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Tumor Cells Upregulate Normoxic HIF-1α in Response to Doxorubicin

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Abstract

Hypoxia-inducible factor 1 (HIF-1) is a master transcription factor that controls cellular homeostasis. While its activation benefits normal tissue, HIF-1 activation in tumors is a major risk factor for angiogenesis, therapeutic resistance and poor prognosis. HIF-1 activity is usually suppressed under normoxic conditions because of rapid oxygen-dependent degradation of HIF-1α. Here we show that under normoxic conditions HIF-1α is upregulated in tumor cells in response to doxorubicin, a chemotherapy used to treat many cancers. Doxorubicin also enhanced VEGF secretion by normoxic tumor cells and stimulated tumor angiogenesis. Doxorubicin-induced accumulation of HIF-1α in normoxic cells was caused by increased expression and activation of STAT1, the activation of which stimulated expression of iNOS and its synthesis of NO in tumor cells. Mechanistic investigations established that blocking NO synthesis or STAT1 activation was sufficient to attenuate the HIF-1α accumulation induced by doxorubicin in normoxic cancer cells. To our knowledge, this is the first report that a chemotherapeutic drug can induce HIF-1α accumulation in normoxic cells, an efficacy-limiting activity. Our results argue that HIF-1α targeting strategies may enhance doxorubicin efficacy. More generally, they suggest a broader perspective on the design of combination chemotherapy approaches with immediate clinical impact.

Keywords

Doxorubicin; HIF-1; STAT1; iNOS; Nitric Oxide; Chemotherapy

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Introduction

Chemotherapy is the most common systemic treatment for human cancers. Although tumors may initially respond to chemotherapy with partial or even complete remission, they often relapse with more aggressive malignant features such as enhanced angiogenesis and chemoresistance. One principal strategy that tumor cells employ to resist chemotherapy is to highjack and exploit important homeostatic signaling pathways originally used by normal cells to adapt, survive, and reconstruct microenvironment. Hypoxia-inducible factor-1 (HIF-1), a heterodimer of HIF-1α and HIF-1β, is such an essential homoeostatic protein (1). HIF-1 is a master transcriptional activator regulating hundreds of vital genes in all steps of tumorigenesis and tumor progression including angiogenesis (1), proliferation / apoptosis (2), therapeutic resistance (1, 3), cancer stem cell maintenance / reprogramming (4), invasion / metastasis (1), and energy metabolism (5). More importantly, high HIF-1α level is an independent prognostic factor for poor chemotherapeutic response, early recurrence, and shortened survival time in many human cancers such as breast cancer (6). Therefore, there is increasing interest to identify the mechanisms of HIF-1α upregulation in cancer cells and to develop novel therapeutic strategies targeting HIF-1, thereby enhancing the efficacy of chemotherapy (1).

Although HIF-1β is constitutively expressed, HIF-1α is rapidly degraded in proteasome after oxygen-dependent ubiquitination by the Von Hippel-Lindau protein (pVHL) complex under normoxic conditions. Binding of pVHL depends on hydroxylation of Pro402 and Pro564 in HIF-1α oxygen-dependent degradation (ODD) domain. Because this hydroxylation requires HIF prolyl hydroxylase (HIF-PH), O_2, and iron (7, 8), hypoxia or iron chelator inhibits ODD-domain prolyl hydroxylation to stabilize HIF-1α. The stabilized HIF-1α heterodimerizes with HIF-1β to form HIF-1, which transactivates downstream gene expression such as vascular endothelial growth factor (VEGF). In addition to prolyl hydroxylation, other posttranslational modifications of HIF-1α such as cysteine S-nitrosylation (Cys533) (9), lysine acetylation (Lys532) (10), or asparagine hydroxylation (Asn803) in HIF-1α C-terminal transactivation domain (CAD) by factor inhibiting HIF-1 (FIH-1) (11) also regulate the stability and transcriptional activity of HIF-1α. Accumulating evidence has proved that hypoxia is one important, but not the only factor stabilizing HIF-1α. Hypoxia-mimetic drug cobalt chloride, iron chelator, nitric oxide (NO), free radicals, and genetic alterations can enhance HIF-1 expression under normoxic conditions (1, 3, 9, 12-14).

Doxorubicin (adriamycin) is a first-line chemotherapeutic drug for treating wide spectrum of cancers. Lee et al elegantly demonstrated that doxorubicin inhibited HIF-1 transcriptional activity by blocking the binding of hypoxia-induced HIF-1 to DNA (15). HIF-1α knock-down or inhibition increases the sensitivity of hypoxic tumor cells to doxorubicin (16, 17). These studies suggest a synergistic anticancer effect by combining chemotherapy with HIF-1 inhibition to target hypoxic tumor cells. In contrast to considerable investigations into the interplay between chemotherapeutic drug and HIF-1α under hypoxic conditions, so far it is unknown whether chemotherapeutic drugs could regulate HIF-1α expression under normoxic conditions. One reason for this dearth is the technical difficulty of sensitively detecting HIF-1α in cells exposed to both normoxia and chemotherapy – two adverse conditions for HIF-1α expression because of oxygen-dependent HIF-1α degradation and impaired protein synthesis machinery due to cytotoxicity. To elucidate the effects of chemotherapy on HIF-1α expression in normoxic tumor cells, we used a 4T1 mouse breast tumor cell line (4T1ODD-luc) which was stably transduced with a fused HIF-1α reporter gene consisting of mouse HIF-1α ODD domain and a firefly luciferase (9). This reporter cell line allows noninvasive monitoring HIF-1α expression with high sensitivity (9). Previous studies have identified that doxorubicin concentrations in 4T1 and MCF-7 tumors ranged

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between 0 – 12 μg/ml (18, 19) and 0 – 8 μg/ml (20), respectively. By treating 4T1ODD-luc and MCF-7 cells with doxorubicin at concentrations within the above ranges, we found increased HIF-1α expression under normoxic conditions. We then investigated the effects of this normoxic HIF-1α accumulation on VEGF secretion and tumor angiogenesis after doxorubicin chemotherapy. More importantly, we identified the underlying mechanism which was the activation of the STAT1-iNOS-NO-HIF-1α signaling pathway. We also explored therapeutic strategies to suppress doxorubicin-induced normoxic HIF-1α accumulation. This work has important implications for trials targeting HIF-1α, which is upregulated not only by hypoxia but also by chemotherapy.

Materials and Methods

Cell Culture 4T1 mouse breast tumor cells and MCF-7 human breast cancer cells were obtained from Duke cell culture facility in 2006. 4T1ODD-luc HIF-1α reporter cells were obtained from Chuan-Yuan Li's laboratory in 2006. 4T1ODD-luc and MCF-7 cells were cultured in DMEM containing 10% FBS and 1% antibiotic-antimycotic under normoxic conditions (95% air, 5% CO_2). 5×10^5 4T1ODD-luc or 1×10^6 MCF-7 cells per 10-cm dish were plated for the Western blots. 1×10^6 4T1ODD-luc cells per 10-cm dish or 2.5×10^5 MCF-7 cells per well of 6-well plates were plated overnight for FACS. The cells were cultured in fresh medium with drugs the next day and continued for 24, 48, or 72 hours. Doxorubicin (Bedford Laboratories) concentrations are provided in figures or 1 μg/ml (0.58 μM) for 4T1 cells and 0.5 μg/ml for MCF-7 cells (0.29 μM). The concentrations of 1400W and EGCG (Cayman Chemical) were 10 μg/ml and 15 μM, respectively. An equal volume of solvent was used as the negative control. Hypoxic culture condition was 0.5% O_2, 5%CO_2 and N_2 balanced for 48 hours.

Bioluminescent Imaging HIF-1α ODD-luciferase reporter activity was quantified by Xenogen IVIS bioluminescence imaging system (9). 1×10^5 4T1ODD-luc cells per well of 12-well plates were cultured with 2 ml culture medium overnight. The cells were treated the next day with fresh medium containing doxorubicin (0.1, 1, or 10 μg/ml; 0.58-5.8 μM) or an equal volume of control vehicle for 24, 48, or 72 hours under normoxic conditions. 1×10^5 4T1ODD-luc cells were treated with 0.1, 1, or 10 mM L-NAME (Sigma-Aldrich) or control vehicle ± 1μg/ml doxorubicin for 24, 48, or 72 hours. Cell reporter activity and tumor volume were measured on day 0 before treatment and at multiple time-points post-treatment. The mice were imaged 10 minutes after i.p. injection of luciferin (150 mg/kg). Tumor bioluminescence intensity was normalized to tumor volume at each time point.

Animal Studies 5×10^6 4T1ODD-luc cells with 200 μl PBS were injected orthotopically in the right thoracic mammary fat pad of each female NCr/nu nude mouse (3). Tumor volume was calculated as: volume = (length × width^2 × π) / 6. When tumor size reached 7 mm in diameter, animals were randomized and injected with 100 μl saline (control) or a maximum tolerated dose (MTD) of doxorubicin (10 mg/kg) via tail vein.

ELISA 1×10^5 4T1ODD-luc cells per well of 12-well plate were cultured with 1 ml medium overnight. The cells were treated with 0, 0.1, 1, or 10 μg/ml of doxorubicin for 24, 48, and 72 hours. VEGF in culture medium was quantified by mouse VEGF ELISA kit (R & D Systems).

Real-time PCR Total RNA was prepared using the miRVana extraction kit (Applied Biosystems). 1 μg of total RNA was reverse transcribed into cDNA using the iScript cDNA synthesis kit (BioRad). Real-time PCR was performed on ABI7900HT Fast Real-Time PCR System using Power SYBRGreen PCR Mix (Applied Biosystems). PCR products were verified by melting curves. The threshold cycle (CT) values for each gene were normalized

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to expression levels of β-actin. The β-actin primers were: forward 5′-GATTACTGCTCTGGCTCCTAGC-3′; reverse 5′-GACTCATCGTACTCCTGCTTGC-3′. The mouse iNOS primers were: forward 5′-CTGTGAGACCTTTGATGTCCGAAG-3′; reverse 5′-CTGGATGAGCCTATATTGCTGTGG-3′.

Western Blots Protein samples from 4T1ODD-luc cells and MCF-7 cells were respectively collected on day 2 and day 3 post-treatment. Nuclear proteins were extracted using NucBuster kit (Novagen). Equal amounts of protein samples were loaded and detected with corresponding antibodies: monoclonal mouse anti-HIF-1α antibody (NB100-105, 1:500 dilution, Novus Biologicals), rabbit polyclonal anti-HIF-1α antibody (NB100-654, 1:500 dilution, Novus Biologicals), mouse anti-α-tubulin antibody (1:10000 dilution, Sigma-Aldrich), monoclonal mouse anti-β-actin antibody (A2228, 1:5000 dilution, Sigma-Aldrich), polyclonal rabbit anti-iNOS antibody (1:500 dilution, Assay Designs), polyclonal sheep anti-histone H1 antibody (NB100-748, 1:500 dilution, Novus), rabbit anti-STAT1 / phosphor-STAT1 (Tyr701) / phosphor-STAT1 (Ser727) antibodies (1:1000 dilution, Cell Signaling Technology), and monoclonal rabbit anti-JAK2 / phospho-JAK2 (Tyr1007/1008) antibodies (1:1000 dilution, Cell Signaling Technology).

Immunohistochemical Staining 5-6 orthotopic 4T1ODD-luc tumors per group were collected on days 0, 1, 4, 7 and 16. Hypoxia marker pimonidazole (NPI) and perfusion marker Hoechst33342 (Sigma-Aldrich) were administered as described (14). 10-μm frozen sections were made. HIF-1α fluorescent staining was performed with anti-HIF-1α antibody (Novus). Direct pimonidazole labeling was performed using Hypoxyprobe (NPI) with Zenon Alexa Fluor555 mouse IgG labeling kit (Invitrogen). Scanned images of each entire tumor section were composited by Metamorph to compare the areas of HIF-1α, pimonidazole, and Hoechst33342. Tumor vasculature was stained with fluorescein-labeled Griffonia simplicifolia lectin I (isolectin B4, Vector Laboratories) as described (21). 7-10 random fields (10×) per frozen section were analyzed to determine the mean value. Tumor vascular fraction is the percentage of vessel area in total tumor area in each field. Tumor activated macrophages (TAMs) were stained with rat anti-mouse CD68 antibody (Serotec) and Alexa488-conjugated anti-rat IgG antibody (Invitrogen).

Fluorescent-Activated Cell Sorting (FACS) Analysis Single cell suspensions were made after mild trypsinization at each time point and incubated with NO-specific probe DAF-FM diacetate following the manufacturer's instructions (Invitrogen). FACS was performed on a FACSCalibur flow cytometer (Becton Dickinson). The cells without DAF-FM probe and the cells exposed to 0.2 mM S-nitrosoglutathione (GSNO) for 4 hours were the negative and positive controls.

Cell Viability Assay Cell Titer-Glo luminescent cell viability assay (Promega): 3×10^3 4T1ODD-luc cells per well of 96-well plates were cultured with 100 μl medium overnight. We replaced the medium with a fresh one containing control vehicle (PBS), 10 μg/ml 1400W, or 15 μM EGCG, ± 1 μg/ml doxorubicin the next day. The treatments continued for 24, 48, and 72 hours. Daily cell viability was quantified and normalized to the value on Day 0. WST-1 cell viability assay (Roche Applied Science): cells were placed in a 96-well plate overnight and were treated with 1 μg/ml doxorubicin, L-NAME (0.1, 1.0, or 10mM), or a combination of these compounds the next day. At 24, 48, and 72 hours post-treatment, WST-1 reagent was added to the wells and cell viability was assessed by a spectrometer according to the manufacturer's instructions.

STAT1 Silencing

1×10^6 4T1 cells were plated in 10-cm dishes and cultured in 5 ml medium overnight. The cells were then transfected with either the STAT1 siRNA (ON-TARGETplus SMARTpool,

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mouse STAT1) or the scrambled siRNA control (ON-TARGETplus Non-targeting siRNA, Thermo Fisher Scientific) with Lipofectamine 2000 transfection reagent (Invitrogen). The cells were treated with 0.5 μg/ml doxorubicin or a vehicle control (PBS). Approximately 24 hours after transfection and drug treatment, the media was replaced with fresh media and drug. Cells were harvested 48 hours post-treatment for assessment of STAT1 and HIF-1 protein expression. These experiments were repeated 4 times.

Statistics Groups were first tested for normality and variance homogeneity. Student *t* test was applied for two-group comparison. One-way ANOVA Student-Newman-Keuls analysis was applied for pairwise multiple comparisons. Difference was considered significant when $P < 0.05$.

Results

Doxorubicin increases HIF-1α level in tumor cells both *in vitro* and *in vivo*

To determine whether doxorubicin affects HIF-1α level in normoxic tumor cells, we treated 4T1ODD-luc reporter cells with increased doxorubicin concentrations (0, 0.1, 1, or 10 μg/ml) for 24, 48, and 72 hours. All three concentrations of doxorubicin induced significant increases in HIF-1α reporter activity 48 hours post-treatment (Fig. 1A and B). 1 μg/ml of doxorubicin induced the most potent upregulation of HIF-1α reporter activity on both 48 hours and 72 hours post-treatment. To confirm doxorubicin-induced increase in normoxic HIF-1α accumulation, we detected HIF-1α protein expression by Western blots, which demonstrated enhanced HIF-1α expression in 4T1ODD-luc cells and MCF-7 cells post-treatment (Fig. 1C). This doxorubicin-induced normoxic HIF-1α accumulation was also verified by a hypoxia-induced HIF-1α control (Supplementary Fig. 1). These results reveal that doxorubicin upregulates HIF-1α expression in tumor cells under normoxic conditions *in vitro*.

To further determine whether doxorubicin may affect HIF-1α level in tumors, we intravenously injected MTD of doxorubicin into female nude mice with orthotopic 4T1ODD-luc tumors. Mean bioluminescence intensities in doxorubicin-treated tumors were significantly higher than controls. The major time window of the increase in HIF-1α reporter activity *in vivo* was from day 3 through day 5 after doxorubicin treatment (Fig. 1D and E). These findings suggest that doxorubicin chemotherapy upregulates HIF-1α level in tumor cells *in vivo*.

Because hypoxia and poor perfusion are common causes for HIF-1α upregulation in tumors, we next sought to identify whether the above doxorubicin-induced *in vivo* HIF-1α upregulation was due to potential changes in tumor hypoxia or perfusion after doxorubicin therapy. We compared the positive-area fractions of HIF-1α, hypoxic marker pimonidazole, and perfusion dye Hoechst 33342 in whole frozen sections of 4T1ODD-luc tumors at multiple time points post-treatment. Doxorubicin significantly increased tumor HIF-1α fraction on post-treatment days 1, 4, and 7 when compared to control treatment (Fig. 2A and B). The increased HIF-1α fractions in doxorubicin-treated tumors confirmed the enhanced HIF-1α reporter activities as described above (Fig. 1 D and E). In contrast, there was no difference in either pimonidazole fraction or perfused tumor fraction (Hoechst 33342 labeling) between the doxorubicin-treated tumors and the saline-treated tumors (Fig. 2C and Supplementary Fig. 2). These results suggest that doxorubicin-induced HIF-1α upregulation was not caused by aggravated tumor hypoxia or decreased perfusion.

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Doxorubicin-induced HIF-1α upregulation stimulates VEGF secretion by tumor cells *in vitro* and tumor angiogenesis *in vivo*

One important consequence of HIF-1α upregulation is to form HIF-1 to promote the expression of VEGF – one of the most potent angiogenic factors (1, 2). Because doxorubicin upregulates tumor cell HIF-1α both *in vitro* and *in vivo*, we asked whether this doxorubicin-induced HIF-1α may stimulate VEGF secretion by tumor cells and promote tumor angiogenesis. Cell counting demonstrated that the 0.1, 1, and 10 μg/ml doxorubicin treatments significantly reduced surviving cell numbers, which were less than 40% of the control cell numbers at all three time points post-treatment (Fig. 3A). However, despite a much smaller surviving cell number, the doxorubicin-treated cells secreted at least 87% of the amount of VEGF secreted by the control cells on day 1 (Fig. 3B). This pattern persisted on days 2 and 3 when, despite < 30% of relative surviving cell number post doxorubicin treatment compared to control treatment, the VEGF secreted by this small fraction of surviving cells was > 60% of the amount of VEGF secreted by the control cells. In other words, the surviving doxorubicin-treated cells secreted an increased amount of VEGF relative to control cells. The other piece of evidence was that 1 μg/ml doxorubicin did lead to higher VEGF secretion than 0.1 μg/ml doxorubicin on both days 1 and 2 (Fig. 3B). Therefore, these data support that doxorubicin stimulates VEGF secretion in surviving tumor cells. Because tumor vascular index rather than tumor size is the most reliable prognostic indicator for tumor relapse after chemotherapy, we then compared the relative tumor vascular fraction in tumors treated with doxorubicin *vs* saline to see whether the doxorubicin-induced increase in VEGF secretion by tumor cells may affect tumor angiogenesis. The relative tumor vasculature fraction in doxorubicin-treated tumors was significantly higher compared to the control tumors 4 days after a single MTD treatment (Fig. 3 C and D). The above findings suggest that doxorubicin not only upregulates HIF-1α expression and promotes VEGF secretion in surviving tumor cells, but also stimulates tumor angiogenesis shortly after treatment.

Nitric oxide (NO) and nitric oxide synthase (NOS) play important roles in doxorubicin-induced normoxic HIF-1α accumulation

Because the above results suggest that hypoxia is not the cause for doxorubicin-induced HIF-1α upregulation, we sought to identify other non-hypoxia factors that may upregulate HIF-1α. Kimura et al. reported that NO donors stimulated HIF-1α expression and VEGF reporter activity in normoxic tumor cells (22). In addition, Metzen et al. found that NO impaired HIF-1α degradation under normoxic conditions (23). Based on this converging evidence, we investigated whether doxorubicin could increase intracellular NO level in normoxic 4T1ODD-luc cells. The NO-specific fluorescent probe DAF-FM diacetate demonstrated that doxorubicin significantly increased the intracellular NO level compared to control treatment (Fig. 4A). Higher concentrations of doxorubicin led to higher intracellular NO level. The above HIF-1α bioluminescent reporter assay (Fig. 1A) and Western blots (Supplementary Fig. 3) also demonstrated that higher concentrations of doxorubicin led to more HIF-1α expression. The parallel changes in intracellular NO level and HIF-1α expression suggest that NO may play an important role in doxorubicin-induced normoxic HIF-1α accumulation. Nitric oxide synthases are enzymes that catalyze the synthesis of NO from L-arginine. The NOS family includes inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS). To identify whether NOS participated in the doxorubicin-induced normoxic HIF-1α accumulation, we treated 4T1ODD-luc cells with increasing concentrations of general NOS inhibitor L-NAME with or without doxorubicin (Fig. 4B). In the control group without doxorubicin, increasing concentrations of L-NAME did not cause any difference in HIF-1α reporter activity at each time point (Fig. 4B and C). Doxorubicin alone significantly increased HIF-1α reporter activity in the normoxic 4T1ODD-luc cells (Fig. 4B and D empty column). This finding is consistent with the results

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in Fig. 1A and B. When L-NAME was combined with doxorubicin, the doxorubicin-induced enhancement of HIF-1α reporter activity was significantly suppressed (Fig. 4D). We also performed a quantitative WST-1 cell viability assay to compare the surviving fraction of tumor cells at 24, 48, and 72 hours post the above treatments (Supplementary Fig. 4). Statistical analysis revealed that the combination of doxorubicin + L-NAME (0.1, 1, 10 mM) treatments did not increase cell death compared to the treatment with doxorubicin alone (Supplementary Fig. 4). This result rules out increased cell death as a possible cause of decreased HIF-1α reporter activity in the cells treated with doxorubicin + L-NAME (Fig. 4D). Therefore, the above experiments suggest that NOS and NO participate in doxorubicin-induced normoxic HIF-1α accumulation.

STAT1 and inducible nitric oxide synthase (iNOS) signaling pathway participates in doxorubicin-induced normoxic HIF-1α accumulation

iNOS is the isoenzyme most commonly associated with carcinogenesis and tumor progression. Because iNOS overexpression led to HIF-1α accumulation in kidney cells (24), we sought to determine the role of iNOS in the doxorubicin-induced normoxic HIF-1α accumulation in tumor cells. Quantitative real-time PCR demonstrated that doxorubicin significantly enhanced iNOS transcription compared to control treatment (Fig. 5A). Western blots further confirmed that doxorubicin stimulated iNOS protein expression under normoxic conditions (Fig. 5B). Because STAT1 is a transcription factor required for iNOS transcription and activation (25), we then sought to determine whether STAT1 is an upstream regulator enhancing iNOS expression in response to doxorubicin treatment. Western blots demonstrated that doxorubicin not only stimulated the expression of STAT1 but also promoted STAT1 activation through the phosphorylation of Tyr701 and Ser727 residues (Fig. 5C). JAK protein family member JAK2 regulates neoplasm, blood cell development, and immune function in response to growth factors and pro-inflammatory cytokines. As an upstream kinase for STAT phosphorylation, JAK2 needs to be activated first by phosphorylation. We performed a Western blot to determine whether JAK2 might be required for the above STAT1 phosphorylation. Because doxorubicin decreases total JAK2 expression and there is no JAK2 phosphorylation after doxorubicin or control treatment (Supplementary Fig. 5), JAK2 does not participate in the doxorubicin-stimulated STAT1 phosphorylation. The roles of other JAK family members or other non-JAK family kinases in this process merit further investigation. To confirm the importance of the STAT1-iNOS signaling pathway in doxorubicin-induced normoxic HIF-1α accumulation, we investigated whether disruption of iNOS or STAT1 would suppress doxorubicin-induced HIF-1α upregulation. To this end, 4T1ODD-luc cells were treated with either the iNOS-specific inhibitor 1400W or the STAT1-interfering chemical EGCG with or without combined doxorubicin treatment (26). Without doxorubicin, 1400W or EGCG only suppressed the basal-level iNOS expression compared to control treatment. When combined with doxorubicin, both 1400W and EGCG effectively suppressed doxorubicin-induced iNOS upregulation in 4T1ODD-luc cells (Fig. 5D, Supplementary Fig. 6 A and B). Western blots further confirmed the doxorubicin-induced upregulations of STAT1 expression and activation were suppressed by EGCG (Fig. 5E). These results suggest that STAT1-iNOS signaling pathway participates in doxorubicin-induced normoxic HIF-1α accumulation.

To determine whether inhibition of iNOS could suppress the doxorubicin-induced increase in intracellular NO synthesis, we treated 4T1ODD-luc and MCF-7 cells with doxorubicin in the presence or absence of 1400W. FACS analysis demonstrated that 1400W significantly suppressed the doxorubicin-induced upregulation of NO synthesis in both cell lines (Fig. 6A and B). To determine whether inhibition of STAT1 could suppress the doxorubicin-induced increase in intracellular NO level, we treated both cell lines with doxorubicin in the presence or absence of EGCG. FACS analysis demonstrated that EGCG also significantly suppressed

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the doxorubicin-induced upregulation of NO synthesis in both cell lines (Fig. 6C and D). Because inhibition of either iNOS or STAT1 can suppress doxorubicin-induced NO upregulation, we then sought to determine whether 1400W or EGCG could suppress doxorubicin-induced normoxic HIF-1α accumulation. Western blots demonstrated that 1400W and EGCG suppressed doxorubicin-induced HIF-1α upregulation in both cell lines under normoxic conditions (Fig. 6E and F). To verify the role of STAT1 in doxorubicin-induced HIF-1α upregulation, we knocked down STAT1 by siRNA. STAT1 siRNA decreased 51% of STAT1 expression and 42% of HIF-1α expression compared to the scrambled siRNA control after doxorubicin treatment (Fig. 6G and H). This finding is consistent with the above result after inhibition of STAT1 by EGCG and confirms that STAT1 is important for doxorubicin-induced HIF-1α upregulation. These results support the conclusion that doxorubicin induces normoxic HIF-1α accumulation by activating the STAT1-iNOS-NO-HIF-1α signaling pathway. Therefore, STAT1 and iNOS are rational targets to suppress the upregulated HIF-1α expression in doxorubicin-treated normoxic tumor cells. Because of the pleiotropic effects of STAT1 and iNOS in apoptosis (27, 28), we took additional caution to estimate the cytotoxicity of doxorubicin combined with EGCG or 1400W against tumor cells. Compared to the initial cell viability right before treatment, the relative cell viabilities of doxorubicin, doxorubicin + 1400W, or doxorubicin + EGCG treatment were individually 37.13 ± 1.60%, 36.54 ± 1.13%, and 87.62 ± 3.77% 48 hours post-treatment; and were 7.83 ± 0.47%, 7.83 ± 0.20%, and 29.77 ± 1.79% 72 hours post-treatment. Doxorubicin combined with EGCG or 1400W still maintained significant cytotoxicity against tumor cells compared to control treatment (Fig. 7A). While there was slightly higher cell viability after Dox + EGCG treatment, there was no attenuation in cytotoxicity after Dox + 1400W treatment compared to Dox treatment.

Discussion

There has been intense interest in developing novel therapeutic strategies to target HIF-1α in cancer therapy for three main reasons: 1) HIF-1α expression has been found in the majority of tumors because of hypoxia, which is usually absent in normal tissues (29). This differential expression of HIF-1α between malignant and normal tissues allows inhibition of HIF-1α to target cancer cells while sparing normal tissues. 2) Because HIF-1 is a master regulator for many aspects in cancer biology, inhibition of HIF-1α leads to the disruption of multiple important mechanisms for tumor cell survival, angiogenesis, and progression. 3) Inhibition of HIF-1α may exploit tumor hypoxia by converting it from a treatment obstacle into a targeting advantage (30). Recent studies have demonstrated that many anti-cancer therapies such as radiotherapy, photodynamic therapy, and hyperthermia can upregulate HIF-1α expression in tumor cells (3, 9, 31, 32). Although these findings improved the understanding of HIF-1α stabilization induced by other factors rather than hypoxia, very little is known about the effects of chemotherapeutic agents on HIF-1α expression in normoxic cancer cells. Yet it is important to address this question, not only because HIF-1α is a major determinant for cancer cell homeostasis under cytotoxic stress (1, 13), but also because normoxic HIF-1α accumulation is sufficient to initiate tumor angiogenesis and to promote cancer cell chemoresistance (14). The majority of chemotherapeutic drugs exert most of their cytotoxic effects on normoxic cells rather than hypoxic cells. The reduced cytotoxicity against hypoxic tumor cells is because of multiple mechanisms including limited penetration distance of drugs in hypoxic tumor regions (33), a low proliferative fraction in hypoxic tumor cell populations, and stimulated detoxification/chemoresistance machinery (30). Therefore, it is generally believed that normoxic tumor cells are more sensitive to chemotherapy than hypoxic tumor cells. However, this paradigm is true only when normoxic tumor cells are exposed to enough high drug concentration to kill them before they activate homeostatic, survival, and anti-apoptotic signaling pathways. Without locally enough high drug concentration, even a normoxic tumor microenvironment does not

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guarantee that all tumor cells are sensitive to chemotherapy especially when treated tumor cells activate survival molecules such as HIF-1. To determine the effects of doxorubicin on normoxic HIF-1α accumulation, we selected several doxorubicin concentrations in the ranges of *in vivo* drug concentrations in 4T1 and MCF-7 tumors (18-20). This study reveals that surviving normoxic tumor cells are able to accumulate HIF-1α which promotes VEGF secretion after doxorubicin treatment. Cell viability assay showed that 37.13% and 7.83% of doxorubicin-treated cells survived 48 hours and 72 hours respectively after doxorubicin treatment (Fig. 7A). Many studies have proven that even less 5% of the entire cancer cell population, when survives, is enough to result in cancer relapse. Because HIF-1 is a key transcriptional factor regulating hundreds of downstream genes, identification of key molecular mechanisms for normoxic HIF-1α accumulation in tumor cells during chemotherapy may provide important insights into how tumor cells respond to chemotherapy, gain survival advantage, and develop therapeutic resistance causing tumor relapse. This study takes a major step toward addressing this very important issue. The significance of doxorubicin-induced normoxic HIF-1α accumulation in tumor cells and the underlying mechanism are explicated below.

First, we found that the STAT1-iNOS-NO-HIF-1α signaling pathway is important for doxorubicin-induced normoxic HIF-1α accumulation (Fig. 7B). STAT1 is a transcription activator for iNOS expression and NO synthesis (25). Previous studies have shown that NO enhances the expression and activity of HIF-1α under normoxic conditions through three mechanisms: 1) NO inhibits prolyl hydroxylase activity to stabilize HIF-1α (23, 34); 2) NO S-nitrosylates the Cys533 in HIF-1α ODD domain to prevents HIF-1α degradation (9); and 3) NO inhibits FIH enzyme activity to abolish the FIH-mediated asparagine hydroxylation of HIF-1α and activates the transcription of HIF-1 downstream genes (35). The time-course of *in vivo* HIF-1α upregulation in 4T1ODD-luc tumors is 3-5 days after doxorubicin treatment, which overlaps the time of HIF-1α upregulation induced by radiotherapy (4-7 days post-radiotherapy) (9). Li et al found that ionizing radiation stimulated tumor associated macrophages (TAMs) to synthesize NO. The exogenous NO produced by TAMs then diffused into 4T1ODD-luc reporter cells and S-nitrosylated Cys533 in HIF-1α ODD domain to stabilize HIF-1α under normoxic conditions (9). The use of the same 4T1ODD-luc reporter cell line allowed us to compare the NO-producing cells between doxorubicin chemotherapy and radiotherapy. The absence of TAMs in our *in vitro* study (Fig. 1 and Fig. 4-6) and the non-significant difference in activated TAM fractions (TAM-marker CD68 staining) between the doxorubicin-treated tumors and the control tumors in our *in vivo* study (Supplementary Fig. 7) strongly suggest that the NO synthesized in doxorubicin-treated tumor cells is sufficient to elicit normoxic HIF-1α accumulation independent of other exogenous NO-producing cells such as TAMs. This work also demonstrates that doxorubicin dosages have different effects on tumor HIF-1 activity. Lee et al elegantly reported that low-dose metronomic doxorubicin therapy (1 mg/kg/day for 5 days) inhibited HIF-1 transcriptional activity in tumor xenografts (15). The dose of doxorubicin in their metronomic therapy was only 1/10 of the single bolus MTD of doxorubicin (10 mg/kg) in this study. Low-dose metronomic doxorubicin therapy inhibits HIF-1 activity, whereas treatment with doxorubicin at maximum tolerated dose induces HIF-1. Therefore, doxorubicin may play multiple roles to regulate HIF-1 expression and activity depending on dosing, timing, and oxygen tensions. In addition to the above mechanism, previous studies suggest that other important mechanisms may also contribute to doxorubicin-induced normoxic HIF-1α accumulation: 1) the degradation of HIF-1α depends on HIF-PH, which requires both Fe^{2+} and molecular oxygen (7, 8). When Fe^{2+} is removed by chelating agents such as desferrioxamine (DFX) or substituted by Co^{2+} or Ni^{2+}, HIF-1α degradation is inhibited. Therefore, DFX and $CoCl_2$ are hypoxia mimetics. Because doxorubicin is a strong iron chelator (36), iron deficiency inhibits HIF-PH activity causing normoxic HIF-1α accumulation. 2) Sinha et al. elegantly demonstrated that iron-mediated electron transfer

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from doxorubicin to molecular oxygen generated free radicals/reactive oxygen species (ROS) (37). Many studies have proven that free radical species and ROS upregulate HIF-1α level (3, 32). We previously provided the first direct *in vivo* evidence showing that free radicals, ROS, and/or reactive nitrogen species upregulate HIF-1α in tumors (3). Therefore, doxorubicin may induce normoxic HIF-1α accumulation through the generation of free radicals and ROS. The regulatory mechanisms of doxorubicin on signaling pathways, cell toxicity, and metabolism are complex because of its multiple effects on diversified molecular targets including iron chelation, generation of free radicals/ROS, DNA binding, alkylation, and inhibition of topoisomerase II (38). Our data add important new content to this body of knowledge – not only is the biology relevant to tumors and to therapeutics, but also exploitation of these findings may hold promise for other clinical benefits such as reduction of cytotoxicity to normal tissues.

Second, this work unveils hypoxia-independent VEGF secretion and tumor angiogenesis induced by doxorubicin. Hypoxia is one important factor for HIF-1 activation, VEGF expression and angiogenesis (1, 30, 39). Other non-hypoxia factors such as radiation therapy and hyperthermia can also upregulate HIF-1α and VEGF (3, 9, 32). VEGF stimulates tumor growth by autocrine and paracrine mechanisms (40, 41) because VEGF is not only a potent proangiogenic factor for tumor endothelial cells but also a key survival factor for tumor cells including 4T1 and MCF-7 cells (40, 42). Here we demonstrate that doxorubicin enhances VEGF secretion in surviving normoxic tumor cells and stimulates post-treatment tumor angiogenesis by activating the STAT1-iNOS-NO-HIF-1α-VEGF signaling pathway (Fig. 7B), which is different from those mechanisms underlying HIF-1α upregulation induced by radiotherapy or hyperthermia (9, 32, 43). These new findings also suggest the importance of combining conventional chemotherapy such as doxorubicin with anti-VEGF/antiangiogenic therapy to inhibit tumor VEGF secretion and angiogenesis not only caused by hypoxia, but also induced by doxorubicin chemotherapy.

Third, our data indicate that the p53 tumor-suppressor gene may not be required in doxorubicin-induced activation of the STAT1-iNOS-NO-HIF-1α signaling pathway. P53 mutation is the most common genetic lesion in human cancers. In a previous clinical study, Yamaguchi et al. demonstrated that iNOS expression was significantly correlated with tumor progression by stimulating angiogenesis and there was no correlation between iNOS and p53 expression (44). The 4T1ODD-luc cell line is p53 null whereas MCF-7 expresses wild-type p53 (45, 46). The HIF-1α upregulation in both p53-null and p53 wild-type tumor cell lines indicates that P53 tumor suppressor might not be necessary for doxorubicin-induced normoxic HIF-1α accumulation (Fig. 1C). In addition, the successful suppression of HIF-1α upregulation by EGCG or 1400W in both cell lines suggests that the STAT1- or iNOS-targeting strategy does not depend on wild-type p53 expression (Fig. 6E and F). Therefore, doxorubicin chemotherapy combined with STAT1 or iNOS inhibitors might be used to treat both p53 wild-type and p53-mutant tumors.

Finally, our findings raise thought-provoking questions about chemoresistance. STAT1 is associated with acquired resistance to doxorubicin (47). However, the molecular mechanism of STAT1-related therapeutic resistance has not been well understood. Unveiling the activation of the STAT1-iNOS-NO signaling pathway in doxorubicin-induced normoxic HIF-1α accumulation may provide new insight into the roles of STAT1 in chemoresistance. Muerkoster et al. found that pancreatic cancer cells acquired chemoresistance when treated with low-dose chemotherapeutic drug etoposide. Etoposide stimulates intracellular synthesis of NO, which inactivates caspases causing chemoresistance (48). Inhibition of iNOS by 1400W rescues caspase activity and enhances tumor cell chemo-sensitivity (48). Therefore, iNOS is a key determinant for acquired chemoresistance. Here we discovered that doxorubicin also upregulates iNOS expression and intracellular NO levels (Fig. 5 and 6).

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Together with Muerkoster's findings, it appears that different types of tumor cells may share this iNOS-NO pathway in response to chemotherapeutic drugs such as doxorubicin and etoposide. Because both doxorubicin and etoposide inhibit topoisomerase II, it is worthwhile to further investigate whether this pathway is a common tumor response to topoisomerase-targeted chemotherapeutic drugs. We also found that 1400W suppressed the expression and activation of STAT1 (Fig. 5E). One potential possibility might be that 1400W inhibited iNOS and lowered intracellular NO levels, thereby restoring caspase activity in a similar manner found in Muerkoster's study (48). The restored caspase activity and apoptosis after 1400W treatment might suppress the expression and activation of STAT1. The other potential possibility is based on the finding that NO stimulates STAT1 activation (49). Therefore, iNOS-specific inhibitor 1400W might attenuate STAT1 activation by suppressing intracellular NO synthesis. It would be intriguing to distinguish and verify the above two hypotheses in future.

The mechanistic elucidation of doxorubicin-induced normoxic HIF-1α accumulation significantly advances understanding of tumor response to cytotoxic drugs, which may help to optimize conventional chemotherapy by the use of HIF-1 inhibitors. This therapeutic strategy is supported by a recent study of Zhang et al showing that combined therapy with HIF-1 inhibitor and doxorubicin achieves significantly improved tumor control (50). With regard to clinical significance, this study suggests that combination chemotherapy with HIF-1α inhibition and doxorubicin would target not only hypoxic tumor cells but also the heretofore overlooked normoxic tumor cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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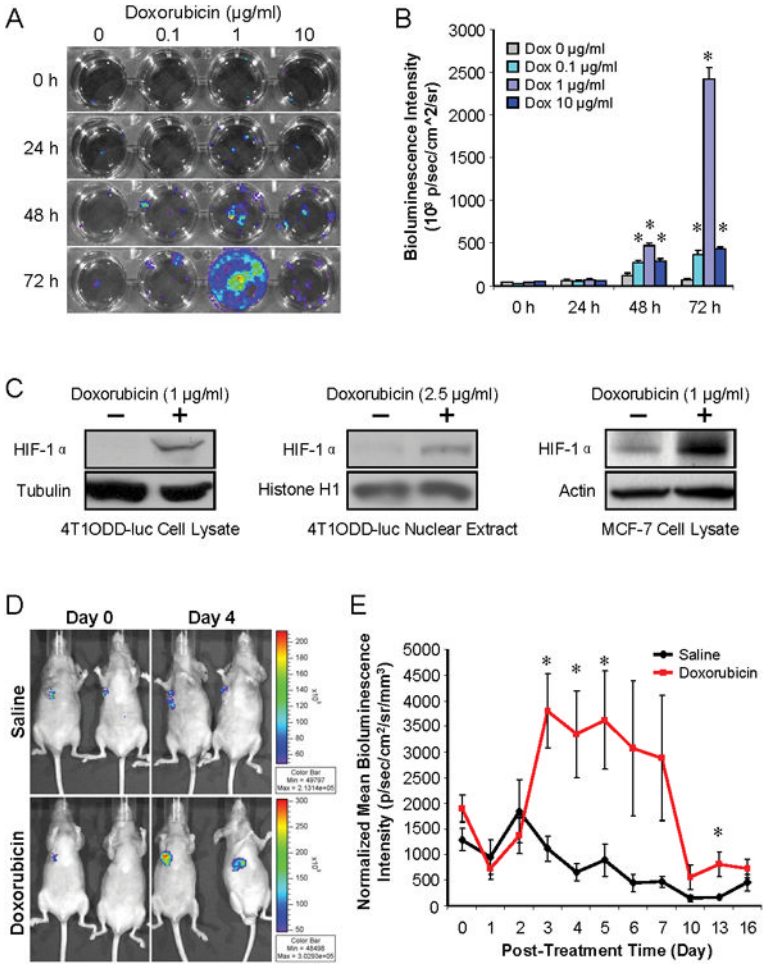

Figure 1.

Doxorubicin upregulates HIF-1α. **A,** HIF-1α reporter activity in normoxic 4T1ODD-luc cells. **B,** quantification of HIF-1α reporter activity in 4T1ODD-luc cells post-treatment (n = 3, mean ± SE). *, $P < 0.05$ compared to control treatment, one-way ANOVA. **C,** Western blots: HIF-1α expression in normoxic 4T1ODD-luc and MCF-7 cells 48 hours post-treatment. Histone H1, loading control for nuclear extracts. **D,** HIF-1α reporter activity in orthotopic 4T1ODD-luc tumors 4 days post-treatment. **E,** quantification of HIF-1α reporter activity in orthotopic 4T1ODD-luc tumors (n = 7, mean ± SE). Doxorubicin (Dox) treatments were as indicated 0.1-10 μg/mL (0.58-5.8 μM). *, $P < 0.01$, Student *t* test.

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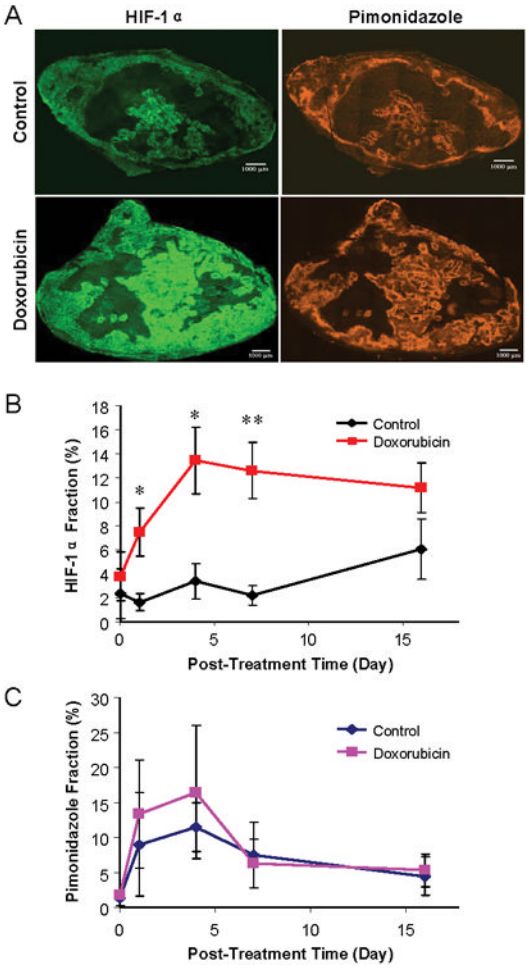

Figure 2.

The upregulated HIF-1α expression in doxorubicin-treated 4T1ODD-luc tumors was not due to hypoxia. **A,** representative immunofluorescent stainings demonstrating the distribution of HIF-1α (green) and pimonidazole (red) in two adjacent entire tumor sections 5 days post-treatment. Scale bars, 1 mm. **B,** HIF-1α fraction in 4T1ODD-luc tumors on days 0, 1, 4, 7, and 16 post-treatment (n = 5, mean ± SE). *, $P < 0.05$, **, $P < 0.01$, Student *t* test. **C,** pimonidazole fraction in 4T1ODD-luc tumors on days 0, 1, 4, 7, and 16 post-treatment (n = 5, mean ± SE). $P > 0.05$, Student *t* test.

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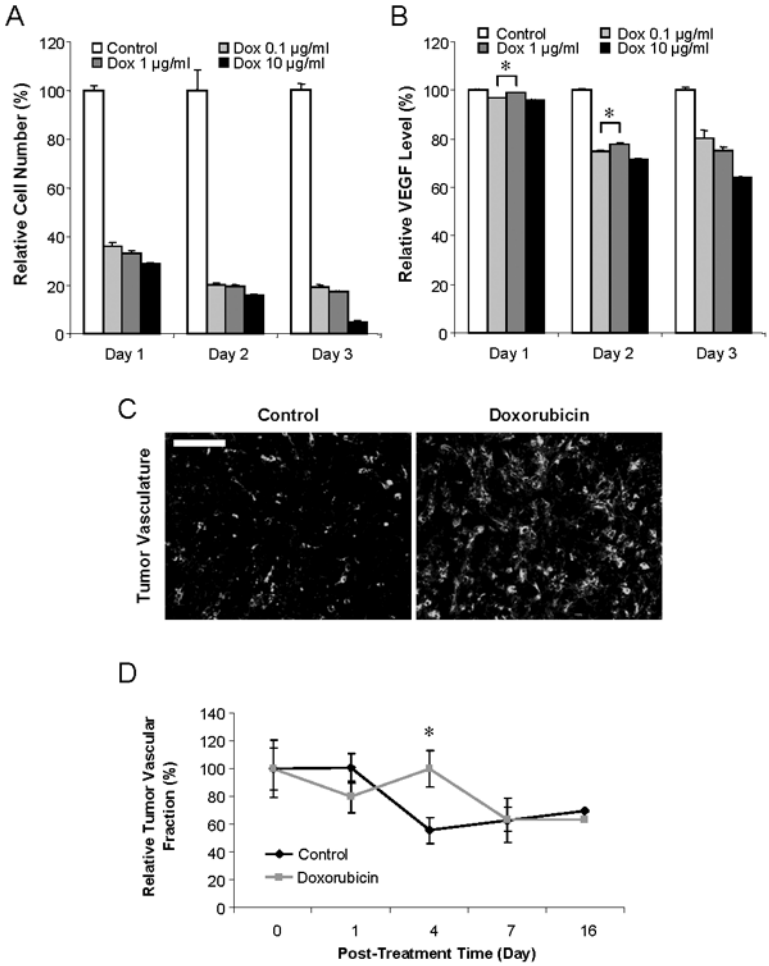

Figure 3.

Doxorubicin stimulates VEGF secretion by 4T1ODD-luc cells and causes a resurgent tumor angiogenesis. **A,** Relative viable cell numbers of all doxorubicin-treated groups were < 40% compared to the control (n = 3, mean ± SE). $P < 0.05$ compared to the control group, one-way ANOVA. **B,** mouse VEGF ELISA showing VEGF concentrations in the cell culture medium of 4T1ODD-luc cells treated with doxorubicin (0.1, 1, and 10 μg/ml) *vs* control treatment (n = 3, mean ± SE). *, $P < 0.005$, one-way ANOVA. **C,** representative fluorescent staining of vasculature in 4T1 ODD-luc tumors 4 days post-treatment. Scale bar, 100 μm. **D,** relative tumor vascular fractions in 4T1ODD-luc tumors (n = 5 in doxorubicin groups on days 0, 4, 7, and 16, n = 6 in all other groups, mean ± SE). *, $P < 0.05$, Student *t* test.

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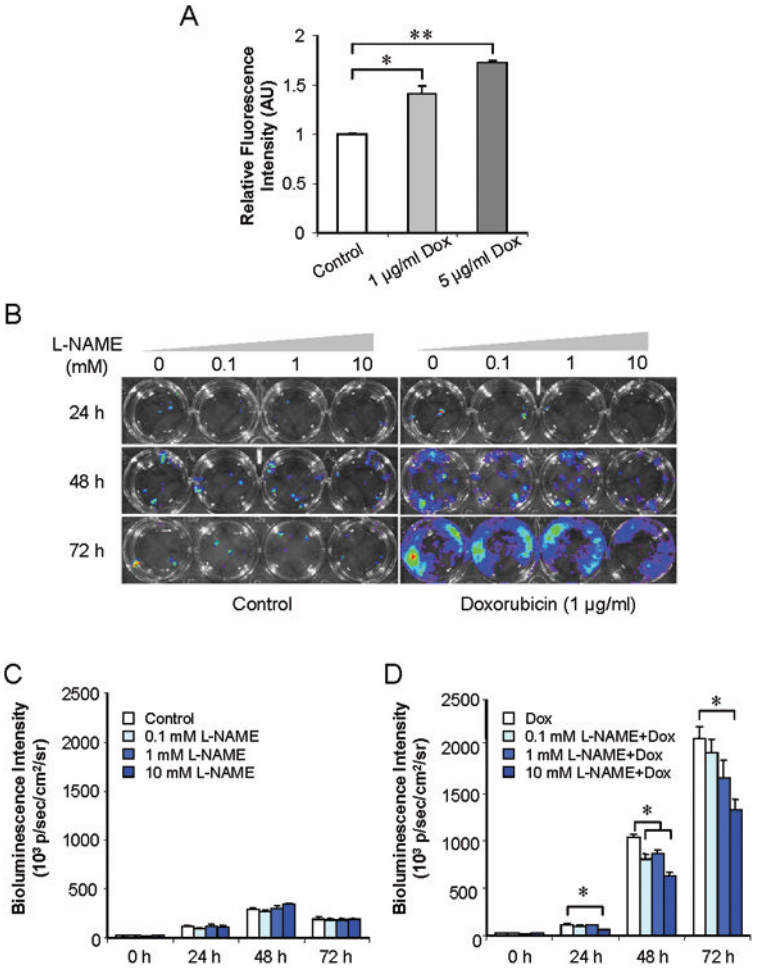

Figure 4.

Doxorubicin increased intracellular NO synthesis in 4T1ODD-luc cells. **A**, FACS analysis: relative fluorescence intensity of NO-specific probe DAF-FM in 4T1ODD-luc cells 48 hours post-treatment (n = 3, mean ± SE). *, $P < 0.05$; **, $P < 0.001$, one-way ANOVA. Dox, doxorubicin. AU, arbitrary unit. **B,** representative bioluminescent images of 4T1ODD-luc cells treated with L-NAME ± Doxorubicin. **C,** HIF-1α reporter activity in 4T1ODD-luc cells treated with L-NAME alone (n = 3, mean ± SE). $P > 0.05$, one-way ANOVA. **D,** HIF-1α reporter activity in 4T1ODD-luc cells treated with Dox ± L-NAME (n = 3, mean ± SE). *, $P < 0.05$, one-way ANOVA. Dox, doxorubicin.

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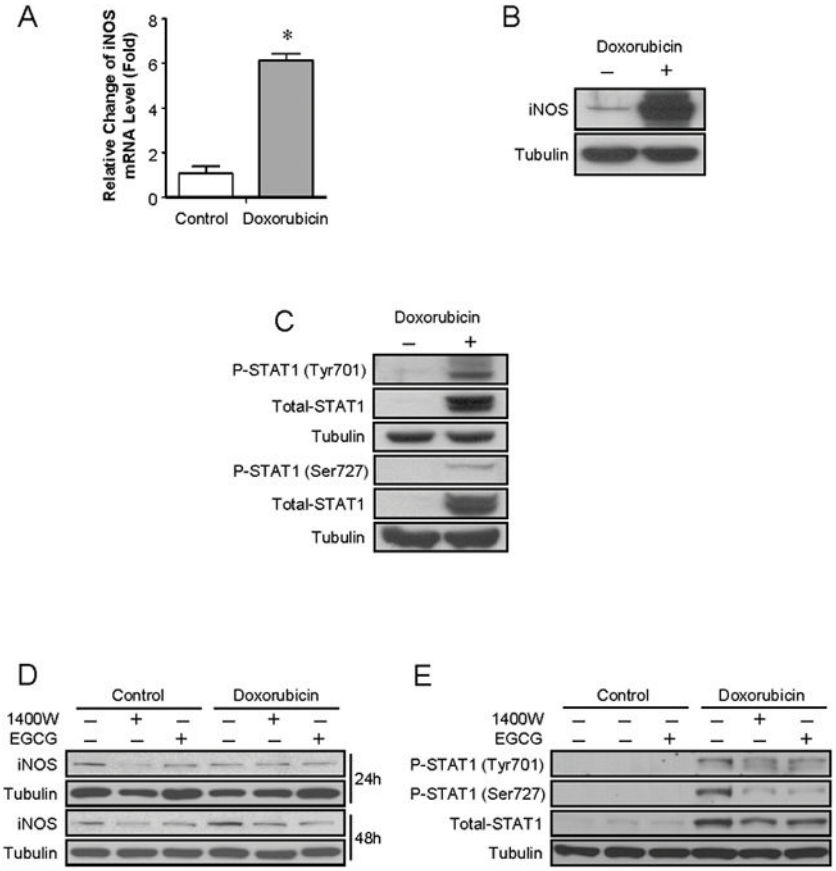

Figure 5.

Doxorubicin upregulates and activates the STAT1-iNOS signaling pathway. **A,** quantitative real-time PCR: iNOS mRNA levels in 4T1ODD-luc cells 2 days post-treatment (n = 3, mean ± SE). *, $P < 0.05$, Student *t* test. **B,** Western blot: iNOS expression in 4T1ODD-luc cells treated with or without doxorubicin. **C,** Western blot: phosphorylated STAT1 (Tyr701 and Ser727) and total STAT1 in 4T1ODD-luc cells 48 hours post-treatment. **D,** Western blot: iNOS expression in 4T1ODD-luc cells treated with or without doxorubicin, 1400W, or EGCG. **E,** Western blot: the expressions of phosphorylated STAT1 (Tyr701 and Ser727) and total STAT1 in 4T1ODD-luc cells 48 hours after treatments with or without doxorubicin, 1400W, or EGCG.

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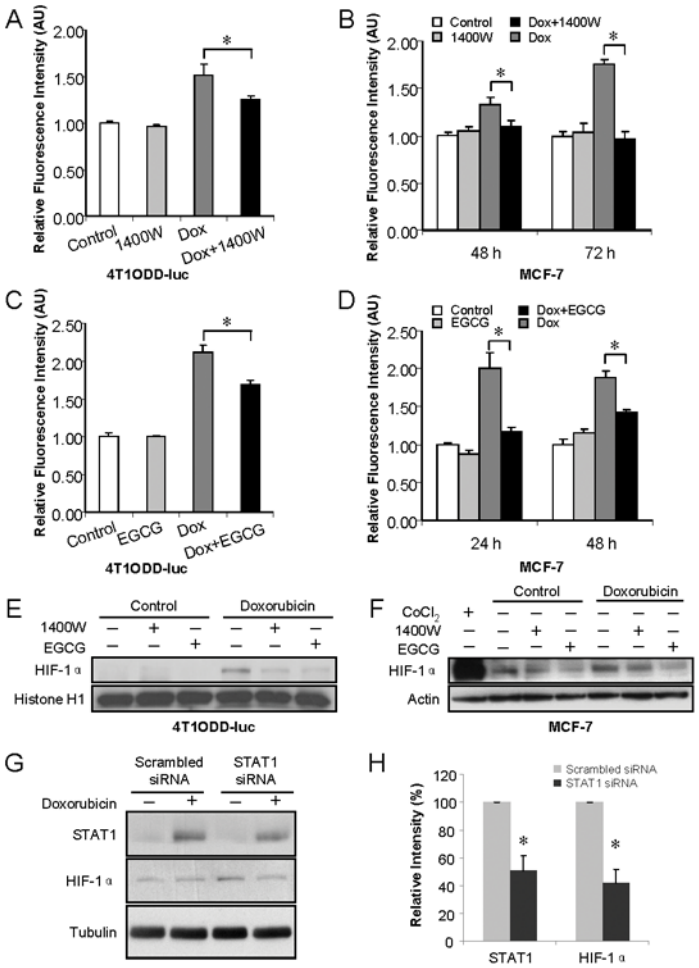

Figure 6.

The iNOS-specific inhibitor 1400W and the STAT1-interfering chemical EGCG suppressed the increased intracellular NO level induced by doxorubicin. Dox, doxorubicin. **A - D,** FACS analysis comparing intracellular NO marker DAF-FM between treated groups and the control group (n = 3, mean ± SE). *, $P < 0.05$, one-way ANOVA. A and C, 72 hours post-treatment. **E,** Western blot of nuclear extract: 1400W and EGCG suppressed the doxorubicin-induced normoxic HIF-1α accumulation in 4T1ODD-luc cells. **F,** Western blot of cell lysate: 1400W and EGCG suppressed the doxorubicin-induced normoxic HIF-1α accumulation in MCF-7 cells. $CoCl_2$, positive control for HIF-1α. **G,** Western blot: knockdown of STAT1 by siRNA decreased the doxorubicin-induced normoxic HIF-1α accumulation in 4T1ODD-luc cells. **H,** quantification of STAT1 and HIF-1α protein expression after knockdown of STAT1 by siRNA in doxorubicin-treated 4T1ODD-luc cells (n = 4, mean ± SE). *, $P < 0.05$ compared to the scrambled siRNA control, Student *t* test.

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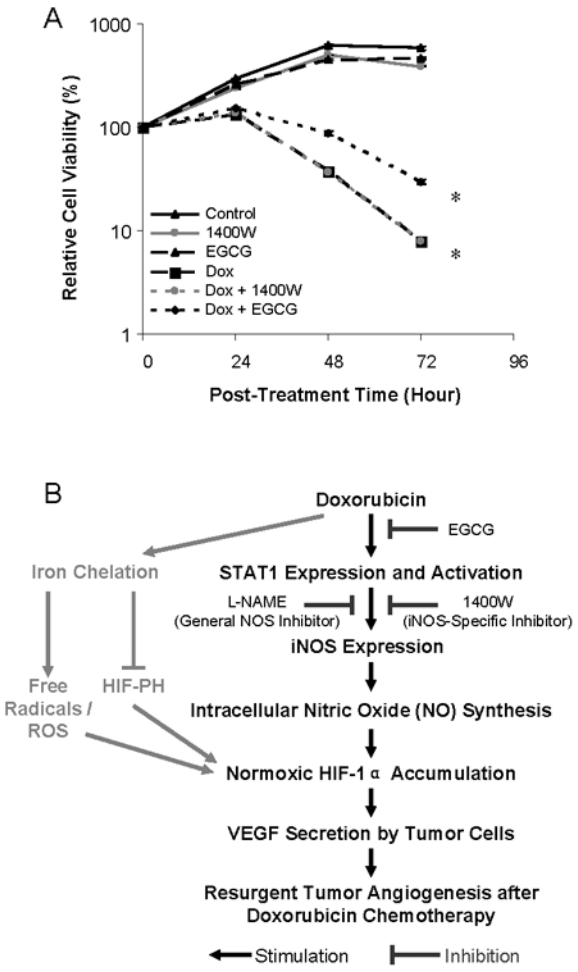

Figure 7.

A, cell viability assay: 4T1ODD-luc cell viability post-treatment (n = 5, mean ± SE). *, $P < 0.05$ compared to the control treatment, one-way ANOVA. Dox, doxorubicin. No difference between Dox and Dox + 1400W treatment at all four time points. **B,** schematic diagram of the mechanisms underlying doxorubicin-induced normoxic HIF-1α accumulation, angiogenesis, and interference strategies. Gray color: other mechanisms suggested by previous studies.