Intermittent Hypoxia Induces Proteasome-Dependent Down-Regulation of Estrogen Receptor α in Human Breast Carcinoma

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ABSTRACT

Purpose: **Hypoxia may influence gene expression to promote malignancy, and acute hypoxia has been shown to transiently repress estrogen receptor (ER)-α expression in breast cell lines. However, the effect of intermittent hypoxia, which is likely more prevalent in breast cancers, remains to be determined.**

Experimental Design: **ER-α expression was assessed by Western blot and immunohistochemistry in a selected cohort of 51 ER-α–positive breast carcinomas, in relation to markers of hypoxia. The effect of acute and intermittent hypoxia on ER-α expression was also determined in MCF7 and ZR-75 breast cell lines, together with the role of proteasome function with the proteasome inhibitor bortezomib.**

Results: **Regional loss of ER-α expression occurs in breast tumors and is consistently present in hypoxic regions defined by the proximity of necrosis and induction of hypoxia-induced genes carbonic anhydrase IX (CA-IX) and glucose transporter 1 (Glut-1), in both *in situ* ($n = 29$; $P < 0.0001$) and invasive ($n = 20$; $P = 0.0001$) carcinomas. In MCF7 and ZR-75 cells, ER-α is transiently down-regulated by acute hypoxia and rapidly restored by reoxygenation. However, intermittent, acute hypoxia can cause a similar down-regulation of ER-α that is not attributable to decreased mRNA and persists in MCF7 cells despite reoxygenation for up to 14 days. This effect occurs with no change in cell viability but a corresponding reduction in growth response to estradiol. However, ER-α expression can be restored by bortezomib.**

Conclusions: **Intermittent hypoxia can cause persistent changes in proteasome function that may contribute to reduced ER-α expression in breast tumors and consequently to diminished response and development of resistance to endocrine therapy.**

INTRODUCTION

Estrogen receptor (ER)-α is a primary target for both chemoprevention and endocrine therapy of breast cancer, and assessment of ER-α status is an important variable in clinical management (1). In pathological lesions associated with increased risk of developing breast cancer, there is a significant increase in the proportion of cells that express ER-α (2). Therefore, it seems that increased and altered ER-α–mediated signaling is important and necessary for early stages of tumorigenesis. However, ER-α–negative status in *in situ* cancer is associated with increased risk of progression to invasive disease, and ER-α–negative invasive breast cancer is a more aggressive subgroup (3). This transition from ER-α positive to negative with progression is thought to reflect emergence of an ER-α–negative clone or phenotypic loss of ER-α.

Hypoxia is a common feature of solid tumors that arises, in part, from the disordered tissue architecture that occurs with progression and contributes to many changes, including altered gene expression (4). Functional assessment of hypoxia within tumors can be accomplished by invasive techniques, but these preclude close correlation with localized gene expression. However, regions of hypoxia can be defined by proximity to necrosis and the expression of hypoxia responsive genes (5).

Recent *in vitro* studies have identified a direct effect of hypoxia on ER-α levels in breast cell lines (6–8); however, ER-α levels seem to be only transiently depressed by acute hypoxia. Therefore, the occurrence of this effect remains to be fully explored in breast tumors *in vivo*, and the implication remains undefined if this effect is only transient. Therefore, our aim was to directly examine human breast carcinomas for evidence of a relationship between ER-α and hypoxia *in vivo* and also to determine whether hypoxia could be a cause of sustained depression of ER-α that might contribute to tumor progression.

MATERIALS AND METHODS

Tissue Specimens. Initially, 700 primary *in situ* and invasive ductal carcinoma cases were reviewed from the database

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Table 1 Clinical-pathological features of the cohort of 51 ER-α – positive/necrosis-positive breast carcinomas

	DCIS	Invasive ductal
Type	31	20
Grade *		
Low	2	3
Moderate	20	11
High	9	6
Necrosis		
<10%	12	7
10–25%	12	10
>25%	7	3
ER-α IHC score		
0–2	2	0
3–4	5	0
5–6	15	4
7–8	9	16

Abbreviations; DCIS, ductal carcinoma *in situ*; IHC, immunohistochemistry.

* Histological grading was according to the Van Nuys Grading system for DCIS and the Nottingham Grading system for invasive breast cancer.

of the Manitoba Breast Tumor Bank (University of Manitoba, Manitoba, Canada; ref. 9) to identify 12 *in situ* and 20 invasive cases that met the following criteria: (*a*) ER ligand binding score >10 fmol/mg; (*b*) regions of necrosis involving >5% of the tumor section, such that perinecrotic and non-necrotic regions could be distinguished; and (*c*) no known previous biopsy or preoperative therapy that might contribute to nonhypoxic causes of necrosis. A second series of 84 ductal carcinoma *in situ* cases were reviewed with the same criteria, and 19 additional *in situ* cases were identified. The final study cohort of 51 cases therefore comprised 20 invasive breast cancers and 31 ductal carcinoma *in situ*. ER levels ranged from 12 to 270 (median 69) fmol/mg protein. Other clinical-pathological features are summarized in Table 1.

Immunohistochemistry. The immunohistochemistry staining was done on serial 5-μm sections from representative blocks from each case for ER-α (ER-6F11, Novocastra Newcastle upon Tyne, UK; 1:50), carbonic anhydrase IX [(CA-IX) M75 antibody, 1:200], glucose transporter 1 [(Glut-1) Chemicon International, Temecula, CA; 1:3,000], progesterone receptor (Novocastra; 1:50), and hypoxia-inducible factor (HIF)1α (H1alpha67, Novus Biologicals, Littleton, CO; 1:750) for selected cases on an automated immunostainer (Ventana, Phoenix, AZ). All immunohistochemistry slides were examined by light microscopy by two observers (Guang-Yu Liu and Peter H. Watson). ER-α and progesterone receptor levels were assessed within the entire tumor section with a semiquantitative scale that combined intensity (scored as 0, none; 1, weak; 2, intermediate; and 3, strong) and proportional expression (scored as 0, no expression; 1, <1%; 2, 1 to 10%; 3, 10% to one-third; 4, one-third to two-thirds; and 5, over two-thirds of cells showing nuclear staining) to obtain an immunohistochemistry score from 0 to 8. Membrane expression of CA-IX and Glut-1 was assessed as either positive (signal in any tumor cells) or negative. Expression of all four proteins was determined as follows: (*a*) within the entire tumor section; and (*b*) within subregions of the section classified as likely to be "hypoxic" and "nonhypoxic." We have previously shown that within solid tumors, hypoxic necrosis typically occurs at 130 μmol/L (range, 80 μm to 200 μm) from the nearest blood vessel, and regions of severe hypoxia, as indicated by induction of CAIX, typically extend from the border of necrosis to within 80 μm (range, 40 μm to 140 μm) of the nearest blood vessel (10). Therefore, we classified subregions within tumors that surrounded and extended up to 50 μm (130 μm to 80 μm) from the edge of necrosis as hypoxic regions. Because we also found that necrosis could occur up to 200 μm from the nearest identifiable blood vessel, subregions within tumors that lay between 100 μm and up to 200 μm from the edge of necrosis were defined as nonhypoxic for comparison. The same definition was used for ductal carcinoma *in situ* cases. As in all of these cases, the ductal carcinoma *in situ* comprised subtypes (solid and cribriform) with sufficient intraductal proliferation and depth of cells to allow distinction between perinecrotic and non-necrotic zones.

Cell Culture. The ER-α–positive human breast cancer cell lines MCF-7 and ZR-75 were cultured in estrogen-deprived medium (comprising phenol red-free DMEM supplemented with 5% twice charcoal-stripped fetal bovine serum) for 3 days before the start point and throughout each experiment. In acute hypoxia experiments, cells were seeded into 10-cm dishes at a low cell density (20,000 cells/cm^2). After 16 to 24 hours, experimental cells were exposed to hypoxic conditions within a 37°C hypoxic incubator [Forma Scientific (Marietta, OH)/anaerobic system] filled with 90% N_2, 5% CO_2, and 5% H_2 to maintain oxygen levels between 0.02 to 0.5% (detected with an oxygen sensor from Alpha Omega Instruments (Cumberland, RI), Series 2000% oxygen analyzer). The control cells were maintained at 5% CO_2 to 95% air. The culture medium for hypoxic cells was placed in the hypoxic incubator for at least 24 hours in advance to equilibrate and was changed every other day. For intermittent hypoxia studies cells were subjected to 3 cycles of hypoxia-reoxygenation, each cycle comprising hypoxia extending for 64 hours and reoxygenation for 8 hours. During the reoxygenation period, cell culture flasks were transferred to a cell culture hood to replenish the media under sterile conditions. After 8 hours reoxygenation, cell culture flasks were then returned to the hypoxic chamber and gradual return to hypoxic conditions. In other experiments, MCF-7 cells were exposed to alternative "stress" conditions that included glucose starvation (glucose media without refreshment for 7 days), low pH (pH = 6.3 to 6.7 for 48 hours), or exposure to medium conditioned by hypoxic MCF-7 cells. In some experiments, bortezomib (VELCADE–a specific proteasome inhibitor kindly provided by Millennium Pharmaceuticals, Inc. (Cambridge, MA); ref. (11) was added 8 hours before harvesting the cells. Bortezomib was dissolved in a regular saline solution (0.9% NaCl) and stored at −70°C before being used at a concentration of 4 μmol/L. For growth experiments, 17β-estradiol (Sigma, St. Louis, MO) was dissolved in 100% EtOH and was added to media at final concentrations of 10 nmol/L. One hundred percent EtOH was used as a vehicle control.

Cell Viability and Growth Assays. Cell viability was assessed by Trypan Blue Viability assay. Normoxic and postintermittent hypoxia MCF7 cells were cultured in parallel, washed twice with PBS day 0 after intermittent hypoxia cycling, trypsinized, and aliquots were mixed 1:1 with trypan blue so-

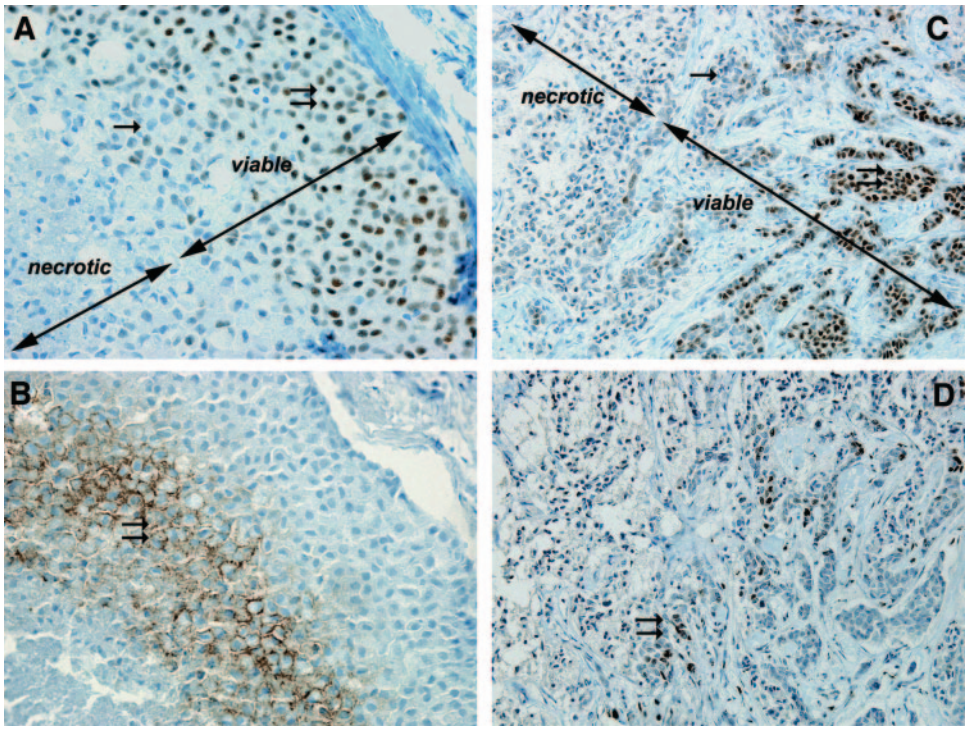

Fig. 1 ER-α expression in ductal carcinoma *in situ* (*left column*, *panels A* and *B*) and invasive carcinoma (*right column*, *panels C* and *D*) in relation to markers of hypoxia. ER-α expression (*A* and *C*) is reduced within morphologically viable regions of tumor cells adjacent to necrotic regions. The same regions of tumor show up-regulation of CA-IX (*B*) and HIF-1 (*D*). *Panels A* and *B* magnification ×400. *Panels C* and *D* magnification ×200. In all of the panels, *single* and *double arrows* indicate groups of tumor cells with low and high gene expression, respectively.

lution (0.04%) before counting cells with a hemocytometer. Viable cells exclude trypan blue, whereas dead cells stain blue because of Trypan Blue uptake. The level of estradiol-induced cell proliferation was determined by AlamarBlue Assay (Serotec, Raleigh, NC) as specified by the manufacturer's instructions. Specifically, the assay is based on the change in color of the redox indicator AlamarBlue in response to metabolic activity resulting from cell growth. Briefly, on completion of the final intermittent hypoxia cycle, MCF7 cells and corresponding normoxic control cells were seeded at 2,000 cells per well in 96-well plate in 5% charcoal-stripped fetal bovine serum. Cells were allowed to adhere for 24 hours, at which point they were grown with either 10 nmol/L E2 or EtOH control. The media was changed every second day, and AlamarBlue was added 8 hours before taking readings on the specified experimental day. Readings were taken at 570 and 600 nm with a SpectraMax 190 plate reader (Molecular Devices, Sunnyvale, CA). Duplicate experiments were done in which six replicates per time point were taken.

Western Blot Analysis. Cells were harvested by scraping and lysed in SDS-Isolation Buffer [50 mmol/L Tris (pH 6.8), 20 mmol/L EDTA, 5% SDS, 5 mmol/L β-glycerophosphate, and protease inhibitors; Boehringer Mannheim, Indianapolis, IN]. Protein lysates (50 μg) were run on a 10% SDS-PAGE gel and then transferred to 0.45-μmol/L Nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA). After blocking in 10% skimmed milk, blots were probed with primary antibodies against ER-α (1:1,000), HIF1α (1:500), and CA-IX (1:2,000), followed by incubation with goat-antimouse secondary antibodies and visualization by incubation with Supersignal (Pierce, Rockford, IL) and exposure to film (Kodak, Rochester, NY). Anti-β–actin antibody (Sigma) was applied as a loading control. Protein band intensities were scanned and quantitated with a MCID image analysis system (Imaging Research, St. Catharines, Ontario, Canada), and ER-α expression level was determined as a ratio to β-actin.

Reverse Transcription-PCR Analysis. Real-time reverse transcription-PCR was used to quantify ER-α transcript levels between MCF-7 treated with intermittent hypoxia and their normoxic controls. Briefly, RNA was extracted with TRIzol (Invitrogen, Burlington, ON, Canada), and 2 μg total RNA was reverse transcribed with Superscript (Invitrogen), with a poly-T 22mer. The following PCR primers sets were designed: ER-α forward, GTGGCTTTGGTCCGTCTCCTCCA and ER-α reverse, TCATCCTCTCCCACATCAGGCACA; and Cyclophilin E forward, CCAGTTCATGTGCCAGGGCGGTGA and Cyclophilin E reverse, AAGAACTGAGAGCCATTGGTGTTTGGG for a two-step PCR protocol (1 × 93.0°C for 2 minutes; 40 × 94°C for 15 seconds, 70°C for 15 seconds; and 25°C hold). Cyclophilin E was used as a housekeeping gene as it was observed not to change between experimental and control conditions (data not shown).

Statistical Analysis. Levels of ER-α were compared between tumors and between hypoxic and nonhypoxic subregions of the same tumor by statistical analysis with Wilcoxon signed rank paired *t* test.

RESULTS

Estrogen Receptor-α Expression Is Reduced within Regions Adjacent to Necrosis in Breast Cancer. To examine the relationship between ER-α and hypoxia, we initially

searched a large tumor bank to identify a sufficiently large series of ER-α–positive tumors that exhibited necrosis (as one readily distinguishable surrogate marker of hypoxia), as overt necrosis is very uncommon in ER-α–positive tumors (12), as opposed to ER-α–negative cases with necrosis that are more common. Overall, 49 of 51 tumors that were ER-α positive by ligand binding assay were also ER-α positive overall by immunohistochemistry assay. The ER-α level within these tumors was consistently lower in perinecrotic areas (Fig. 1, *A* and *C*), and there was a significant difference between perinecrotic and non-necrotic tumor subregions in both *in situ* and the invasive tumors ($P < 0.0001$ and $P = 0.0001$, Wilcoxon test; Fig. 2*A*). Overall, 42 of 49 (86%) of ER-α immunohistochemistry-positive cases showed significant down-regulation in the perinecrotic zone, and this was independent of the overall ER-α ligand binding level in both *in situ* and invasive tumors. Among these were all 15 of 15 CA-IX–positive cases and 21 of 25 Glut-1–positive cases.

Estrogen Receptor-α and Progesterone Receptor Down-regulation Is Associated with Markers of Hypoxia, CA-IX, and Glut-1. Expression of the two hypoxia-induced proteins, CA-IX and Glut-1 (5, 13, 14), were also examined in adjacent tissue sections. A total of 31 cases showed positive staining with one (6 CA-IX positive only, 16 Glut-1 positive only) or both proteins (9 cases). In most cases, expression of both CA-IX and Glut-1 was limited to perinecrotic hypoxic areas (Fig. 1*B*). ER-α levels in these specific CAIX-positive/Glut-1–positive hypoxic regions adjacent to necrosis were significantly lower than the ER-α levels in the adjacent nonhypoxic regions in these cases ($P < 0.001$, Wilcoxon test; Fig. 2*B*). Progesterone receptor expression was also examined as a marker of activation of ER-α. In 16 progesterone receptor-positive cases, lower progesterone receptor was observed in the same regions adjacent to necrosis and where CA-IX and/or Glut-1 expression was present. HIF1α protein expression was often difficult to detect but when present was seen preferentially in the same hypoxic regions adjacent to areas of necrosis (Fig. 1*D*).

Acute Hypoxia Causes Transient Down-regulation of Estrogen Receptor-α Expression in Human Breast Cancer Cell Lines. To confirm the impact of hypoxia on ER-α expression, Western blot analysis was done on both MCF-7 and ZR-75 cell lines cultured under hypoxic conditions. In MCF-7 cells subjected to acute hypoxia, ER-α was reduced in a time-dependent fashion and in parallel with induction of HIF1α and CA-IX (Fig. 3*A*). Similar results were obtained with ZR-75 cells

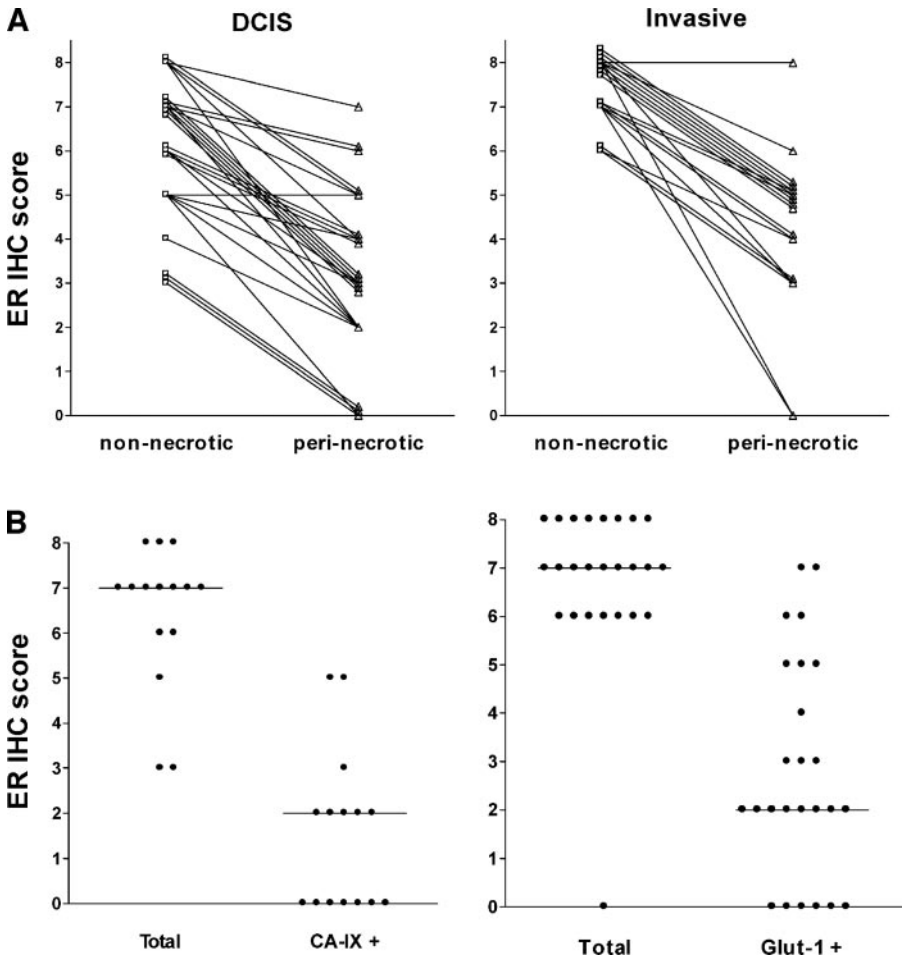

Fig. 2 Variation in the level of expression of ER-α in breast carcinoma correlates with indicators of hypoxia including the proximity to areas of necrosis and the expression of CA-IX and Glut-1. *Panel A* shows a consistent difference in the ER-α immunohistochemistry score between perinecrotic zones (0 to 50 μm from necrosis) and non-necrotic zones (100 to 200 μm from necrosis) in 20 ductal carcinoma *in situ* (*left*; $P = 0.0001$) and 29 invasive carcinoma (*right*; $P < 0.0001$). *Panel B* shows the difference in overall ER-α immunohistochemistry total score in each tumor and ER-α immunohistochemistry score within those specific areas showing expression of CA-IX (*left*; $n = 15$; $P < 0.0001$) or Glut-1 (*right*; $n = 25$; $P < 0.0001$). (*IHC*, immunohistochemistry; *DCIS*, ductal carcinoma *in situ*)

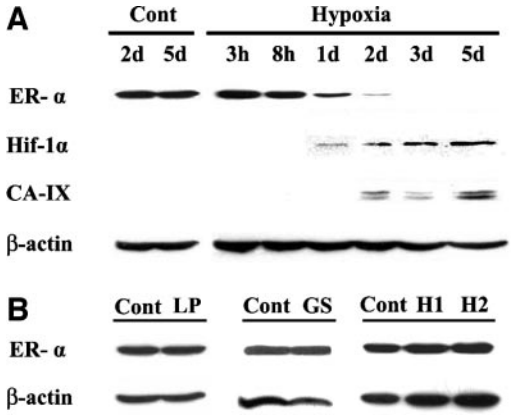

Fig. 3 The effect of acute hypoxia and other stress factors on ER-α expression measured by Western blot in MCF-7 breast cancer cells. *Panel A* shows reduction of ER-α in cells subjected to hypoxia in parallel with induction of hypoxia response genes HIF1α and CA-IX. *Panel B* shows no effect on ER-α expression under several other stress conditions, including low pH (pH = 6.3 to 6.7 for 48 hours), low glucose (glucose starvation for 7 days), or exposure to medium conditioned by hypoxic cells (H1, 24 hours and H2, 48 hours) relative to control cells and actin. (*LP*, low pH; *GS*, low glucose; Cont, control cells)

with reduction of ER-α evident at 24 hours exposure and absence after 48 to 72 hours (data not shown). We were not able to detect progesterone receptor expression in these estrogen-depleted culture conditions or assess its response to hypoxia. However, it is probable that the concurrent reduction in progesterone receptor expression observed *in vivo* in tumors represents reduced ER-α activity.

We also examined ER-α expression in MCF-7 cells grown under three additional stress conditions other than hypoxia. These included low pH, low glucose, and exposure to medium conditioned by hypoxic cells. Western blot analysis failed to show any detectable change in the ER-α level under any of these conditions (Fig. 3*B*).

Intermittent Hypoxia Causes Persistent Depression of Estrogen Receptor-α Expression. To discover if hypoxia could be a cause of loss of ER-α expression with progression, we initially examined the effect of sustained acute hypoxia for up to 48 hours followed by reoxygenation and recovery under normoxic conditions. In both MCF7 and ZR-75 cells, there was a similar time-dependent but complete recovery of ER-α expression, with detectable ER-α protein within 24 hours and restoration to control levels within 7 days of reoxygenation (Fig. 4*A*). We then examined the effect of repeated or intermittent acute hypoxia over a period of 9 days followed by reoxygenation and recovery under normoxic conditions. During each cycle of intermittent acute hypoxia, cells were exposed to a period of hypoxia that was equivalent to the single period of sustained acute hypoxia that had been studied initially. This is because the nominal period of 64 hours hypoxia in each cycle included an initial equilibration period of ~24 hours after the cells were returned to the hypoxic chamber before reaching severe hypoxia for a period of 48 hours. After this period of intermittent hypoxia, ER-α expression was down-regulated in both cell lines. However, in contrast to ZR-75 (where recovery soon occurred to control levels), in MCF7 cells, ER-α remained depressed after 14 days, ranging from 2 to 9% relative to control levels in multiple independent experiments (Fig. 4*B*).

Cell Viability and Growth Potential Is Maintained after Both Acute and Intermittent Acute Hypoxia. Cell viability was examined by trypan blue exclusion and growth assays. After three cycles of intermittent acute hypoxia, cell viability was comparable with control cells grown in parallel under normoxic conditions, as measured by the percentage of nonvi-

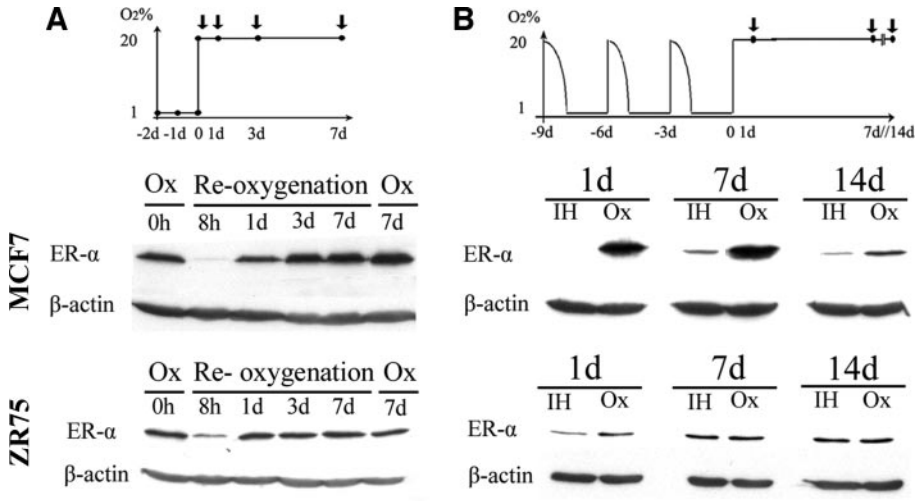

Fig. 4 The effect of reoxygenation on ER-α expression after either acute or intermittent acute hypoxia. *Panels A* and *B* show the experimental profiles (*top*) and ER-α levels (*bottom*) after acute hypoxia (*A*) and intermittent acute hypoxia (*B*). Western blots shown are representative of three independent experiments. The time points at which cells were harvested during subsequent periods of reoxygenation and return to normoxic conditions are indicated (*arrows*). *Panel A* shows the recovery of ER-α levels within 7 days after 48 hours of hypoxia in both cell lines. *Panel B* shows persistent depression of ER-α levels in MCF7 but not in ZR-75 cells, up to 14 days postintermittent hypoxia (*IH Lanes*) compared with control cells maintained under normoxic conditions. (*d*, day; h, hour; *IH*, intermittent hypoxia; *Ox*, cells maintained under normoxic conditions)

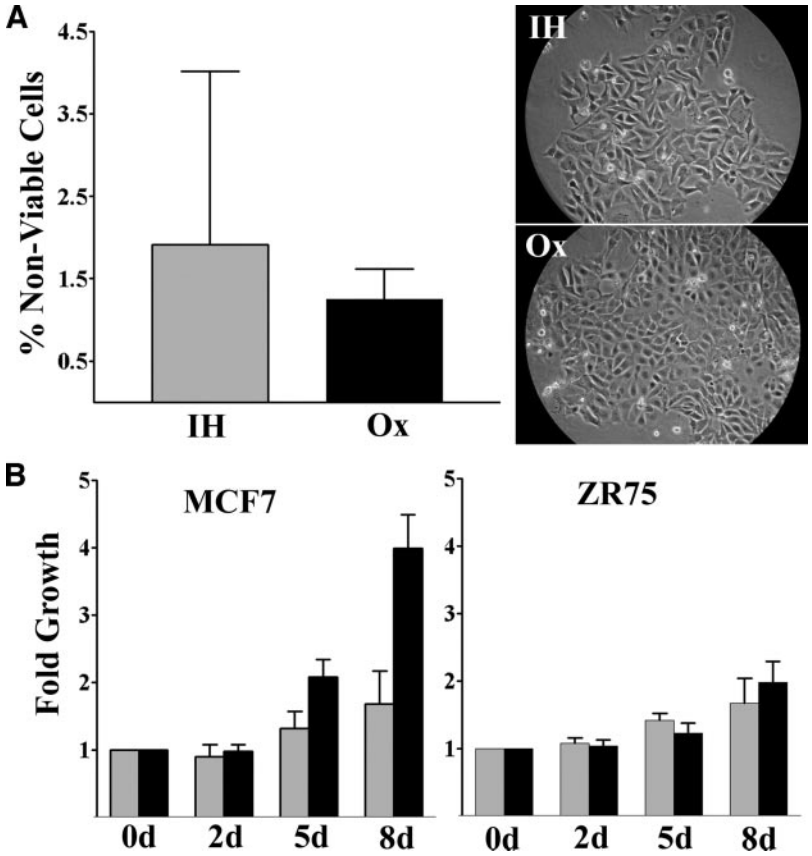

Fig. 5 The effect of intermittent hypoxia on cell viability and estradiol-induced cell proliferation. *Panel A* (*left*) shows the percentage of nonviable MCF7 cells after three cycles of intermittent hypoxia (see Fig. 4*B* for details) as compared with control cells maintained under normoxic conditions. Triplicate samples in which a minimum of 300 cells were counted per replicate; *bars*, ±SD. *Panel A* (*right*) shows comparable MCF-7 cell morphology after intermittent hypoxia as compared with control cells (*Ox*) by phase-contrast light microscopy. *Panel B* shows the fold induction of growth stimulated by estradiol compared with EtOH control for MCF7 (*left*) and ZR75 (*right*) cells that have been subjected to three cycles of intermittent hypoxia (▨) as compared with control cells (■). Representative graphs from duplicate experiments are shown. *Bars*, ±SD of six replicates. (*IH*, intermittent hypoxia; *Ox*, cells maintained under normoxic conditions)

able cells and by direct assessment of morphology by light microscopy (Fig. 5*A*). Growth assays done in parallel showed that MCF7 cells were capable of an intact growth response to estradiol after three cycles of intermittent acute hypoxia. However, this response was significantly reduced compared with control cells and was directly proportional to, and likely a reflection of, the persistently reduced ER-α protein levels. In contrast, ZR-75 cells assessed after three cycles of intermittent acute hypoxia showed a similar growth response to estradiol to that of control cells (Fig. 5*B*).

Depression of Estrogen Receptor α by Intermittent Hypoxia Is Proteasome Dependent. To explore possible mechanisms for sustained depression of ER-α, we first examined ER-α mRNA expression by reverse transcription-PCR in MCF7 and ZR-75 cells after intermittent hypoxia and reoxygenation. We found no significant difference in ER-α mRNA levels at any time point in either cell line. We next examined the possible role of altered proteasomal degradation in maintaining suppressed levels of ER-α protein by inhibiting proteasome function with bortezomib (11). Preliminary experiments were done to determine the optimal dose in MCF7 cells based on the maximal stabilization of HIF1α (a protein that is rapidly degraded by the proteasome under normoxia) and confirmation of minimal cytotoxic effects as determined by Trypan Blue viability assay (data not shown). Administration of bortezomib to MCF7 cells after 1, 7, and 14 days of reoxygenation (Fig. 6*A*) caused significant stabilization and induction of HIF1 protein and restored ER-α levels, measured by Western blot analysis, to comparable levels to control normoxic cells (Fig. 6*B*).

DISCUSSION

An effect of acute hypoxia on ER-α expression has previously been identified in breast cell lines and is thought to involve the proteasome, at least in some cells (7, 8). However, the existence of a relationship *in vivo* is poorly defined, and the connection between this transient effect of acute hypoxia and any sustained effect on ER-α expression that might have implications for estrogen-mediated cellular responses has not been determined.

We have shown that down-regulation of ER-α in breast tumors *in vivo* is closely correlated with several cellular markers of hypoxia (5) and is therefore consistently present in those regions of tumors that are shown to be hypoxic at the time of surgical resection. Using breast cell lines, we have also shown that the effect of hypoxia may be relatively specific, given the absence of an effect of glucose starvation or media conditioned by severely hypoxic cells, which represent other stress factors that are likely to be present within perinecrotic regions of tumors. Nevertheless, we have also confirmed that the effect of acute hypoxia on ER-α levels in cell lines is transient. Therefore, if depression of ER-α is not sustained and restricted to relatively uncommon and localized regions of necrosis in invasive breast tumors, then it would seem unlikely to be a cause of

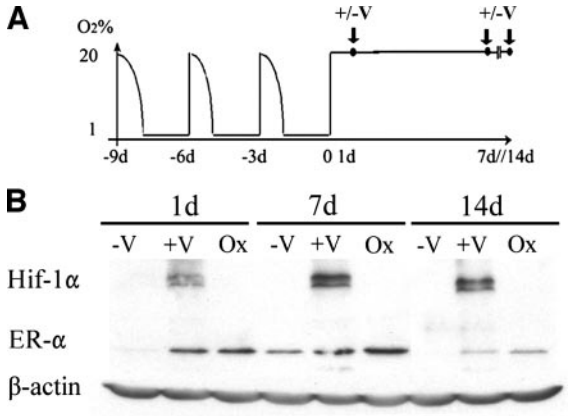

Fig. 6 Proteasome inhibition with bortezomib (VELCADE) restores ER-α levels after intermittent hypoxia. Western blot analysis showing ER-α protein level in MCF7 cells at day 1, day 7, and day 14 after three cycles of intermittent hypoxia (as shown in *Panel A*). Persistent depression of ER-α levels in MCF7 cells (*-V*) is restored by incubation with bortezomib (*+V*; 4 μmol/L for 8 hours) to the levels seen in control cells (*Ox*). Induction of HIF1α under normoxic conditions (*+V Lanes*) provides a positive control for proteasomal inhibition by bortezomib. (*d*, day; *Ox*, cells maintained under normoxic conditions)

persistent reduction or loss of ER-α expression in other areas of the tumor that do not show features of acute hypoxia. However, our finding that intermittent hypoxia can cause sustained depression of ER-α in MCF7 cells raises the possibility that reduced ER-α in other regions of tumors may reflect previous or intermittent hypoxia elsewhere and may contribute more widely to a durable reduction of ER-α expression.

HIF1α is a key component of the cellular response to hypoxia. If hypoxia is a dominant cause of altered ER-α expression, then a close relationship between HIF1α and ER-α might be expected. Interactions between HIF1α and both ER-α and ER-β pathways have also been proposed (15, 16). However, different studies involving cell lines and tumors have found either no relationship (12, 17, 18), or positive (19) or negative associations (6, 20). Explanations for this may be that HIF1α expression is very tightly regulated and only transient, or that HIF1α can also be induced by other factors (4, 5). Also, most studies relating HIF1α to factors including ER-α have also only considered overall associations rather than specific relationships at the cellular level. This is underscored by a recent study (6) that included eight ER-α–positive ductal carcinoma *in situ* lesions and examined geographical localization of HIF1α expression, which showed that loss of differentiation manifested by morphologic changes and down-regulation of ER-α was associated with HIF1α expression, at least in ductal carcinoma *in situ*. However, the consistency of this relationship with necrosis and within individual lesions was only documented for the morphologic features.

CA-IX and Glut-1 are key components of the HIF1α-mediated hypoxia response that, by comparison with HIF1α itself, are relatively stable proteins and therefore have a more sustained profile of response and are likely to persist in tumor regions that experience hypoxia and subsequently undergo reoxygenation (5, 14). This assumption is supported by the observation that although the majority of HIF1α-positive tumors are also necrosis, CA-IX, and Glut-1 positive, the latter markers are much more frequently detected (5). Therefore, in the present study, we elected to define hypoxic regions by three persistent indicators of hypoxia, represented by necrosis and induction of CA-IX and Glut-1 (5, 12, 13, 17). In both *in situ* and invasive tumors, morphologically viable but hypoxic regions defined by these criteria exhibited loss of ER-α and progesterone receptor expression compared with adjacent nonhypoxic regions. This is consistent with our previous studies, in which a strong relationship between overall ER-α–negative status and these indicators of hypoxia has also consistently emerged.

The level of ER-α (in addition to the status) is a significant determinant of response to endocrine therapy (1). The earlier literature, based primarily on ER-α determined by ligand binding assay done on whole tissue samples, suggested that in many cases ER-α status does not change with progression to metastasis (21) and that resistance to endocrine therapy is usually associated with a maintained ER-α expression and function. This has focused attention on alterations in other mechanisms including altered coregulators and signal transduction pathways. However, immunohistochemical studies have shown that ER-α is reduced in the majority of tamoxifen-resistant tumors (22). Also, in a minority of cases (~25%), spontaneous loss of ER-α expression occurs between the primary ER-α–positive breast cancer and its recurrence (23), accompanied by poor response to tamoxifen therapy. The mechanisms by which this occurs are largely unknown but in some instances may reflect hyperactivation of other signaling pathways (24). Therefore, reduced levels of ER-α can be associated with reduced sensitivity or resistance to endocrine therapies, in a small but significant number of cases.

Hypoxia within specific regions of tumors may be acute, chronic, or intermittent (25). The latter may be attributable to many causes but in some instances may reflect the release of angiogenic factors from hypoxic tumor cells (4), leading to reoxygenation at the margins of hypoxic regions, subsequent recovery of tumor cells in these regions, renewed proliferation and demand, and resulting hypoxia. Therefore, whereas acute hypoxia adjacent to necrosis may be associated with only local loss of ER-α expression, intermittent hypoxia and reoxygenation in other regions that is known to occur (25), may lead to more sustained depression of ER-α levels distant from areas of frank necrosis, even after an extended period of normoxia.

During acute hypoxia, an increased degradation of ER-α protein through altered proteasomal degradation has already been identified as a mechanism for ER-α down-regulation (8). Changes at the ER-α mRNA level have also been identified in some cells (7). Our results confirm that acute hypoxic ER-α down-regulation occurs in both MCF7 and ZR-75 cells and can be attributed to a transient alteration of proteasome function. But more importantly, we have shown that this alteration can persist for many days, even after restoration of normoxic conditions, when some cell lines are subjected to repeated acute intermittent hypoxia. It remains to be determined if this alteration is permanent or if there is a full recovery to normal levels over time. However, testing this over longer periods, where other factors (*e.g.*, clonal selection) might come into play, will require development of a different experimental system. Nevertheless, this

observation is consistent with the fact that acute hypoxia and intermittent hypoxia can have differential effects on gene expression (26) and enzyme activity (27). Possible explanations for this phenomenon include changes in proteasome composition or subunit function. The proteasome activity changes with age (28, 29), in response to immunologic challenge (29), and after ischemia/reperfusion injury, where lipid peroxidation occurs secondary to accumulation of free radicals and oxidative damage, leading to modification of 20S proteasome α-subunit components (30).

In conclusion, our findings suggest that intermittent hypoxia can cause persistent changes in proteasome function that may contribute to reduced ER-α expression in breast tumors. Our study focused on a relatively rare subset of ER-α–positive tumors that manifest severe regional hypoxia and necrosis to establish a relation between hypoxia and ER-α down-regulation *in vivo*. Additional study will be required to determine whether this is a common mechanism in breast tumors. Nevertheless, hypoxia is prevalent at both early *in situ* and later invasive stages. Therefore, hypoxia may play a role in progression from ER-α–positive to ER-α–negative status and so contribute to reduced response to endocrine therapies.

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