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ARTICLES

Hypoxia/Reoxygenation: A Dynamic Regulator of Lysyl Oxidase-Facilitated Breast Cancer Migration

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Abstract Fluctuating oxygen levels characterize the microenvironment of many cancers and tumor hypoxia is associated with increased invasion and metastatic potential concomitant with a poor prognosis. Similarly, the expression of lysyl oxidase (LOX) in breast cancer facilitates tumor cell migration and is associated with estrogen receptor negative status and reduced patient survival. Here we demonstrate that hypoxia/reoxygenation drives poorly invasive breast cancer cells toward a more aggressive phenotype by up-regulating LOX expression and catalytic activity. Specifically, hypoxia markedly increased LOX protein expression; however, catalytic activity (β-aminopropionitrile inhibitable hydrogen peroxide production) was significantly reduced under hypoxic conditions. Moreover, poorly invasive breast cancer cells displayed a marked increase in LOX-dependent FAK/Src activation and cell migration following hypoxia/reoxygenation, but not in response to hypoxia alone. Furthermore, LOX expression is only partially dependent on hypoxia inducible factor-1 (HIF-1α) in poorly invasive breast cancer cells, as hypoxia mimetics and overexpression of HIF-1α could not up-regulate *LOX* expression to the levels observed under hypoxia. Clinically, LOX expression positively correlates with tumor progression and co-localization with hypoxic regions (defined by HIF-1α expression) in ductal carcinoma in situ and invasive ductal carcinoma primary tumors. However, positive correlation is lost in metastatic tumors, suggesting that LOX expression is independent of a hypoxic environment at later stages of tumor progression. This work demonstrates that both hypoxia and reoxygenation are necessary for LOX catalytic activity which facilitates breast cancer cell migration through a hydrogen peroxide-mediated mechanism; thereby illuminating a potentially novel mechanism by which poorly invasive cancer cells can obtain metastatic competency. J. Cell. Biochem. 103: 1369–1378, 2008.

Key words: motility; breast cancer; lysyl oxidase; hypoxia; reoxygenation

Breast cancer is the most prevalent malignancy in women with most breast cancer deaths caused by metastatic tumor burden. As the mechanisms of metastasis are multi-faceted and incompletely understood, the process by which tumor cells interact with their microenvironment, alter their invasive potential, and metastasize both regionally and distantly has been intensely investigated in recent years [Rofstad, 2000; Paszek et al., 2005; Anderson et al., 2006; Rinker-Schaeffer et al., 2006; Steeg, 2006].

Lysyl oxidase (LOX) is a copper-dependent amine oxidase that was thought to function only in the extracellular milieu by cross-linking collagens or elastin to increase extracellular matrix tensile strength [Lucero and Kagan, 2006]. However, catalytically active LOX can

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function intracellularly—influencing cell signaling and gene transcription, suggesting novel functions for LOX in both normal and disease-related biological processes [Lucero and Kagan, 2006; Jansen and Csiszar, 2007]. For example, *LOX* expression is up-regulated in several cancer types including invasive/metastatic breast cancer cell lines and metastatic breast tumors [Kirschmann et al., 2002; Payne et al., 2005, 2007; Erler et al., 2006].

Tumor hypoxia promotes metastasis in many cancers including breast [Rofstad, 2000; Postovit et al., 2002; Subarsky and Hill, 2003; Cairns and Hill, 2004; Hussain et al., 2007; Rofstad et al., 2007] via the up-regulation of many genes, including *vascular endothelial growth factor*, *urokinase receptor*, *c-MET*, and *CXCR4*, which are integral to metastatic tumor progression [Semenza et al., 2000; Pouyssegur et al., 2006]. Recently, hypoxia has been shown to up-regulate *LOX* expression, via HIF-1 binding to hypoxia responsive elements in the LOX promoter, leading to enhanced invasion in an invasive/metastatic breast cancer cell line, MDA-MB-231 [Erler et al., 2006]. Conversely, inhibition of *LOX* expression and catalytic activity in this cell line significantly reduced the number of distant metastases to the lung and liver in vivo [Erler et al., 2006]. Furthermore, overexpression of LOX in poorly invasive breast cancer cell lines resulted in an increase in in vitro migration and invasion [Kirschmann et al., 2002; Payne et al., 2005]. The mechanism by which LOX facilitates a migratory phenotype in breast cancer cells is attributed to hydrogen peroxide-mediated FAK/Src activation, Rac-1 activation, and Rho inactivation (hallmarks of a migratory phenotype)[Payne et al., 2005, 2006; Siesser and Hanks, 2006].

Our current investigation elucidates the relationship between hypoxia/reoxygenation, LOX catalytic activity, and LOX-induced migration in poorly invasive breast cancer cell lines, MCF-7 and T-47D. We demonstrate that LOX mRNA and protein expression is greatly increased in poorly invasive breast cancer cells by hypoxia and that reoxygenation induces LOX-dependent FAK/Src activation and cell migration beyond that observed with hypoxia alone. Finally, we demonstrate that up-regulation of LOX is positively correlated with HIF-1α expression in ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC); however, this relationship is lost in metastatic tumors where LOX protein was the most heavily expressed. This body of work provides evidence that hypoxia and reoxygenation are important factors in regulating LOX activity and LOX-mediated tumor progression, and that manipulation of the tumor microenvironment serves as a potential therapeutic target for breast cancer.

MATERIALS AND METHODS

Cells and Culture Conditions

Cell lines were maintained as previously described [Kirschmann et al., 2002]. Hypoxic and hypoxia/reoxygenation conditions were achieved as previously described [Postovit et al., 2002]. Where indicated, cells were treated with deferoxamine (DFO; 100 μM; Sigma), cobalt chloride ($CoCl_2$; 100 μM; Sigma), catalase (100 U/ml; Sigma) or β-aminopropionitrile (βAPN; 100, 200, or 300 μM; Sigma) for 24 h prior to assay.

Real-Time PCR Analysis

RNA extraction, reverse transcription, and real-time PCR were performed as previously described [Topczewska et al., 2006] using TaqMan® gene expression primer/probe sets (Hs00184700_m1 *LOX*; Hs00173626_m1 *VEGF*; Applied Biosystems). Expression was normalized to *RPLPO* (4333761F; Applied Biosystems). Statistical significance was evaluated using a one-way ANOVA.

Semiquantitative PCR Analysis

RNA extraction, reverse transcription, PCR amplification, and verification of PCR amplicons were performed as previously described [Kirschmann et al., 2002]. Specific primers used were: (*LOX*: forward: 5′-GATCCTGCTGATCCGCGACAA-3′; reverse: 5′-GGGAGACCGTACTGG AAGTAGCCAGT-3′ plus 5% DMSO), (*VEGF*: forward: 5′-GCCTCCGAAACCATGAACTTTCTG-3′; reverse: 5′-CGGCTTGTCACATCTGCAAGT ACG-3′), and (*18S rRNA*: forward: 5′-TTGGAGGGCAAGTCTGGTGCCAGCAGC-3′; reverse: 5′-TCTGTCAATCCTGTCC GTGTCGGGCC-3′).

In Vitro Migration Assay

Analysis of in vitro cell migration was performed as previously described [Payne et al., 2005]. Statistical significance was evaluated using a one-way ANOVA.

Lysyl Oxidase Activity Assay

Breast cancer cells were plated onto six-well tissue culture plates in normal culture medium and grown to approximately 60% confluence. Twenty-four hours prior to assay, culture medium was replaced with phenol-free RPMI-1640 supplemented with Mito+ serum extender (Collaborative Research), with or without 300 μM βAPN. Cells (1×10^6, 80% confluence), were harvested with trypsin/EDTA, and incubated with 1 μM dichlorodihydrofluorescein diacetate (DCFDA, Invitrogen) in either 20 or 1% O_2 for 45 min in the presence or absence of βAPN (300 μM). DCFDA is a cell permeable fluorescent dye (492–495 nm excitation; 517–527 nm emission) that is primed via an esterase-dependent cleavage within the cell, and fluoresces in response to reactive oxygen species [Wood et al., 2006]. Cells were washed twice with PBS and fluorescence (FITC) of untreated and βAPN-treated cells were analyzed by flow cytometry. LOX activity was defined as total mean fluorescence—fluorescence in βAPN treated cells. Statistical significance was evaluated using a Student's *t*-test.

Electrophoresis and Immunoblotting

Cell lysis and immunoblotting were conducted as previously described (Payne et al., 2005). The antibodies used were: anti-FAK ($pTyr^{576}$ or $pTyr^{397}$, 1:1000, Biosource), anti-FAK (1:500, BD Pharmingen), anti-Src ($pTyr^{418}$, 1:1,000, Biosource), anti-Src (1:250, Upstate), anti-actin (1:10,000, Chemicon), anti-HIF-1α (1:250, BD Transduction Laboratories), anti-LOX 1:1,000, or anti-GAPDH (1:1,000, Abcam), and the appropriate horseradish peroxidase-labeled secondary antibodies. Rabbit anti-LOX antibody was generated to the peptide KYSDDNPYYNYYDTYERPRPGG (amino acids 176–197 of LOX; Zymed Laboratories) and specificity was evaluated in rat brain tissues [Li et al., 2004].

HIF-1α Expression and siRNA Constructs

HIF-1α expression (pCEP4-HIF-1α) and control (pCEP4) plasmid vectors have been described previously [Forsythe et al., 1996; Maxwell et al., 1999] and were kindly provided by Dr. Gregg Semenza (Johns Hopkins University, MD). Validated HIF-1α-specific siRNA (ID #42840) or non-specific siRNA were purchased from Ambion. FuGENE 6 (Roche Diagnostics) or siPORT NeoFx (Ambion) were used for transfections according to manufacturer's specifications.

Immunohistochemistry

Archival tissue was obtained from the pathology core facility (Robert H. Lurie Comprehensive Cancer Center, Northwestern University) under the approval of the Institutional Review Board at Children's Memorial Hospital. Immunohistochemical staining was performed as previously described [Topczewska et al., 2006] using rabbit anti-LOX (1:50), anti-HIF-1α (1:150, Calbiochem), or rabbit IgG isotype control (DAKO), and the appropriate biotinylated secondary antibody. Sections were scored blinded as no staining, weak (<25%), moderate (25–75%), or strong (>75%). Statistics were conducted using the Spearman Rank Order Correlation (SigmaStat).

RESULTS

LOX Expression Is Induced by Hypoxia in Poorly Invasive Breast Cancer Cells

Exposure of T-47D and MCF-7 breast cancer cells to decreasing levels of oxygen (20–0.5%) for 24 h resulted in a dose-dependent increase in *LOX* mRNA levels (Fig. 1A). As compared to untreated control (20% O_2), *LOX* expression was maximally increased at 0.5% and 1% O_2 for T-47D and MCF-7 cells, respectively. At 1% O_2 conditions, a time course indicated that *LOX* mRNA is initially up-regulated after 4 h and reached maximum by 16–24 h (Fig. 1B). The increase in hypoxia-induced *LOX* expression was transient as expression levels returned to that of control cells (20% O_2) by 24 h of reoxygenation at 20% O_2 (Fig. 1C). Similar to mRNA levels, proLOX (50 kDa) protein was greatly increased following exposure to 1% O_2 for 24 h (Fig. 1D). Mature LOX (32 kDa) protein was also increased, albeit to a lesser extent (Fig. 1D). These results demonstrate that hypoxia induces LOX expression in poorly invasive/non-metastatic breast cancer cells and that these cells process LOX to the active 32 kDa form.

Hypoxia-Induced LOX Requires Oxygen for Catalytic Activity

Recent studies have indicated that LOX protein can have biological functions independent

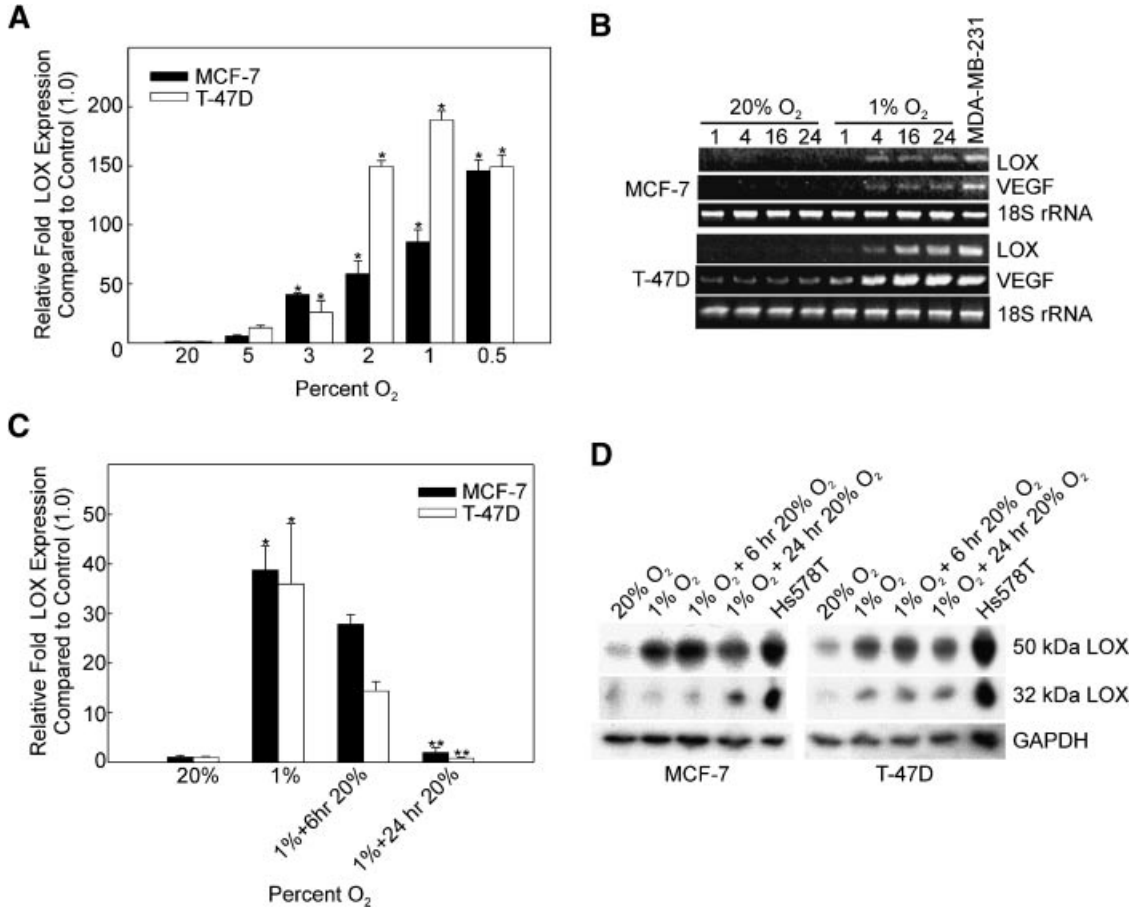


Fig. 1. Hypoxia induces LOX expression in poorly invasive breast cancer cells. **A**: *LOX* mRNA expression in poorly invasive/non-metastatic MCF-7 and T-47D breast cancer cells in response to decreasing levels of oxygen for 24 h as analyzed by real-time PCR and normalized to an endogenous control, RPLPO. Relative fold LOX expression was determined by comparison to cells cultured under 20% O_2 and arbitrarily set at 1.0. **B**: *LOX, VEGF,* and *18S rRNA* mRNA expression in MCF-7 and T-47D cells in response to 1% O_2 for 1, 4, 16, and 24 h as analyzed by semiquantitative PCR. Hypoxia-induced *LOX* expression was compared to endogenous *LOX* expression in invasive/metastatic MDA-MB-231 cells. **C**: *LOX* mRNA expression in MCF-7 and T-47D breast cancer cells upon reoxygenation in 20% O_2 for 6 and 24 h following 24 h treatment at 1% O_2. *LOX* mRNA expression was analyzed by real-time PCR and normalized to an endogenous control, RPLPO. Relative fold LOX expression was determined by comparison to cells cultured under 20% O_2 and arbitrarily set at 1.0. **D**: Immunoblot analysis of pro-LOX (50 kDa) and mature LOX (32 kDa) expression in MCF-7 and T-47D treated for 24 h at 20% O_2, 1% O_2, or reoxygenation conditions (24 h at 1% O_2 followed by 6 and 24 h 20% O_2). Rabbit anti-LOX antibody was generated to the peptide KYSDDNPYYNYYD-TYERPRPGG (amino acids 176-197 of LOX; Zymed Laboratories) and specificity was evaluated in rat brain tissues (Li et al., 2004). GAPDH expression was used as an equal loading control and Hs578T cell lysates (invasive breast cancer cell line) were used as a positive LOX control. *$P < 0.05$ compared to 20% O_2 controls (ANOVA). **$P < 0.05$ compared to 1% O_2 treated cells (ANOVA).

of the catalytic domain [Fogelgren et al., 2005; Bouez et al., 2006; Lucero and Kagan, 2006; Min et al., 2007; Polgar et al., 2007]. Therefore, in this study βAPN (a specific inhibitor of LOX catalytic activity) was used to address the role of active LOX rather than LOX-specific siRNAs (knockdown of LOX protein). Using a biologically relevant assay that detects hydrogen peroxide generated by LOX enzymatically interacting with a naturally occurring substrate(s), we observed little to no cell-associated LOX activity (defined as βAPN-inhibitable hydrogen peroxide generation) in MCF-7 and T-47D cells cultured in 20% O_2, compared to endogenous LOX expressing MDA-MB-231 cells (Fig. 2A). These results demonstrate that this flow cytometric LOX assay reproduced the same results as the conventional Amplex Red LOX activity assay we have previously used [Payne et al., 2005]. As is shown in Figure 2B, βAPN-inhibitable LOX activity was significantly reduced when either MCF-7 (poorly

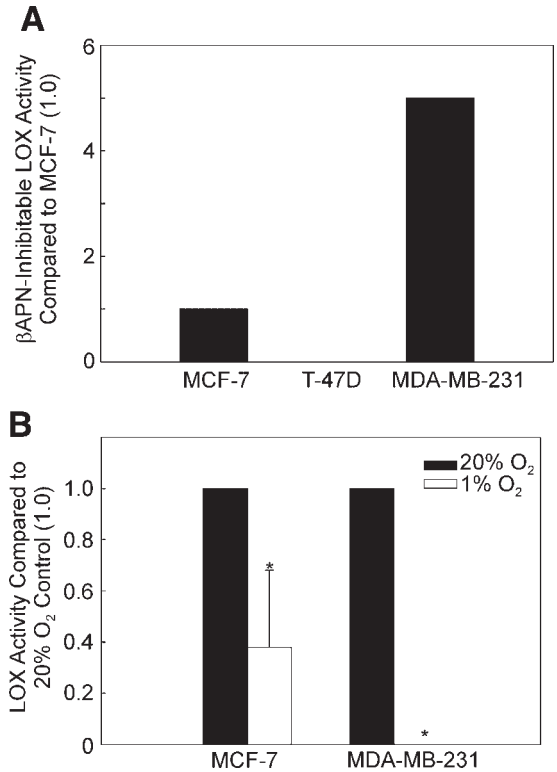


Fig. 2. **A**: LOX catalytic activity in breast cancer cell lines. Cell-associated LOX activity was measured in MCF-7, T-47D (little to no LOX expression) and MDA-MB-231 (endogenous LOX expression) using DCFDA. DCFDA is a cell permeable fluorescent dye that is primed via an esterase-dependent cleavage within the cell, which then fluoresces in response to reactive oxygen species. Fluorescence (FITC) of untreated cells and cells pretreated for 24 h and assayed in the presence of 300 μM βAPN were analyzed by flow cytometry. LOX activity was defined as total mean fluorescence—fluorescence in βAPN treated cells. **B**: LOX activity (defined as βAPN inhibitable hydrogen peroxide production) in MCF-7 and invasive MDA-MB-231 cell lines cultured and assayed in either 20% O_2 or 1% O_2 conditions using a DCFDA fluorogenic assay. Data were normalized to 20% O_2 controls. *$P < 0.05$ compared to 20% O_2 controls (ANOVA).

invasive) or MDA-MB-231 (highly invasive) cells were cultured in a 1% O_2 microenvironment, confirming that cell-associated LOX absolutely requires the presence of oxygen for its catalytic reaction. Although MCF-7 and T-47D cells exposed to hypoxia/reoxygenation have increased 50 and 32 kDa LOX protein expression, we were unable to measure a detectable increase in cell-associated LOX activity. This suggests that LOX is secreted extracellularly by these cells and therefore its activity is not detected with our cell-associated LOX activity assay.

Hypoxia/Reoxygenation Leads to LOX-Induced Increases in Cell Migration Through a Hydrogen Peroxide-Dependent Mechanism

We examined whether hypoxia-induced LOX was functional in poorly invasive breast cancer cells, with regard to promoting a migratory phenotype, in a hypoxic microenvironment. A significant increase in cell migration was observed when MCF-7 and T-47D cells were cultured in 1% O_2 followed by 6 h reoxygenation in 20% O_2, as compared to cell migration observed in 1% O_2 or 20% O_2 alone (Fig. 3A). The significant increase in cell migration was dependent on LOX activity since treatment with βAPN dose-dependently decreased migration to levels observed under hypoxia alone (Fig. 3A). In contrast, the increase in cell migration observed with hypoxia alone was independent of LOX activity as treatment with βAPN did not significantly alter migration in the absence of reoxygenation (data not shown). Furthermore, the increase in hypoxia/reoxygenation-induced migration was due in part to hydrogen peroxide production since catalase treatment led to a significant decrease in migration when MCF-7 and T-47D cells were exposed to hypoxia/reoxygenation conditions (Fig. 3B). Similar to βAPN, catalase treatment did not significantly alter cell migration under hypoxic conditions in which LOX is catalytically inactive. These data support the hypothesis that hypoxia/reoxygenation induces LOX-facilitated cell migration via a hydrogen peroxide-dependent mechanism.

We have previously shown that changes in the FAK/Src signaling pathway accompanied LOX-induced migration of invasive/metastatic breast cancer [Payne et al., 2005]. Therefore, we examined whether LOX-induced cell migration under hypoxia/reoxygenation also induced phosphorylation of FAK and Src. When MCF-7 and T-47D cells were cultured under hypoxia/reoxygenation conditions, there was an increase in the phosphorylation of FAK [Y576] and Src [Y418], beyond that observed with hypoxia alone, that was reversed upon βAPN treatment (Fig. 3C,D). In MCF-7 cells, as opposed to T-47D cells, treatment with hypoxia alone induced phosphorylated FAK [Y576] and Src [Y418]; this is in accordance with previous studies which demonstrate that hypoxia can induce FAK/Src phosphorylation in certain cell types by other mechanisms [Seko et al., 1999]. In

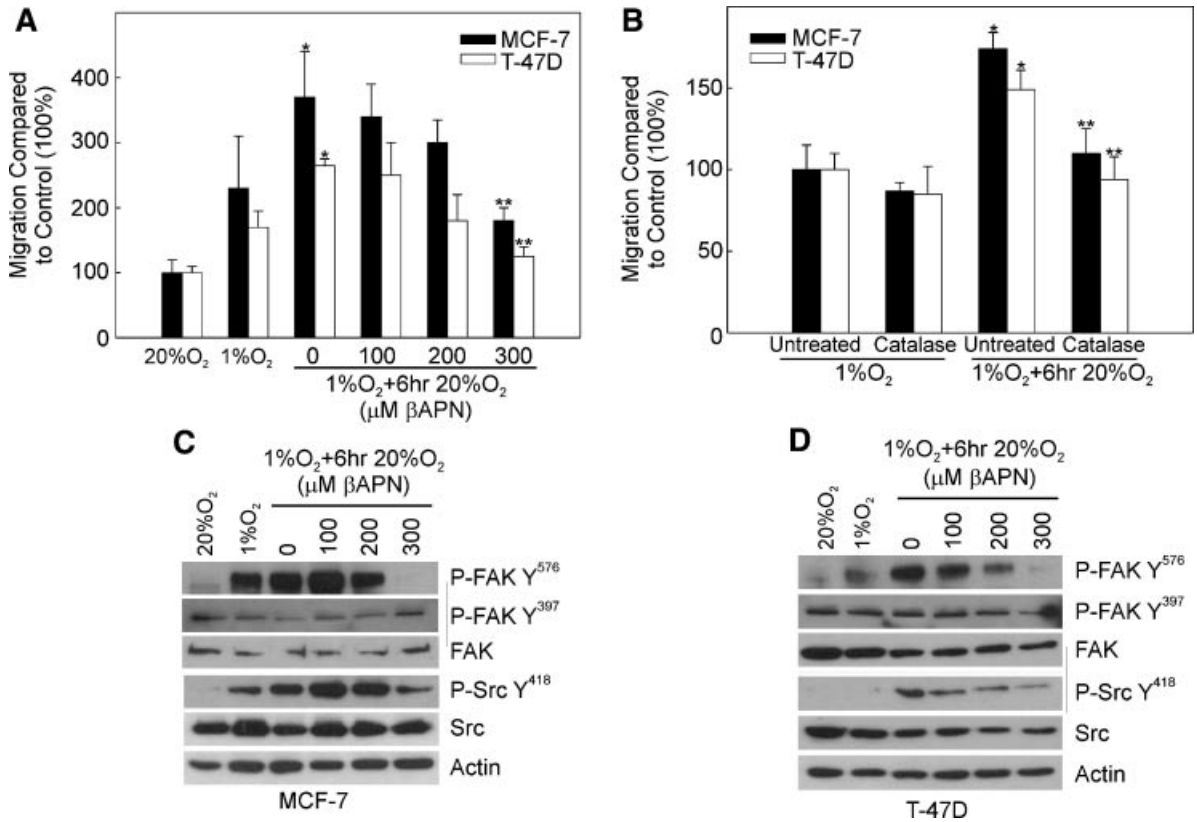


Fig. 3. Hypoxia/reoxygenation, but not hypoxia alone, induces LOX-dependent migration in poorly invasive breast cancer cells. **A**: Migration of MCF-7 and T-47D cells cultured in 20% O_2, 1% O_2, or reoxygenation conditions (24 h at 1% O_2 followed by 6 h 20% O_2). Where indicated cells were treated with increasing doses of βAPN for 24 h prior to and during the migration assay (6 h). Cell migration was calculated as the total number of cells that migrated through a 0.01% gelatin-coated polycarbonate filter (10 μm pore size) within 6 h and normalized to 20% O_2 controls (100%). **B**: Migration of MCF-7 and T-47D cells cultured in 1% O_2 for 24 h alone or with reoxygenation in 20% O_2 for 6 h in the absence or presence of 100 units/ml catalase. Data were normalized to 1% O_2, untreated controls (100%). Immunoblot analysis of FAK, autophosphorylated FAK Y^{397}, activated FAK Y^{576}, Src, and activated Src Y^{418} expression in (**C**) MCF-7 (**D**) and T-47D cells cultured in 20% O_2 or 1% O_2 for 24 h alone or with reoxygenation conditions (20% O_2 for 6 h) in the absence or presence of increasing concentrations of βAPN. $^*P < 0.05$ compared to 20% O_2 controls (ANOVA). $^{**}P < 0.05$ compared to untreated cells cultured under reoxygenation conditions (ANOVA).

MCF-7 cells, LOX appears to further increase FAK/Src phosphorylation upon reoxygenation. Levels of FAK autophosphorylation at tyrosine 397, as well as overall FAK and Src expression remained relatively constant regardless of oxygen concentration. These data demonstrate that hypoxia/reoxygenation-induced LOX activity is correlated with activation of the FAK/Src signaling pathway to promote a migratory phenotype.

Hypoxia-Induced *LOX* Expression Is Partially HIF-1 Mediated

We next investigated how *LOX* transcription was induced in poorly invasive breast cancer cells under hypoxic conditions. One of the major mechanisms of hypoxia-induced transcription is through a heme-dependent mechanism [Semenza, 2001]. In this regard, DFO and $CoCl_2$, both heme disruptors, can mimic hypoxia-induced gene transcription through an iron-dependent mechanism. Treatment of MCF-7 and T-47D cells with DFO or $CoCl_2$ for 24 h significantly increased *LOX* expression by approximately 15-fold (Fig. 4A). However, these hypoxia-mimetics did not induce *LOX* expression to the same extent as hypoxia alone (approximately 120-fold; Fig. 4A vs. C, respectively), suggesting that the hypoxic induction of *LOX* occurs via both heme-dependent and independent pathways.

In order to determine whether *LOX* expression was specifically induced by HIF-1, we utilized a HIF-1α expression construct and

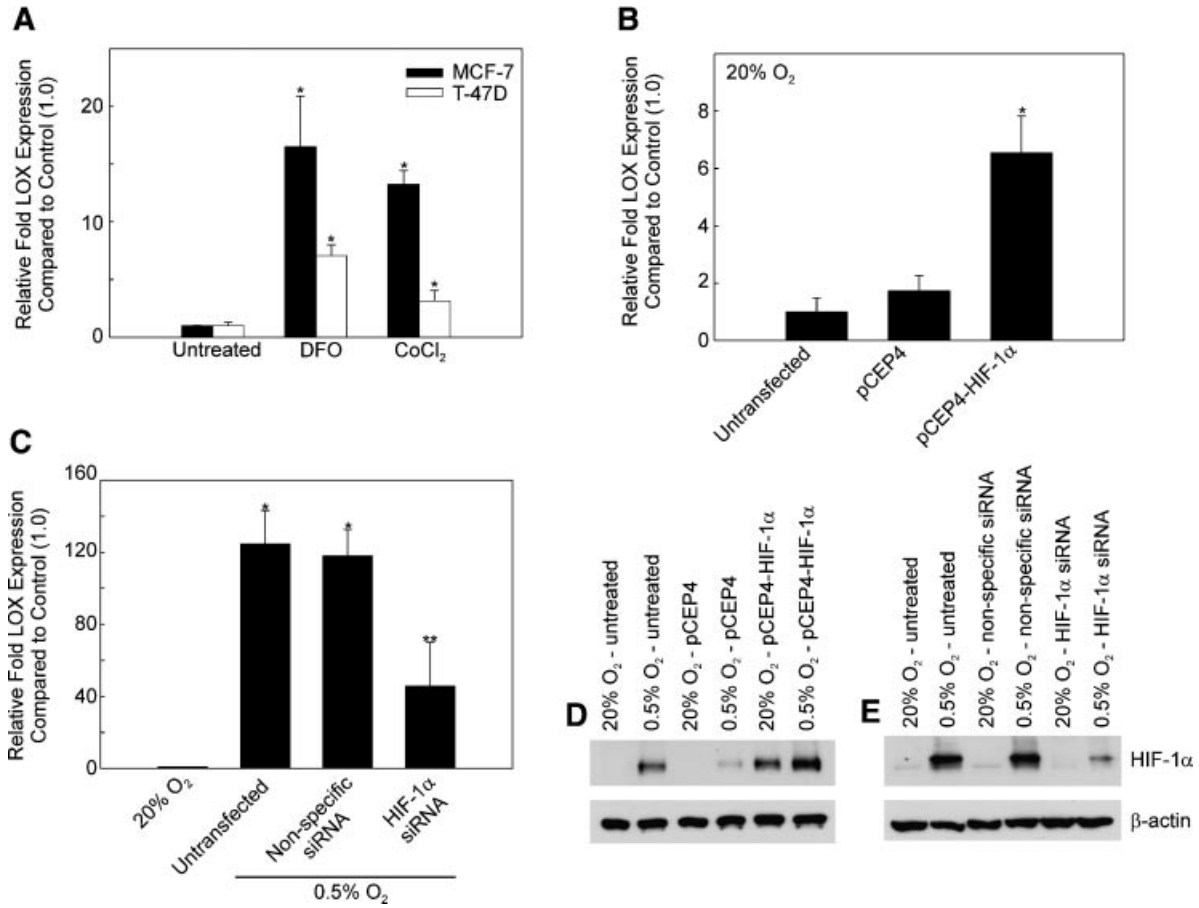
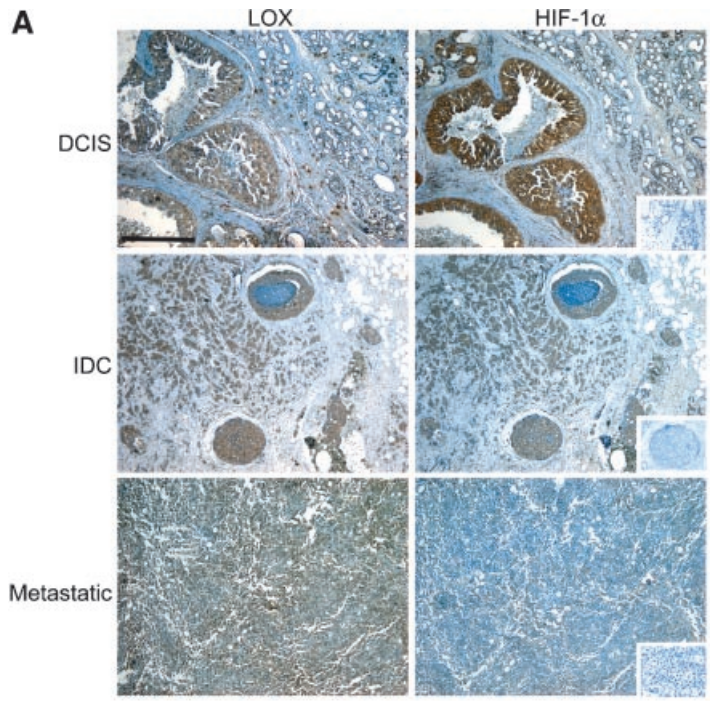


Fig. 4. HIF-1α is required but not sufficient for the hypoxia-induced *LOX* expression in poorly invasive breast cancer cells. MCF-7 cells were either treated with **A**: 100 μM DFO or 100 μM $CoCl_2$ for 24 h, **B**: transfected with a control (pCEP4) or HIF-1α expression construct (pCEP4-HIF-1α), or **C**: transfected with a non-specific siRNA control or HIF-1α-specific siRNA 24–48 h prior to isolation of RNA. *LOX* mRNA expression in untreated and treated cells was analyzed by real-time PCR and normalized to an endogenous control, RPLPO. Relative fold LOX expression was determined by comparison to untreated cells cultured under 20% O_2 and arbitrarily set at 1.0. $^*P < 0.05$ compared to untreated controls (ANOVA). $^{**}P < 0.05$ compared to untransfected cells cultured in 1% O_2 (ANOVA). Cell lysates were collected and analyzed for HIF-1α expression using immunoblot analysis in (**D**) MCF-7 cells transfected with control (pCEP4) or HIF-1α expression vectors (pCEP4-HIF-1α) and subjected to 20% or 0.5% O_2 conditions for 24 h, and **E**: MCF-7 cells transfected with either non-specific or HIF-1α-specific siRNA for 48 h and subjected to 20% or 0.5% O_2 conditions for 24 h. Blots were stripped and reprobed for β-actin expression to assess equal loading.

HIF-1α-specific siRNA in order to activate or inhibit HIF-1α function, respectively. MCF-7 cells overexpressing active HIF-1α (pCEP4-HIF-1α) had increased *LOX* expression (sevenfold; Fig. 4B). The fold induction of *LOX* expression is reminiscent to that induced by DFO or $CoCl_2$ treatment (Fig. 4A). MCF-7 cells transfected with pCEP4-HIF-1α demonstrated HIF-1 protein expression regardless of oxygenation status (Fig. 4D). Conversely, the hypoxic up-regulation of *LOX* expression was significantly muted (60% or threefold decrease) when HIF-1α expression was knocked down with HIF-1α-specific siRNA, but not with a non-specific siRNA negative control (Fig. 4C,E). Taken together, these data suggest that *LOX* expression is only partially regulated by HIF-1.

LOX Expression Correlates With Tumor Hypoxia Only in Primary Breast Tumors

As a clinical correlate, we examined the expression of LOX and HIF-1α in serial sections of human primary and metastatic breast carcinoma tumors. Immunohistochemistry revealed that LOX protein expression was positively correlated with tumor grade ($P < 0.05$) in that in DCIS and IDC there was similar HIF-1α and LOX staining ($P < 0.05$; Fig. 5B). Specifically, LOX expression was greatest in hypoxic areas (defined by HIF-1α expression) in these tumors.



B

		None	Weak	Moderate	Strong	Total
DCIS	LOX	1	0	2	3	6
	HIF-1α	1	2	0	3	
IDC	LOX	0	2	3	6	11
	HIF-1α	1	3	1	6	
Metastatic	LOX	0	0	1	6	7
	HIF-1α	3	2	2	0	

Fig. 5. LOX expression is positively correlated with hypoxia in poorly aggressive breast carcinoma, but is expressed independently in metastatic disease. **A**: Immunohistochemical analysis of HIF-1α and LOX expression was performed on serial sections of formalin-fixed, paraffin-embedded ductal carcinoma in situ (DCIS), invasive ductal carcinoma (IDC), and metastatic breast tumors. Bar equals 500 μm and isotype controls (rabbit IgG, DAKO) are in the insets. **B**: The expression and prevalence of LOX and HIF-1α staining was enumerated in tissues from each type of breast carcinoma. Staining was evaluated blindly and designated as no staining, weak (<25%), moderate (25–75%), or strong (>75%). Statistics were conducted using the Spearman Rank Order Correlation (SigmaStat).

However, in metastatic tumors this relationship was lost as LOX protein was heavily expressed regardless of HIF-1α expression (Fig. 5A). These data suggest that LOX expression in tumor cells with metastatic capabilities is no longer under the regulation of HIF-1α.

DISCUSSION

LOX has become increasingly studied in cancers as its importance in tumor progression becomes more thoroughly realized [Payne et al., 2007]. We and others have shown that: LOX is expressed in invasive/metastatic breast cancer cell lines and in recurrent/metastatic breast tumors [Kirschmann et al., 2002; Payne et al., 2005]; LOX expression is correlated with decreased overall survival in breast cancer patients [Erler et al., 2006]; LOX enzymatic inhibition promotes a less migratory/invasive and metastatic phenotype [Erler et al., 2006]; and LOX facilitates breast cancer cell migration via a hydrogen peroxide-dependent mechanism [Payne et al., 2005].

Here, we demonstrate that poorly invasive breast cancer cells, which express little to no

endogenous LOX, aberrantly express high levels of LOX when exposed to hypoxic conditions. These findings are in accordance with those previously described by Erler et al., who demonstrated that LOX is up-regulated by hypoxia in MDA-MB-231 breast carcinoma cells, which endogenously express LOX, and are inherently invasive and metastatic [Erler et al., 2006]. Therefore, in addition to making invasive/metastatic breast cancer cells more invasive, hypoxia can induce LOX expression in poorly invasive breast cancer cells, thereby enabling them to acquire invasive competency.

Since fluctuating oxygen levels, resulting from episodes of ischemia followed by reperfusion, characterize rapid metastasis [Rofstad, 2000; Cairns and Hill, 2004; Rofstad et al., 2007], it was essential to determine whether hypoxia-induced LOX in poorly invasive breast cancer cells was active under hypoxic conditions. Importantly, our results reveal that under hypoxic conditions LOX is not catalytically active. This finding is in accordance with the LOX mechanism of action in that LOX requires oxygen in order to regenerate catalytic activity [Lucero and Kagan, 2006]. As a consequence of its dependency on oxygen, hypoxia-induced LOX was unable to induce cell migration until the microenvironment was reoxygenated. In a manner similar to that described previously, this increase in migration was also correlated with an increase in hydrogen peroxide production and activation of Src and FAK. These novel findings reiterate the role of LOX in tumor metastasis, and illuminate the importance of reoxygenation when studying hypoxia-induced phenomena. Of interest, the LOX activity assay employed in this study did not account for extracellular LOX activity. Future studies will be needed to examine the role of extracellular LOX induced by hypoxia/reoxygenation on matrix–tumor cell and/or stromal–tumor cell interactions, as well as tumor cell metastatic progression.

Also explored was the transcriptional mechanism(s) governing hypoxia-induced *LOX* expression. Overexpression of HIF-1α or treatment with heme-disrupting agents in poorly invasive breast cancer cells correspondingly resulted in only a marginal increase in *LOX* expression. These results demonstrate that hypoxia-induced *LOX* expression in poorly invasive breast cancer cells is partially, but not completely, HIF-1 mediated and suggest that HIF-1α is required, but not sufficient, for the complete hypoxic up-regulation of *LOX*. Erler et al., [2006] similarly demonstrated that the hypoxic up-regulation of *LOX* in MDA-MB-231 cells is HIF-dependent. They did not, however, illuminate the possibility that HIF-independent signaling pathways are also integral to *LOX* expression, as exemplified by the ability of cells, including MDA-MB-231, to express LOX even in the absence of hypoxia.

The association between hypoxia and increased LOX expression was validated in clinically relevant breast cancer tissues. Using HIF-1α as a surrogate marker of hypoxia in malignant tissue, we observed a significant correlation between hypoxia and LOX expression in in situ and locally invasive breast tumors. However, in metastatic tissue this correlation was lost as the metastatic tumors examined strongly expressed LOX regardless of HIF-1α expression. Based on differential LOX expression in breast tumors and cell lines, we hypothesize that breast cancer cells with a high metastatic potential constitutively overexpress LOX regardless of surrounding oxygen tensions, whereas breast cancer cells with a low metastatic potential are susceptible to induction of LOX expression by low oxygen tension.

Perhaps most significantly, these data are vital for developing appropriate therapeutic strategies to target both poorly and highly invasive cells in primary breast tumors. While inhibiting LOX activity has potential in decreasing tumor burden, an alternative possibility would be to manipulate the tumor microenvironment to prevent hypoxia/reoxygenation and thus mitigate LOX-facilitated tumor progression.

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