibb), 100 units/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). Twenty-four hours before exposure to hypoxic conditions, the medium was replaced with medium without phenol red and steroids (achieved by charcoal-filtering of FBS; this medium was used for all experiments, hereafter referred to as "experimental medium"). Cells used

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Table 1. Nucleotide sequences of oligonucleotide probes used in EMSA analysis

Element (abbreviation)	Sequences
Consensus HRE (HRE)	F: CGCTACCG***AGTA*****CG**TGACCG
	R: ggcgCGGTC***ACGTAC***TCGGTAGCG
HRE1	F: GCTAGGA***GACGCA***GAGCCAC
	R: ggcgGTGGCT***CTGCGT***CTCCTAGC
HRE1 Mutated (HRE1 Mut)	F: GCTAGGAGA**TCT**AGAGCCAC
	R: ggcgGTGGCTCT**AGA**TCTCCTAGC
HRE2	F: ACCTCTCCGC***GCGTCC***CAAA
	R: ggcgTTTG***GGACGC***GCGGAGAGGT
HRE2 Mutated (HRE2 Mut)	F: ACCTCTCCGC**GA**GT**A**CTAAA
	R: ggcgTTTAG**T**AC**TC**GCGGAGAGGT
HRE3	F: TGCTCTGCGC***TGCAGCC***TCC
	R: ggcgGGA***GGCTGCA***GCGCAGAGCA
HRE4	F: TCCCCG***GGACGC***GGGTCCGGGA
	R: ggcTCCCGGACCC***GCGTCC***CGGGGA

NOTE: Consensus HRE and the putative C/EBPα promoter HRE nucleotides are represented in bold italic; bold nucleotides represent mutated bases; and unrelated nucleotides are shown in lowercase.

in biochemical studies were grown to 40% to 60% confluence in 100-mm cell culture dishes or in six-well plates. The volumes of cell culture media used during hypoxia experiments were 8 mL for cells grown in 100-mm cell culture dishes and 2 mL/well for cells cultured in six-well plates.

Desferrioxamine and hypoxia treatment. Hypoxia was achieved by placing the cell culture dishes in a RSBiotech MiniGalaxy A Oxygen (O_2) control incubator, kept at 1% O_2, 5% CO_2, 94% N_2, 37°C, and constant humidity. All oxygen concentrations reported are nominal oxygen concentrations, representing the oxygen concentration in the cell culture atmosphere. For transfection experiments, we used the iron chelator desferrioxamine at a final concentration of 100 μmol/L to mimic hypoxia.

Transcriptional inhibition and mRNA stability assay. The transcription inhibitor 5,6-dichlorobenzimidazole-1-β-D-ribofuranoside (DRB; Sigma) was added directly to T-47D breast cancer cells cultured in experimental medium (stock solution, 100 mmol/L in DMSO; final concentration, 100 μmol/L; ref. 20). The cells were then exposed to hypoxia (1% O_2) for 1, 2, 4, or 8 h. At these time points, the cells were harvested for PCR analysis of C/EBPα mRNA levels.

Quantitative real-time RT-PCR. Total RNA was extracted from breast cancer cell lines with NucleoSpin RNA II kit (Macherey-Nagel EURL). First-strand cDNA was generated with iScript cDNA Synthesis kit (Bio-Rad Laboratories), and real-time PCR was carried out with the iCycler iQ System (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instruction. All PCRs were done in duplicate. The sequences of the HIF-1α and C/EBPα primers were designed with Oligo Analyzer 1.0.2 (Kuopio University, Finland) based on human HIF-1α and C/EBPα mRNA sequences, respectively, as follows: HIF-1α, forward 5′-TGCTAATGCCAC-CACTACC-3′ and reverse 5′-TGACTCCTTTTCCTGCTCTG-3′; C/EBPα, forward 5′-CCTTGTGCAATGTGAATGTGC-3′ and reverse 5′-CGGA-GAGTCTCATTTTGGCAA-3′. The primers of vascular endothelial growth factor (VEGF) and RPL13A were as follows: VEGF, forward 5′-CTTTCTGCTGTCTTGGGTG-3′ and reverse 5′-ACTTCGTGAT-GATTCTGCC-3′; RPL13A, forward 5′-CCTGGAGGAGAAGAGGAAAGAGA-3′ and reverse 5′-GAGGACCTCTGTGTATTTGTCAA-3′. Up-regulation and down-regulation of HIF-1α, VEGF, and C/EBPα were determined by the $2^{-\Delta\Delta Ct}$ method (21) and normalized to RPL13A levels. The results are given as relative fold increase or decrease compared with cells cultured under normoxia.

Plasmids and transient transfection. Firefly luciferase reporter plasmids containing various regions of the CEBPα promoter (C/EBPluc-576, C/EBPluc-416, C/EBPluc-119, and C/EBPluc-76) and the control plasmid without the C/EBPα promoter (pXP1-luc; ref. 22) were kindly provided by G.J. Darlington (Baylor College of Medicine, Houston, TX). Three new plasmids were generated from C/EBPluc-576 by mutating three HIF consensus sequences in this region [hypoxia-responsive element (HRE)-1 Mut, HRE2 Mut, HRE3 Mut] with QuickChange II Site-Directed mutagenesis kit (Stratagen) according to the manufacturer's recommendations. The primers used to mutate the sites are the following: HRE1 Mut-F, GAGATCAGAGC-TAGGAGATCTAGAGCCACCGCGCTCAG; HRE1 Mut-R, CTGAGCGCGGTGGCTCTAGATCTCCTAGCTCTGATCTC; HRE2 Mut-Fm CTCAGGACCTCTCCGCGAGTACTAAACGGCCCCCCACC; HRE2 Mut-R, GGTGGGGGGCCGTTTAGTACTCGCGGAGAGGTCCTGAG; HRE3 Mut-F, CCCCGCTGCTCTGCGCTTACGCCTCCCCGGGACGCGG; HRE3 Mut-R, CCGCGTCCCGGGGAGGCGTAAGCGCAGAGCAGCGGGG. The PCR was done using the C/EBPluc-576 plasmid as DNA template and *Pfu* Ultra polymerase for 18 amplification cycles (denaturation at 95°C, 30 s; annealing at 55°C, 1 min; elongation at 68°C, 7 min). The amplification product was incubated with *Dpn*I, a methylated DNA–specific enzyme, to digest the parental DNA template. Using a heat shock transformation, the plasmid was introduced into pXL1-blue competent bacteria, which were selected with ampicillin.

Transient transfection of plasmids was carried out with the Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. Briefly, 24 h before transfection, 4×10^5 T-47D cells were seeded in experimental medium in six-well plates. Plasmids (3 μg) were transfected under normoxia (21% O_2) with 8 μL of Lipofectamine for 6 h before the transfection medium was replaced. After 16 h, cells were exposed to hypoxia (1% O_2) or to desferrioxamine (100 μmol/L) for 24 h. After harvesting, cell extracts were assayed for luciferase activity by using firefly D-luciferin (Euromedex) as a substrate and measuring luminescence in a Berthold(α) CliniLumat luminometer. Transfections were done in duplicate and the activity of the firefly luciferase was normalized to the

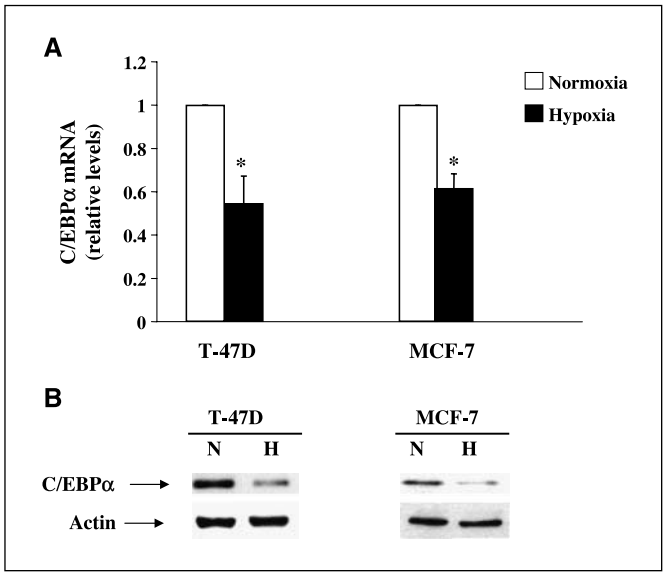

Figure 1. Hypoxia reduces C/EBPα expression in breast cancer cells. *A,* real-time PCR analysis of C/EBPα mRNA expression in T-47D cells (*left*) or MCF-7 cells (*right*). Cells were cultured under 1% O_2 (hypoxia) or 21% O_2 (normoxia). C/EBPα mRNA expression was analyzed by real-time PCR and expression was recorded as the fold decrease normalized to normoxia. *, $P < 0.05$, significantly reduced compared with cells cultured under normoxia (Mann-Whitney *U* test). *Columns*, mean of at least three independent experiments; *bars*, SE. *B,* Western blot showing C/EBPα protein levels in T-47D cells (*left*) and MCF-7 cells (*right*) after 24 h of culture under 1% O_2 (*H*) or normoxia (*N*). Representative of at least three experiments.

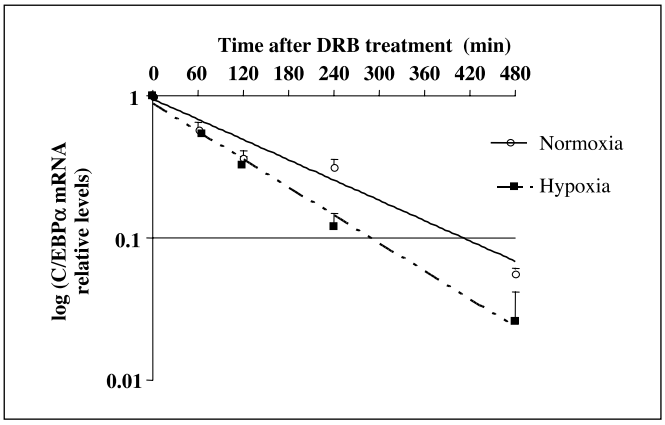

Figure 2. Hypoxia reduces C/EBPα mRNA stability. Cells were seeded (5×10^5 per well) and incubated for 24 h before exposure to DRB (final concentration, 100 μmol/L). Total RNA was isolated and C/EBPα mRNA was determined in triplicate samples by quantitative RT-PCR and normalized relative to the corresponding RPL13A mRNA level. *Bars,* SE of three independent experiments. The results are expressed relative to C/EBPα values at time 0. The equations of the curves, fitted by exponential regression, are as follows: for 21% O_2, $y = 0.8947^{e^{-0.0055x}}$ min, $r^2 = 0.9649$ ($t_{1/2} \approx 105.80$ min); for 1% O_2, $y = 0.8812^{e^{-0.0075x}}$ min, $r^2 = 0.9931$ ($t_{1/2} \approx 75.56$ min).

protein levels of the samples (assessed by Bio-Rad detergent-compatible protein assay).

siRNA transfection. Stealth RNAi (Invitrogen) targeted to HIF-1α or siRNA oligonucleotides directed toward HIF-1α ligated into the pTer vector were transiently transfected into T-47D cells to knock down HIF-1α gene expression. To construct pTer-HIF, gene-specific oligonucleotides were chosen as previously described (23). The HIF-1α oligonucleotides targeted nucleotides 1,521 to 1,541 of the HIF-1α mRNA sequence (NM001530) and were as follows: forward, 5′-CUGAUGACCAGCAACUUGAdTdT-3′; reverse, 5′-UCAAGUUGCUGGUCAUCAGdTdT-3′. The sequences were ligated into the pTer vector that had been digested with *Eco*R1 and *Xho*1.

For knockdown of HIF-1α, T-47D cells were seeded 24 h before transfection in six-well plates (4×10^5 per well, for transfection with stealth RNAi) or in 10-cm cell culture dishes (2×10^6 per dish, for transfection with pTer-HIF) in experimental medium. Transfections were done in serum-free Opti-MEM transfection medium with 150 pmol RNAi and 5 μL of Lipofectamine for 5 h for stealth RNAi, or with 10 μg plasmid and 25 μL of Lipofectamine for 24 h for pTer-HIF. The transfection medium was then replaced with experimental medium, and the cells were incubated for 16 h to allow time for HIF-1α knockdown. The T-47D cells were then exposed to hypoxia (1%) for 24 h. Controls were transfected with the scrambled siRNA or the empty vector pTer. BLOCK-iT Fluorescent Oligo (Invitrogen) was used to monitor the efficiency of the stealth RNAi transfection.

Protein extraction, subcellular fractionation, and Western blot analysis. Total protein extracts were used for most of the experiments done. Cells were placed on ice and washed with PBS without Ca^{2+} and Mg^{2+} and subsequently lysed in cold lysis buffer containing 90 mmol/L Tris-HCl (pH 6.8), 3% SDS, and 15% glycerol.

To assess the C/EBPα content in nuclear and cytosolic extracts, subcellular fractionation was done as previously described (24). Equal amounts of protein (30–40 μg) were separated on 7.5% (HIF-1α) or 10% (VEGF and C/EBPα) SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Perkin-Elmer). Actin and lamin B were used as controls for loading and cellular fragmentation and were done in parallel on 10% SDS-polyacrylamide gels. After blocking with 0.2% casein (I-Block reagent, Tropix) in PBS with 0.1% Tween 20, the membranes were incubated overnight at 4°C with the primary antibodies specific for C/EBPα (rabbit polyclonal, 1:1,000; Santa Cruz Biotechnology), HIF-1α (mouse monoclonal, 1:1,000; Novus Biologicals, Inc.), VEGF (rabbit polyclonal, 1:1,000; Santa Cruz Biotechnology), actin (rabbit polyclonal, 1:1,000; Sigma), and lamin B (mouse monoclonal, 1:1,000; Oncogene Research Products). Blots were washed and incubated with antimouse or antirabbit antibodies conjugated to alkaline phosphatase (1:20,000; Tropix) for 1 h at room temperature, and subsequently washed and revealed with CDP Star chemoluminescence reagent (Perkin-Elmer).

EMSAs. We performed EMSA to determine whether HIF-1α could bind to the C/EBPα promoter. The consensus binding site for HIF-1α, the putative HRE sequences in the C/EPBα promoter (HRE1, HRE2, HRE3, and HRE4), and their mutated counterparts (HRE1 Mut and HRE2 Mut) were synthesized and used as oligonucleotide probes for EMSA (Table 1). Probes were obtained by annealing the coding and noncoding strands and labeling the duplex molecules with the Klenow fragment of DNA polymerase I in the presence of [α-^{32}P]dCTP (>3,000 Ci/mmol; 1 Ci = 37 GBq).

First, EMSA was done to assess binding of the HRE and the putative HRE sequences to nuclear extract from T-47D cells maintained at 21% O_2 or at 1% O_2. Reactions were carried out as previously described with 10 μg of nuclear extract and 0.1 ng of each radiolabeled oligonucleotide probe (25).

Binding specificity was assessed by (*a*) competitive EMSA, by incubating the radiolabeled putative HRE sequences with nuclear extracts from hypoxic T-47D cells in presence of 10- or 100-fold excess of unlabeled consensus HRE oligonucleotide; and (*b*) antibody competition, by preincubating nuclear extracts with 4 and 8 μg of rabbit polyclonal HIF-1α ODDD antibody for 2 h on ice before incubating with HRE1 and HRE2 probes for 30 min at room temperature. Eight micrograms of purified polyclonal rabbit IgG were used as negative control and were similarly treated. Complexes were resolved by electrophoresis as described above, then dried and autoradiographed with PhosphoImager.

EMSA was also done to assess binding of wild-type and mutated HRE1 and HRE2 to nuclear extract from T-47D cells maintained at 21% O_2 or at 1% O_2. After electrophoretic separation of nonshifted and shifted oligonucleotide probes, results were quantified with PhosphoImager (ImageQuant software). This experiment was paralleled with the detection of nucleoprotein complexes with the same oligonucleotides as nonradioactive probes and the same concentration of nuclear extracts. After electrophoretic separation, nucleoprotein complexes were electrotransferred onto nitrocellulose sheets, which were probed with a rabbit polyclonal HIF-1α ODDD antibody (provided by Dr. Jacques Pouyssegur, CNRS UMR 6543, Centre Antoine Lacassagne, Nice, France) diluted at 1:1,000. Detection was carried out with antirabbit antibodies conjugated to alkaline phosphatase (1:20,000; Tropix), and subsequent revelation with CDP Star chemoluminescence reagent (Perkin-Elmer).

Patients and immunohistochemical study. Ten ductal breast carcinoma samples, obtained from patients surgically treated for breast cancer in the gynecologic oncology department of Georges Pompidou European Hospital were studied. Samples were formalin fixed, paraffin embedded, and stained with H&E. Breast carcinomas were classified according to the Scarff-Bloom-Richardson (SBR) breast cancer grading system modified by Elston and Ellis (26). All these tumors express estrogen receptor (Table 1). An immunohistochemical study was done on freshly cut 4-μm serial sections with estrogen receptor monoclonal antibody (NCL-ER-6F11/2; Novocastra), glucose transporter 1 (Glut-1) polyclonal antibody (used as a marker for hypoxia; DAKO), and C/EBPα polyclonal antibody (Santa Cruz Biotechnology). Positive controls consisted of normal breast tissue for estrogen receptor and C/EBPα antibodies and red blood cells for Glut 1 antibody.

Immunocytochemistry studies. T-47D cells were seeded at a density of 4×10^4/mL of experimental medium in a four-well Lab-Tek II chamber slide. The cells were then incubated for 24 h under 21% O_2 or 1% O_2. Cells were washed with PBS, fixed on ice with 4% paraformaldehyde-PBS for 15 min, and permeabilized for 5 min in 0.1% Triton-PBS followed by three PBS washes. Nonspecific sites were saturated with 5% bovine serum albumin-PBS for 30 min before exposure for 1 h to an anti-C/EBPα goat polyclonal antibody (Santa Cruz Biotechnology; 1:250 dilution in 1% donkey serum-PBS). Negative controls were obtained by omitting the primary antibody. The cells were washed twice in PBS-Tween and incubated for 1 h with donkey anti-goat IgG Alexa 488 (1:600 in PBS; Molecular Probes, Invitrogen). Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). After final rinses in 0.1% PBS/Tween and PBS, slides were mounted with Vectashield containing DAPI (Vector Lab) and fluorescence

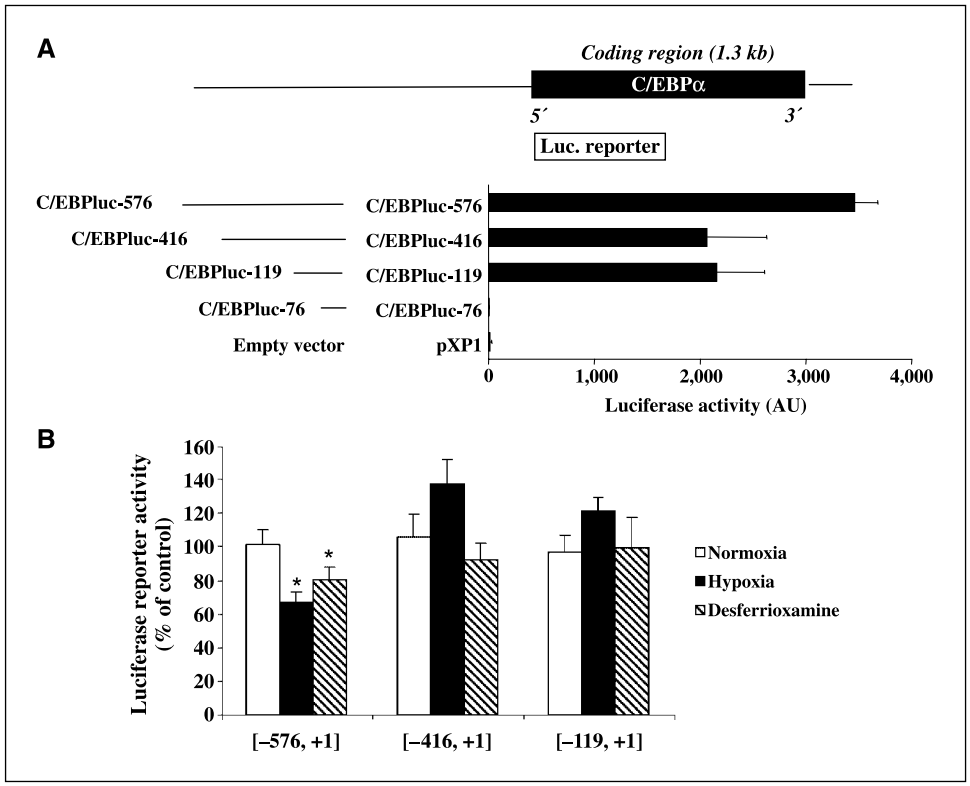

Figure 3. Hypoxia reduces luciferase activity in a C/EBPα luciferase reporter assay. *A,* pXP1 plasmids containing progressive deletions of the C/EBPα promoter fragments coupled to firefly luciferase (*left*) were transfected into T-47D cells and luciferase activity was assessed under normoxia (*right*). *AU,* arbitratry units. *B,* T-47D cells were transfected with firefly luciferase reporter pXP1 plasmids containing different regions of the C/EBPα promoter and incubated under normoxia, hypoxia (1% O_2), or normoxia with desferrioxamine (100 μmol/L) for 24 h before luciferase activity was assessed. *, $P < 0.001$, significantly reduced compared with cells cultured under normoxia. *Columns,* mean of three independent experiments; *bars,* SD.

was assessed by fluorescence microscopy combined with confocal analysis in a Nikon Labophot 2 equipped with a FITC filter.

Proliferation and cell cycle analysis. T-47D cells were cultured under 1% O_2 or 21% O_2 in experimental medium for 24 h before harvesting for fluorescence-activated cell sorting analysis of S-phase fraction. Bromodeoxyuridine (BrdUrd; 10 μmol/L) was added to the cultures for pulse labeling of S phase 20 min before harvesting. The cells were then trypsinized, fixed in 70% ice-cold ethanol for 3 h, and labeled with propidium iodide. Cell cycle progression was analyzed by dual-parameter flow cytometry with a FACSCalibur flow cytofluorometer (Becton-Dickinson Biosciences) and CellQuest Pro software (BD Biosciences). The proliferative index (PI; ref. 27) and S-phase fraction (SPF) were calculated using the following formulas: SPF = [S / (G_0-G_1 + S + G_2-M)] × 100, and PI = [(S + G_2-M) / (G_0-G_1 + S + G_2-M)] × 100.

Statistical analysis. Mann-Whitney *U* test was used to compare the mean C/EBPα mRNA relative levels in cells cultured under normoxia and hypoxia. For all other studies, means of treatment groups were compared with one-way 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. $P \leq 0.05$ was considered statistically significant.

Results

Hypoxia reduces C/EBPα expression in breast cancer cells. Using quantitative real-time RT-PCR, we found that hypoxia significantly reduced C/EBPα mRNA levels in T-47D and MCF-7 breast cancer cells by ~60% (Fig. 1*A*). The down-regulation of C/EBPα mRNA levels was paralleled by a similar decrease in C/EBPα protein in both cell lines (Fig. 1*B*).

Hypoxia decreases C/EBPα mRNA stability. C/EBPα mRNA stability in T-47D cells cultured under hypoxia or normoxia was examined after treatment with the transcriptional inhibitor DRB (Fig. 2). Half-lives of C/EBPα mRNA were estimated by exponential regression. Hypoxia reduced the half-life of C/EBPα mRNA by ~30% [105 ± 6.67 minutes (r^2 = 0.96) for 21% O_2 and 74.34 ± 4.44 minutes (r^2 = 0.99) for 1% O_2; $P < 0.05$; Fig. 2].

Hypoxia and desferrioxamine reduce the transcriptional activity of the C/EBPα promoter. The effect of hypoxia and desferrioxamine on the regulation of C/EBPα transcription was investigated by transfecting T-47D cells with a reporter plasmid coupled to different fragments of the C/EBPα promoter (Fig. 3*A*). Cells transfected with C/EBPluc-576 displayed an ~40% reduction in luciferase reporter activity under hypoxia when compared with normoxic controls ($P < 0.001$; Fig. 3*B*). Desferrioxamine caused a similar but less potent effect ($P < 0.01$). In cells transfected with the smaller promoter fragments (C/EBPluc-416 and C/EBPluc-119), luciferase activity was not influenced by hypoxia or desferrioxamine. The smallest promoter fragment, C/EBPluc-76, did not give any measurable luciferase activity when transfected into cells even under normoxic conditions and was not used for the studies of hypoxia effects (Fig. 3*A*).

EMSAs. Analysis of the C/EBPα promoter sequence (available in the human genome database Ensembl) revealed the presence of four putative binding sites for HIF-1α in the −576/−416 region, here referred to as HRE1, HRE2, HRE3, and HRE4 (see Fig. 4*A*).The ability of the putative HREs found in the −576/−416 region of the C/EBPα promoter sequence to bind to nuclear extracts prepared from T-47D cells maintained at 21% O_2 or at 1% O_2 was assessed by EMSA analysis. A consensus HRE was used as a control for all experiments.

A shifted complex was observed for the HRE, HRE1, HRE2, and HRE3 sequences when incubated with nuclear extracts from cells cultured under hypoxic conditions (Fig. 4*B*). In some cases, a shifted band was also observed after incubation with extracts from cells cultured under normoxia; however, these bands were always

weaker than those observed in the corresponding hypoxic conditions. In contrast, a little oligonucleotide shift was observed for HRE4 under either condition. The binding specificity of the sequences was investigated by competition experiments. The five sequences, including the consensus sequence, were incubated with excess (10- or 100-fold) unlabeled HRE. All sequences showed decreased binding after incubation with extracts from cells cultured under hypoxia in the presence of increasing amounts of competitor (10- to 100-fold; Fig. 4*C*).

HRE1 is critical for down-regulation of C/EBPα promoter activity in hypoxia. Additional reporter plasmid constructs carrying C/EBPα promoter fragments mutated in each of the three HREs that were found to bind to HIF-1α (HRE1, HRE2, or HRE3) were prepared and the promoter activity of these fragments was assessed under normoxia or hypoxia after transfection into T47-D cells.

Mutation of the HRE3 site did not alter the effect of hypoxia, and therefore this site was not studied further. When HRE2 was mutated, basal activity was slightly increased and the effect of hypoxia was even more pronounced than in the wild-type fragment. Interestingly, mutation of the HRE1 site resulted in a complete loss of the hypoxia effect (Fig. 4*D*). Subsequently, EMSA analysis was carried out with mutated HRE1 and HRE2 sequences, which were compared with the corresponding wild-type sequences.

To investigate whether HIF-1α was present in the nucleocomplex, we tested if HIF-1α antibodies could supershift it. When HRE, our positive control, was used as a probe, the HIF-1α ODDD antibody (applied at dilutions of 1:10 and 1:5) was able to inhibit the formation of the nucleocomplex but did not supershift it (Supplementary Fig. S1*A, lanes 3* and *4*), probably because the binding of the antibody to nuclear HIF-1α prevented it from binding to DNA. The same result was observed for HRE1 and HRE2 (respectively *lanes 8, 9* and *13, 14* for HRE1 and HRE2). Polyclonal rabbit IgG (*lanes 5, 10*, and *15*) used at 1:5 dilution did not affect the complex formation. Taken together, these results suggest the presence of HIF-1α in the shifted complexes.

We next assessed the binding of mutated HRE1 and HRE2 to the nuclear extracts. As seen in Supplementary Fig. S1*B* (*higher panel*), mutations in the HRE1 and HRE2 sequences caused considerably decreased binding of nuclear extract from cells cultured under

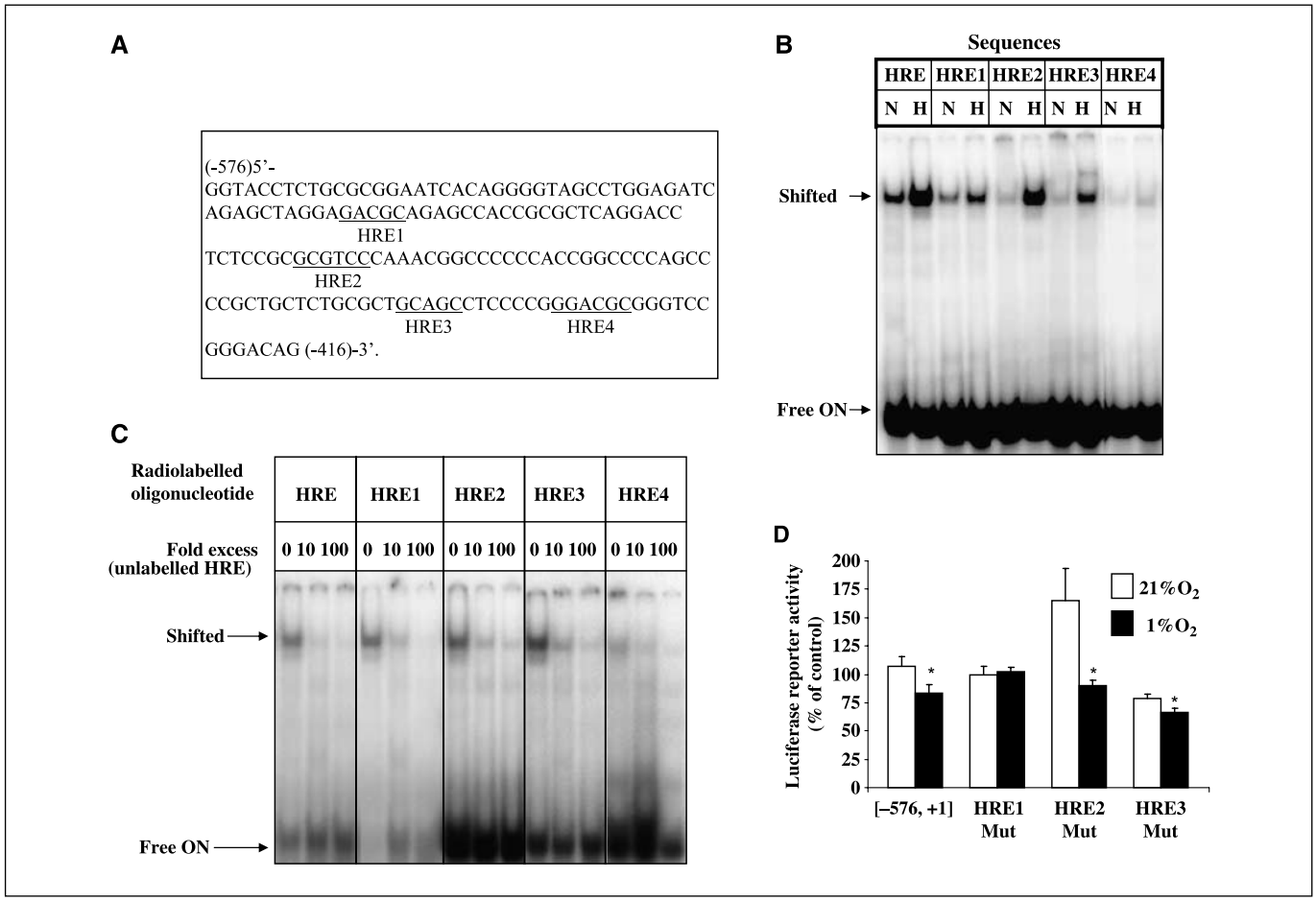

Figure 4. HIF-1α binds to C/EBPα promoter HREs and abolishes C/EBPα down-regulation in hypoxia. *A,* analysis of the C/EBPα promoter sequence revealed the presence of four putative binding sites for HIF-1α in the −576/−416 region, here referred to as HRE1, HRE2, HRE3, and HRE4 (*underlined*). *B,* EMSA was done with 0.1 ng of radiolabeled HRE and wild-type HRE1, HRE2, HRE3, and HRE4 oligonucleotides in the presence of 10 μg of nuclear extracts from cells maintained at 21% O_2 (*N*) or 1% O_2 (*H*). *ON,* oligonucleotide. *C,* EMSA was done with 10 μg of nuclear extract from T-47D cells maintained at 1% O_2 and 0.1 ng of each radiolabeled oligonucleotide (HRE, HRE1, HRE2, HRE3, and HRE4). The specificity of protein binding to HREs was assessed by adding increasing amounts of unlabeled HRE (10- and 100-fold excess) to the reactions. Representative of at least three independent experiments. *D,* T-47D cells were transfected with firefly luciferase reporter pXP1 plasmids containing C/EBPluc-576 and C/EBPluc-576 with mutated HRE1, HRE2, or HRE3. The cells were incubated under normoxia or hypoxia (1% O_2) for 24 h before luciferase activity was assessed. *, $P < 0.05$, significant change compared with cells transfected under normoxia (ANOVA; Bonferroni's test). *Columns,* mean of at least three independent experiments; *bars*, SD.

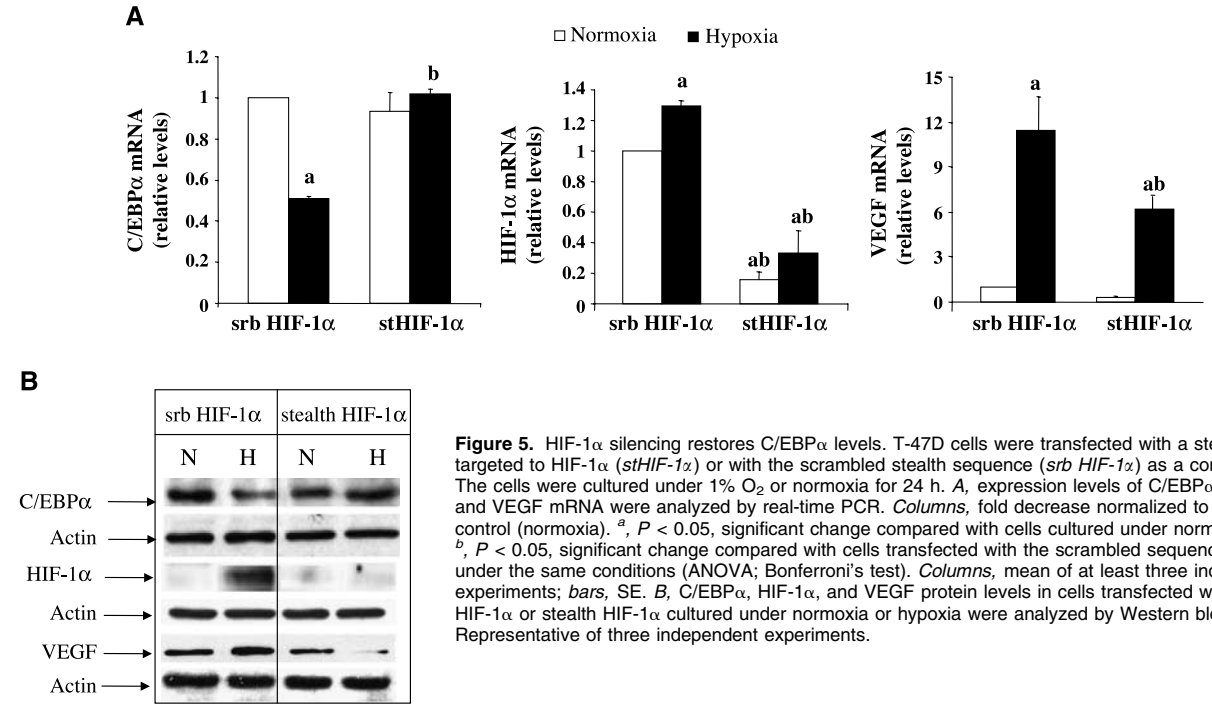

Figure 5. HIF-1α silencing restores C/EBPα levels. T-47D cells were transfected with a stealth RNAi targeted to HIF-1α (*stHIF-1α*) or with the scrambled stealth sequence (*srb HIF-1α*) as a control. The cells were cultured under 1% O_2 or normoxia for 24 h. *A,* expression levels of C/EBPα, HIF-1α, and VEGF mRNA were analyzed by real-time PCR. *Columns,* fold decrease normalized to untreated control (normoxia). [a], $P < 0.05$, significant change compared with cells cultured under normoxia; [b], $P < 0.05$, significant change compared with cells transfected with the scrambled sequence cultured under the same conditions (ANOVA; Bonferroni's test). *Columns,* mean of at least three independent experiments; *bars,* SE. *B,* C/EBPα, HIF-1α, and VEGF protein levels in cells transfected with srb HIF-1α or stealth HIF-1α cultured under normoxia or hypoxia were analyzed by Western blot. Representative of three independent experiments.

hypoxic conditions (Supplementary Fig. S1*C*). The oligonucleotide-protein complexes were then transferred onto nitrocellulose membranes and shift-Western blot analysis was done with a specific HIF-1α antibody. The results showed the presence of HIF-1α bound to the wild-type sequences (HRE, HRE1, and HRE2) after incubation with nuclear extracts of cells cultured under hypoxic conditions, whereas no bands were present when the cells had been cultured under normoxic conditions. No HIF-1α immunoreactive band was observed for the mutated HRE1 Mut and a weak band was detected for HRE2 Mut, showing that mutation of HRE1 and HRE2 considerably abolishes or decreases binding of HIF-1α to these sequences (Supplementary Fig. S1*B, bottom*).

HIF-1α knockdown restores C/EBPα levels in T-47D cells cultured under hypoxia. Next, we investigated whether HIF-1α silencing with stealth RNAi would affect the down-regulation of C/EBPα observed in breast cancer cells cultured under hypoxia. Silencing of HIF-1α in T-47D cells cultured under hypoxia led to complete restoration of C/EBPα mRNA levels when compared with cells grown under normoxia (Fig. 5*A, left*). HIF-1α knockdown by stealth RNAi led to a reduction in HIF-1α mRNA levels by 70% to 80%, showing knockdown efficiency (Fig. 5*A, middle*). HIF-1α silencing also reduced VEGF induction under hypoxia (Fig. 5*A, right*).

Silencing of HIF-1α also prevented the down-regulation of C/EBPα protein levels in T-47D cells (Fig. 5*B, top*). Furthermore, in cells where HIF-1α was silenced with stealth RNAi, the induction of HIF-1α in hypoxia was greatly reduced, and hypoxia could no longer increase VEGF protein levels, showing the efficacy of HIF-1α silencing on the protein level (Fig. 5*B*).

The experiment was repeated in T-47D cells transfected with plasmid siRNA targeted to HIF-1α (pTer-HIF). Again, we observed restitution of C/EBPα mRNA and protein levels, reduced HIF-1α mRNA and protein levels, and reduced induction of VEGF in cells transfected with siRNA targeted to HIF-1α cultured under hypoxic conditions (Supplementary Fig. S2).

Immunohistochemistry. Immunohistochemical study was done for C/EBPα and Glut-1 (a marker of hypoxia) on 10 tumor samples, all of which were estrogen receptor positive (Supplementary Fig. S3*A*). Two SBR2 tumors had a homogeneous pattern of C/EBPα immunostaining with >50% immunostained cells. Two tumors (SBR2 and SBR3) presented a heterogeneous pattern of C/EBPα immunostaining with 10% to 15% immunostained cells. Finally, six of the ten tumors were C/EBPα negative or very weakly positive (<10% immunostained cells). These six tumors were composed of the three SBR1 tumors, two of the five SBR2 tumors, and one of the two SBR3 tumors. Glut-1 membrane immunostaining (5–20% of immunostained cells) was encountered in four tumors, all measuring at least 20 mm. This percentage of Glut-1–positive tumors in breast cancers was in agreement with previous reports (28). Comparison of C/EBPα and Glut-1 expressions showed that positive Glut-1 areas were always associated with negative C/EBPα areas (Supplementary Fig. S3*B* and *C*) except in one case in which positive Glut-1 expression was associated with some weakly positive C/EBPα cells (tumor sample 10). These observations on tumor samples are in agreement with the negative effect of hypoxia on *C/EBPα* gene expression.

Hypoxia modifies the subcellular localization and protein level of C/EBPα. Immunocytochemistry experiments were done to determine the subcellular localization of endogenous C/EBPα proteins under different conditions. As seen in Fig. 6, the localization of C/EBPα was highly regulated by oxygen concentration. C/EBPα was mainly nuclear under normoxia (Fig. 6*A*) but relocated to become mostly cytoplasmic under hypoxia (Fig. 6*B*). C/EBPα protein levels were also assessed in the nuclear and cytoplasmic fractions of cells cultured under normoxia or hypoxia

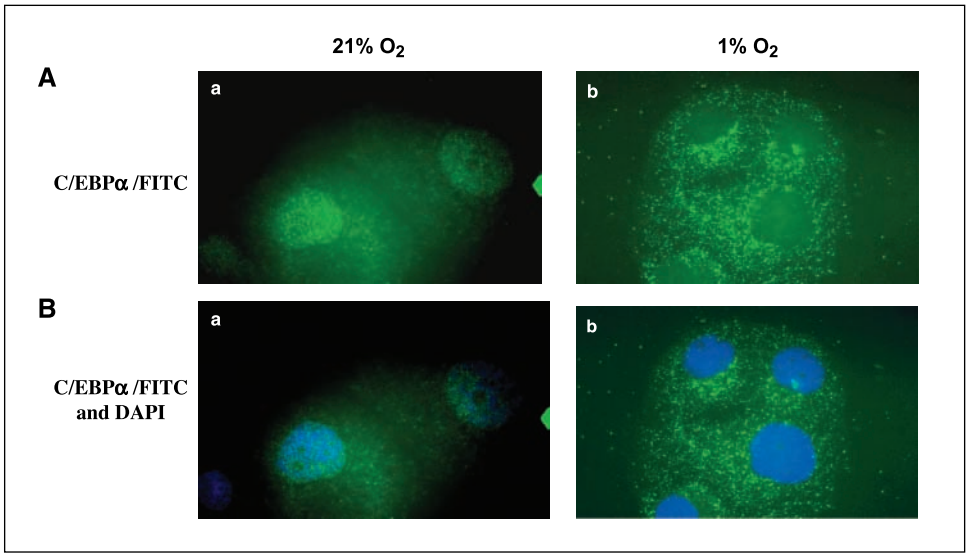

Figure 6. Hypoxia modifies the subcellular localization of C/EBPα. *A,* T-47D cells were incubated for 24 h under 21% O_2 (*a*) or 1% O_2 (*b*), fixed, and stained for C/EBPα with anti-C/EBPα antibodies/Alexa 488–conjugated secondary antibody (*green*). *B,* cell nuclei were stained with DAPI (*blue*) and fluorescence was assessed by fluorescence microscopy combined with confocal analysis. Representative images of three independent experiments. Magnification, ×100.

by Western blots (Supplementary Fig. S4). The C/EBPα protein levels were slightly increased in the cytosol and markedly decreased in the nuclear fraction of hypoxic cells as compared with control cells, confirming the relocalization of this protein to the cytosol in hypoxia.

Proliferation and cell cycle analysis. A small but significant increase in cell proliferation was observed in cells cultured under 1% O_2 for 24 hours when compared with cells cultured under normoxic conditions (Supplementary Table S1). Furthermore, cell cycle progression analysis showed a small but significant increase in the percentage of cells found in S and G_2-M phases after 24 hours of culture under hypoxic conditions as compared with normoxic culture conditions. The proliferative index was thus increased from 38.85 ± 0.86 in normoxia to 42.67 ± 0.64 in hypoxia, indicating mildly increased proliferation of cells cultured under short-term hypoxic conditions.

Discussion

Hypoxia is common 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 radiotherapy and chemotherapy (29). In the present study, we have shown that the transcription factor C/EBPα, which is involved in cellular differentiation and cell cycle control, is also a target of hypoxia regulation. This regulation could play a role in cancer progression and resistance to therapy.

In the present study, we have shown that hypoxia caused a robust reduction in C/EBPα mRNA and protein levels in both T-47D and MCF-7 breast cancer cells. Therefore, we examined the mechanisms responsible for the down-regulation of the *C/EBPα* gene expression by hypoxia. One mechanism is the decreased stability of C/EBPα mRNA in hypoxic cells. Previously, hypoxia has been shown to reduce mRNA stability of other genes with different half-lives such as MCP-1 (half-life of 35 minutes; ref. 30) and endothelial nitric oxide synthase (half-life of 13 ± 2 hours; ref. 31). The second effect of hypoxia is a decrease in promoter activity, which seems to be mediated by a HRE sequence and HIF-1α. Indeed, both EMSA analysis and mutational functional studies led us to focus on two putative responsive sequences called HRE1 and HRE2. Binding of both sequences to nuclear proteins was increased in hypoxia. Shift-Western blot analysis and antibody competition experiments suggested that these sequences could bind to HIF-1α, and this binding was shown to be disrupted in mutated HREs. Mutation of HRE2 (GCGTCC), which is a conserved HRE, did not reduce the effect of hypoxia on luciferase activity. However, when HRE1 (an imperfect reverse HRE sequence) was mutated, the negative effect of hypoxia was disrupted. This result shows that the HRE1 sequence of the C/EBPα promoter, located between 522 and 527 bp upstream of the transcription start site, is responsible for the HIF-1α–mediated down-regulation of C/EBPα in hypoxia.

Next, we investigated whether down-regulation of C/EBPα in hypoxia could be prevented by knocking down HIF-1α with stealth RNAi or with a siRNA plasmid targeted to HIF-1α. We found that in cells where HIF-1α was silenced, hypoxia-induced down-regulation of C/EBPα was completely inhibited. This finding confirms that HIF-1α is responsible for the down-regulation of C/EBPα seen in T-47D breast cancer cells cultured under hypoxia. Our results are in agreement with previous studies in adipocytes and leukemic cells, where hypoxia and desferrioxamine reduced C/EBPα levels (32, 33). However, in those studies the contribution of HIF-1α was not assessed.

To assess the relevance of the present data to breast cancer, we examined 10 tumor samples from patients who had undergone surgery for ductal breast carcinoma to determine C/EBPα and hypoxia status. We found that C/EBPα is less expressed in higher-grade tumors, which is in agreement with a recent study by Gery et al. (7). Most importantly, colocalization of Glut-1 and C/EBPα was not observed in any of the samples examined, except in one sample in which the expression of C/EBPα was very low. Although there are several limitations to this study due to the small sample size, the fact that no colocalization of Glut-1 and C/EBPα was observed is consistent with the hypothesis that hypoxia leads to down-regulation of C/EBPα.

Hypoxia also altered the cellular distribution of C/EBPα in T-47D cells and relocated this protein mainly to the cytosol. Both the reduction in C/EBPα protein and its relocation to the cytosol could act in concert to decrease the functional activity of this protein in the nucleus. These observations seem to be particularly relevant in

light of the study by Gery et al. (7). These authors showed that C/EBPα was down-regulated in a large number of breast carcinoma samples compared with normal tissue. They further showed that the localization of this factor shifted from a nuclear localization in normal epithelial cells to a mixed cytoplasmic and nuclear localization in cancer cells. The extranuclear localization of C/EBPα in T-47D cells driven by hypoxia suggests an inactive state of this transcription factor. Moreover, it has been shown that hypoxia leads to a dedifferentiated phenotype in ductal carcinoma *in situ* and in breast cancer cell lines (16), a finding that is in line with the reduction of C/EBPα expression shown in the present study.

C/EBPα induces growth arrest and terminal differentiation *in vitro* and *in vivo* in various cells types, including breast cancer cells (5–7, 34, 35). C/EBPα may also interact with $p21^{WAF1/CIP1/sdi1}$, an important regulatory protein, to bring about cell cycle arrest (4). Interestingly, we have observed a decrease in p21 protein levels in T-47D cells cultured under hypoxia.[10] Because p21 is crucial for the orderly progression of the cell cycle, one possible consequence of its down-regulation by hypoxia is the modulation of cell proliferation, which we have indeed observed in T-47D cells. These results suggest that short-term hypoxia moderately favors cell proliferation, and are in line with the down-regulation of the cell cycle control factors C/EBPα and p21.

In conclusion, the findings of the present study show that hypoxia leads to down-regulation of C/EBPα in breast cancer cells by several mechanisms, including transcriptional repression mediated by HIF-1α binding to a HRE in the C/EBPα promoter and posttranscriptional effects such as reduced stability of C/EBPα mRNA and altered cellular distribution of C/EBPα protein. All these mechanisms can contribute to the decreased functional activity of this transcription factor. The down-regulation of C/EBPα in hypoxic regions of high-grade ductal breast carcinomas indicates that this finding could have physiopathologic relevance, and justifies further examination of C/EBPα as a possible therapeutic target in breast cancer.

[10] Unpublished results.

Acknowledgments

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