Anthryacycline Inhibits Recruitment of Hypoxia-inducible Transcription Factors and Suppresses Tumor Cell Migration and Cardiac Angiogenic Response in the Host*

Received for publication, April 21, 2012, and in revised form, August 20, 2012 Published, JBC Papers in Press, August 20, 2012 DOI 10.1074/jbc.M112.374587

Tetsuhiro Tanaka,† Junna Yamaguchi,§ Kumi Shoji, and Masaomi Nangaku*

From the ‡Division for Health Service Promotion, University of Tokyo and §Division of Nephrology and Endocrinology, University of Tokyo School of Medicine, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan

Background: Anthracycline is an antitumor agent of the topoisomerase inhibitor family. Doxorubicin inhibits the expression of hypoxia-inducible genes, suppresses HIF-dependent migration of target tumors, and dampens angiogenic response of the host heart. Doxorubicin blocks recruitment of HIF heterodimers to the enhancer and inhibits hypoxia response. The pleiotropic effect of doxorubicin on HIF signaling provides a clue for understanding efficacy and toxicity of cancer chemotherapy.

**Results:** Doxorubicin inhibits the expression of hypoxia-inducible genes, suppresses HIF-dependent migration of target tumors, and dampens angiogenic response of the host heart. Doxorubicin blocks recruitment of HIF heterodimers to the enhancer and inhibits hypoxia response. The pleiotropic effect of doxorubicin on HIF signaling provides a clue for understanding efficacy and toxicity of cancer chemotherapy.

**Conclusion:** Doxorubicin blocks recruitment of HIF heterodimers to the enhancer and inhibits hypoxia response. The pleiotropic effect of doxorubicin on HIF signaling provides a clue for understanding efficacy and toxicity of cancer chemotherapy.

**Significance:** The pleiotropic effect of doxorubicin on HIF signaling provides a clue for understanding efficacy and toxicity of cancer chemotherapy.

Solid tumors are characterized by continuous proliferation and unlimited expansion, which is associated with an increase in metabolic demand. The adaptive cellular responses include facilitation of glycolysis and a release of angiogenic factors. Despite this, insufficient ATP production and unmet oxygen and nutrient supplies by the vasculature almost always follow, and the tumor environment becomes hypoxic.

Hypoxia influences the efficacy of tumor chemotherapy and radiotherapy in various ways. It causes cells to cycle more slowly and selects cells with reduced susceptibility to apoptosis. Tumor hypoxia predicts radiation response (1) and adversely affects prognosis in head and neck tumors (2) and in advanced cancers of the uterine cervix (3). Likewise, the expression of hypoxia-inducible factor (HIF)3–1α also predicts prognosis in patients undergoing curative radiation therapy for squamous cell cancer of the oropharynx (4). This can be predicted by the principal function of HIF-1 to allow cells to adapt to the hypoxic environment. However, it remains undetermined how the expression and the functional operation of HIF could be influenced by environmental factors in patients undergoing chemotherapeutic and radiotherapy.

Anthryacyclines such as doxorubicin (DXR) and daunorubicin (DNR) are chemotherapeutic agents of the topoisomerase inhibitor family and are widely used for the treatment of cancers of the bladder, breast, stomach, lung, and ovaries, as well as malignancies of hematopoietic organs. They interact with DNA by intercalation, inhibit DNA polymerase, RNA polymerase, and topoisomerase II reactions, and suppress DNA and RNA biosynthesis, thus exhibiting antitumor action. It is important to note that the targets of cancer chemotherapy are usually hypoxic for the aforementioned reasons.

HIF is a heterodimeric transcription factor belonging to the basic helix-loop-helix-per-arnt-sim family (5). It is composed of an oxygen-labile α subunit and a constitutively expressed β subunit (also referred to as aryl hydrocarbon receptor nuclear translocator, ARNT). The expression of HIF-1α and HIF-2α, the two major isoforms of HIF-α, is mainly determined by oxy-

*This work was supported by Grants-in-aid for Scientific Research 24390213 (to M. N.) and 22790781 (to T. T.) from the Japan Society for the Promotion of Science and a research grant from the Takeda Science Foundation (to T. T.).

†To whom correspondence may be addressed. Tel: 81-3-3815-5411 (Ext. 33128); Fax: 81-3-5800-8806; E-mail: tetsu-tky@umin.ac.jp.

§To whom correspondence may be addressed. Tel: 81-3-3815-5411 (Ext. 33128); Fax: 81-3-5800-8806; E-mail: mnangaku-tky@umin.ac.jp.

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gen-dependent hydroxylation of the conserved proline residues, followed by recruitment of the von Hippel-Lindau (VHL) tumor suppressor protein and proteasomal degradation. Lack of molecular oxygen prevents these degradation processes, and the HIF-α protein accumulates, translocates to the nucleus, forms a heterodimer with ARNT, and transactivates its target genes. To date, ~100-200 HIF target genes have been identified, and they control a variety of cellular hypoxia responses such as anaerobic metabolic switch, angiogenesis, and erythropoiesis.

In this study, we investigated the connection between cancer chemotherapy and the cellular hypoxia response. In particular, we report that DXR impairs the cellular hypoxia response mediated by HIF, and we present the molecular mechanisms that inactivate HIF. Furthermore, we show possible implications in the migration of tumor cells and the hypoxic adaptation of tumors and the tumor-bearing host.

EXPERIMENTAL PROCEDURES

Cell Culture—HK-2, a human proximal tubular cell line, was cultured in DMEM/F-12 supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin. HeLa cervical cancer cells, A498, KMRC-3 VHL-defective renal cell carcinoma (RCC) cells, and Caki-1 VHL-competent RCC cells were maintained in DMEM containing 10% FCS. KMRC-3 and Caki-1 cells were purchased from the Health Science Research Resources Bank (Osaka, Japan). Chemical hypoxia was induced with an iron chelator, 2,3-dipyridyl. Hypoxic stimulation was accomplished by exposing cells to 1% oxygen in a multigas incubator, APM-30D (ASTEC, Fukuoka, Japan).

Transient Transfection and the Reporter Assay—Transcriptional activity of HIF-1 was measured by luciferase reporter assays. The pGL3 (Promega, Madison, WI) vector driven by 7X hypoxia-responsive elements (HREluc) (6) was transiently transfected using polyethyleneimine, and hypoxic induction was measured using the luciferase assay system (Promega) and a Lumat 9507 luminometer (EG and Berthold, Bad Wildbad, Germany). A Renilla luciferase vector (pRL-CMV) was simultaneously transfected to correct for transfection efficiency. For the measurement of transcription of an authentic HIF target gene, a minimal promoter of the carbonic anhydrase 9 (CA9) gene was synthesized (sense 5'-GATCTCTCCCTCCACCCGCTTGTTCCTCAATGACGTACAGCGCGAGAAGAATTT; and reverse 5'-gtctgacgctgtatctgctatgctcgccctgagcttagac). cDNAs for hLOX and hLOXL2 were then subcloned into the BglII-HindIII site of the pGL3-basic vector. Expression plasmids coding for normoxia-stable, constitutively active mouse HIF-1α and HIF-2α were constructed by PCR-based mutagenesis at two proline and one asparagine residues (P402A/P577A/N813A of HIF-1α; P405A/P530A/N851A of HIF-2α), as described previously (7).

Real Time PCR—RNA was isolated using RNeasy Plus (Takara, Shiga, Japan) and reverse-transcribed with the ImProm-II reverse transcription system (Promega). One-fifth (v/v) of the synthesized cDNA was used as a template for PCR quantification. PCR was performed on an iCycler (Bio-Rad) with THUNDERBIRD SYBR qPCR mix (Toyobo, Osaka, Japan). Data were analyzed using the 2^(-ΔΔCt) method. Relative expression levels were calculated using β-actin mRNA as reference. Primers for quantification are listed in Table 1.

Immunoblotting—Cells were lysed in extraction buffer (7 M urea, 10% glycerol, 10 mM Tris-HCl, pH 6.8, 1% SDS, 5 mM dithiothreitol) supplemented with Complete Protease Inhibitor Mixture (Roche Applied Science), and aliquots were resolved by SDS-PAGE, transferred onto PVDF membranes, and probed for proteins of interest. The primary antibodies used were as follows: anti-HIF-1α (Novus Biologicals, CO); anti-ARNT (Abcam, MA); anti-BNIP3 (Santa Cruz Biotechnology); anti-CA9 (Santa Cruz Biotechnology); anti-LOX (Abnova, Taipei City, Taiwan); anti-LOXL2 (GeneTex), and anti-β-actin (Sigma). Following incubation with HRP-conjugated secondary antibodies, signals were detected with the ECL Plus reagent (Thermo Scientific, IL).

Chromatin Immunoprecipitation—Binding of HIF-1α to the target gene promoter was assessed by chromatin immunoprecipitation (ChIP) assay. Cells were fixed with 1% formaldehyde, lysed with 1% SDS, and sonicated. Fragmented DNA in complex with HIF-1α was immunoprecipitated with an anti-HIF-1α rabbit polyclonal antibody (Novus Biologicals) and protein-Sepharose beads (GE Healthcare). Precipitated DNA was used as a template for PCR, with the following primers: hVEGF promoter forward 5’-GGACGATCTCAAGGTGTTCTCAATA and reverse 5’-GAGCAGGAGGAGAAGATT; hCA9 promoter forward 5’-CCACGCTCTGGTTCAAT and reverse 5’-AGCACGATGTGCACATGAG.

Migration Assay—Tumor cell migration was evaluated with the scratch assay as described previously (8). In brief, 0.5–1.0 × 10^6 cells were seeded onto coated 60-mm dishes and incubated for 6 h. Then the monolayer was scratched using a P200 pipette tip, and the cells were allowed to migrate for the indicated time. Distances between the edges of the scratch were measured to quantitatively evaluate cell migration.

Retrovirus Transduction—A498 clones stably overexpressing LOX and LOXL2 were generated by retrovirus transduction (Platinum Retrovirus Expression System, Pansendia. Cell Biolabs, CA). cDNAs for human (h)LOX and hLOXL2 were PCR-amplified using following primers: hLOX forward, 5’-aggccagctggttttcaatctt; and reverse 5’-ttgacgtaacccggtgctgtgg; hLOXL2 forward, 5’-ggctggctgctgctgctgctg; and reverse, 5’-gtcttctgctgctgctgctgctg. cDNAs for hLOX and hLOXL2 were then subcloned to pBLeuScript II SK(+) (Agilent Technologies, CA) at the Smal site in the reverse orientation, and the BamHI-EcoRI (hLOX) and BamHI-EcoRV (hLOXL2) fragments were further cloned at the BamHI-EcoRI or BamHI-SnaBI site of the retroviral vector, pMXs-IRE-PURO. Plasmids were transfected to the packaging cell line, and the culture supernatants were collected 48 h later, passed through a 0.45-μm filter, and added to cells in the presence of 8 μg/ml Polybrene. Infected cells were selected and maintained with 4 μg/ml puromycin.

In Vivo Experiments—In the first set of experiments, the effect of DXR on the expression of HIF target genes in vivo was investigated in nude mouse tumor xenografts. In brief, 1 × 10^7 viable HeLa cells were subcutaneously injected into the flanks of nude mice (n = 10). At 4 weeks, mice were subjected to daily administration of either DXR (0.5 mg/kg) or vehicle for 3 days,
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| Target gene                                      | Species | Strand | Sequence (5’-3’)              |
|-------------------------------------------------|---------|--------|------------------------------|
| Hypoxia-inducible protein 2 (HIG2)              | Human   | Forward| ACTCCAGACACTGCCCGCTCT        |
| Glucose transporter 1 (GLUTI)                   | Human   | Reverse| GCCACATCCTGAGAAGAAAA         |
| β-Actin                                         | Human   | Reverse| CGACGACCCTCTCAAAGAA          |
| Lysyl oxidase (LOX)                             | Human   | Reverse| AGCTCTGCTGCTGCTGTA           |
| Lysyl oxidase-like 1 (LOXLI)                    | Human   | Reverse| AACTGAAGAGCCCTCTCAC          |
| LOXL2                                           | Human   | Reverse| TGCTGCTGCTGCTGCTGC           |
| LOXL3                                           | Human   | Reverse| CTCGCTGCTATCCACGCCC          |
| LOXL4                                           | Human   | Reverse| CGACCTGCAAGCCTTCCCT          |
| GAPDH                                           | Human   | Reverse| ACACTCGACACTTGCGAAGAA        |
| CXC chemokine receptor type 4 (CXCR4)           | Human   | Reverse| GCCACAGACACTGAGCACC          |
| Glucose transporter 1 (Glut1)                   | Mouse   | Reverse| CTCTCCTGCTGCTGCTCCT          |
| Hypoxia-inducible protein 2 (Hig2)              | Mouse   | Reverse| ACACTCGACACTTGCGAAGAA        |
| BNP                                             | Mouse   | Reverse| GCCACAGACACTGAGCACC          |
| Heme oxygenase 1 (Ho-1)                         | Mouse   | Reverse| GCCACAGACACTGAGCACC          |
| VEGF                                            | Mouse   | Reverse| GCCACAGACACTGAGCACC          |
| β-Actin                                         | Mouse   | Reverse| GCCACAGACACTGAGCACC          |
| Vegf                                            | Rat     | Forward| GAAGAGCCGAGAATGCTTG          |
| Epo                                             | Rat     | Reverse| CTCTCCTGCTGCTGCTCG           |
| Bnp                                             | Rat     | Reverse| CAAGGAGAGAACAGCACCGA         |
| Angiopoietin 1 (Ang1)                           | Rat     | Reverse| CTCTCCTGCTGCTGCTCG           |
| Ang2                                            | Rat     | Reverse| CAAGGAGAGAACAGCACCGA         |
| Heme oxygenase 1 (Ho-1)                         | Rat     | Reverse| CTCTCCTGCTGCTGCTCG           |
| Prostaglandin-endoperoxide synthase 2 (Ptgs2)   | Rat     | Reverse| CTCTCCTGCTGCTGCTCG           |
| β-Actin                                         | Rat     | Reverse| GAAGGAGAGAACAGCACCGA         |

After which tissues were removed, and the expression of HIF target genes was evaluated by real time PCR or by immunohistochemistry. In a separate set, nude mice carrying xenografts were injected with 60 mg/kg pimonidazole (Chemicon, CA) to detect loci of hypoxia.

In the second set of experiments, the role of DXR on hypoxic gene induction of the host organ was further investigated in rats with acute anemia. Hemolytic anemia was induced in 6-week-old male SD rats by three daily administrations of phenylhydrazine (PHZ) (50 mg/kg intraperitoneal). DXR (2 mg/kg) or vehicle was co-administered with the third dose of PHZ. Six hours after the final treatment, rats were euthanized; organs were removed, and Vegf, Epo, and Bnp mRNAs were quantified by real time PCR. Each treatment group consisted of 6–8 rats.

In the third set of experiments, the effect of DXR on the induction of HIF target angiogenic genes was evaluated in an experimental heart failure model. A single subcutaneous injection of 150 mg/kg isoproterenol (ISP) was administered to 6-week-old female SD rats, as described previously (9). Heart failure was evidenced by increases in left ventricular end-diastolic dimension and posterior wall thickness and an accumulation of extracellular matrix. Rats were enrolled in the following four treatment groups: 1) vehicle; 2) vehicle + DXR; 3) ISP, and 4) ISP + DXR. Each group consisted of 6–8 rats. Three days after treatment, the rats were euthanized, the hearts were removed and subjected to further study. Animals were housed in individual cages in a temperature- and light-controlled environment and were allowed free access to chow and water. All experiments were performed in accordance with the guidelines of the Committee on Ethical Animal Care and Use at the University of Tokyo.

Immunohistochemistry—Tissues were immersion-fixed in methyl Carnoy’s or buffered formalin solutions and embedded in paraffin. Sections (3 μm thickness) were dewaxed and rehydrated through a graded ethanol series. Immunohistochemistry for CA9, HO-1, PTGS2, ED1, and aminopeptidase P (PG12) was performed by an indirect immunoperoxidase method. The primary antibodies used were anti-HO-1 (StressGen, British Columbia, Canada), anti-PTGS2 (Cayman Chemical, MI), anti-ED1 (Chemicon), and anti-JG12 (Bender MedSystems, Vienna, Austria). Immunodetection for HIF-1α was performed as
TABLE 2
Effect of DXR on cell viability

| Cell line | Doxorubicin concentration | p value |
|-----------|---------------------------|---------|
| HEK293   | 0.1 µg/ml                 | 0.01    |
|           | 1 µg/ml                   | 0.01    |
|           | 10 µg/ml                  | 0.01    |
| Hela     | 0.1 µg/ml                 | 0.01    |
|           | 1 µg/ml                   | 0.01    |
|           | 10 µg/ml                  | 0.01    |
| HepG2    | 0.1 µg/ml                 | 0.01    |
|           | 1 µg/ml                   | 0.01    |
|           | 10 µg/ml                  | 0.01    |
| HK-2     | 0.1 µg/ml                 | 0.01    |
|           | 1 µg/ml                   | 0.01    |
|           | 10 µg/ml                  | 0.01    |
| KMRC-3   | 0.1 µg/ml                 | 0.01    |
|           | 1 µg/ml                   | 0.01    |
|           | 10 µg/ml                  | 0.01    |

Table 2 shows the effect of doxorubicin concentration on cell viability.

RESULTS

DXR is an anthracycline chemotherapeutic agent widely used for the treatment of various tumors. However, chemotherapeutic resistance to DXR, which is mediated by altered availability of the drug and its inactivation and modification of the pathways causing DNA repair and death (11), varies significantly among cell lines. Therefore, we first screened cell lines for viability against DXR treatment (Table 2). As expected, susceptibility to DXR varied among cell lines, with the highest levels observed in HEK293, moderate levels in Hela and HepG2, and mild levels in HK-2 and KMRC-3 cells. In subsequent studies, we predominantly used cell lines with relative resistance to DXR to minimize the effect of cell toxicity as a contributing factor to the study results.

DXR Decreases Transcriptional Activity of HIF-1—Cellular hypoxia response is primarily mediated by hypoxia-inducible transcription factors. In this regard, we tested the effect of DXR on HIF-1-mediated gene transcription using an HREluc reporter assay. DXR produced dose-dependent inhibition of the reporter activity induced by chemical hypoxia in HK-2 cells (Fig. IA). In contrast, DXR did not significantly influence HRELuc activity in normoxia or pGL3-CMV activity in either normoxia or hypoxia, suggesting that the effect of DXR is specific to HIF-1. The inhibitory effect of DXR was also observed in the promoter activity of a naturally occurring, authentic HIF-1 target gene, CA9 (Fig. IB). To test whether this is a general observation, we further investigated whether the effect could be reproduced using other anthracycline agents in different cell lines. DNR, another anthracycline agent, similarly inhibited the hypoxic induction of HRELuc, although the effective concentration range varied in comparison with DXR (Fig. 1C). In contrast, etoposide (VP-16), a topoisomerase II inhibitor, which does not belong to the anthracycline family, did not influence the hypoxic induction of the reporter (Fig. 1D). The effect of DXR was also tested in HeLa cells, and although the concentration range used was lower by 1 order of magnitude due to high cellular susceptibility, DXR again inhibited the induction of HRELuc (Fig. 1E). Additionally, DXR suppressed the HRELuc activity stimulated by genetic overexpression of HIF-1α (Fig. 1F), further raising a possibility that this is a specific effect. Collectively, these results indicate that DXR impairs the cellular hypoxia response mediated by HIF-1, an effect that is likely to be specific to this class of anthracycline antitumor agents.

The hypoxic induction of HIF-1 target genes is mediated through binding of the HIF-1α/ARNT heterodimer to the consensus hypoxia-responsive element (HRE), coded by the conserved -R(A/G)CGTG- motif. In contrast, the pharmacological effect of DXR is mediated by causing DNA structural changes at both ends of the intercalated sites marked by the -GC- sequence. Therefore, it could be predicted that DXR influences a variety of transcription factors whose cis-elements are similarly marked by the core -GC- motif. To test this possibility, we examined whether the xenobiotic response, which is also mediated through binding of the aryl hydrocarbon receptor/ARNT heterodimer to the -RCGTG- motif, could be similarly influenced by DXR. Cells transfected with the xenobiotic-responsive element (XRE)-driven luciferase (XRELuc) were stimulated with a dioxin agonist, 3-methylcholanthrene, in the presence or absence of DXR. In contrast to its effect on HRELuc, DXR did not influence XRELuc activity (Fig. 1G), nor did it affect XRELuc stimulated by genetic overexpression of the constitutively active aryl hydrocarbon receptor (Fig. 1H) (12). Therefore, intercalation and structural changes to DNA alone are unlikely to explain the observed effect of DXR on HIF, which is probably specific to the HIF response.

The effect of DXR on the promoter activity of the HIF target genes was reflected in their expression changes. In HK-2 cells, the hypoxic induction of representative HIF targets, HIG2 and GLUT1, was decreased by DXR treatment (Fig. 2A). Consistent with this, the hypoxic induction of HIF target proteins, BCL2/adenovirus E1B 19-kDa protein-interacting protein 3 (BNIP3) and CA9, was also blunted by DXR in HeLa cells (Fig. 2B). In contrast, DXR did not significantly influence the expression of HIF-1α and ARNT (discussed below). We also tested the effect of DXR on a VHL-defective cell line, KMRC-3. The expression of CA9 was strikingly decreased by increasing the concentration of DXR, further illustrating the negative impact of DXR on HIF signaling.

DXR Inhibits Recruitment of the HIF Heterodimer—In an attempt to identify molecular mechanisms through which DXR decreases the transcriptional activity of HIF-1, we checked the protein expression of HIF-1α and its binding partner, ARNT. In HK-2 cells, the expression of the HIF-1α and ARNT proteins was not changed appreciably by DXR treatment, over the concentration range at which the hypoxic induction of HRELuc was significantly blunted (Fig. 3, A, upper panel, and quantified in B). Similarly, the expression of the HIF-1α protein remained unchanged by DNR, over the concentration range sufficient to cause functional derangement of HIF-1 (Fig. 3A, lower panel). The validity of this finding was investigated further. To exclude the possibility that the lack of quantitative differences observed by immunoblotting arose from a technical procedure, for example, from excessive protein loading and saturation of the
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A. HRELuc (HK-2)

B. CA9 promoter luc (HK-2)

C. HRELuc (HK-2)

D. HRELuc (HK-2)

E. HRELuc (HeLa)

F. HRELuc (HK-2)

G. XRELuc (HK-2)

H. XRELuc (HK-2)
signal by overexposure, we repeated the experiments using ser-" 

serially diluted aliquots of hypoxic, DXR(H11002), or DXR(H11001) cells. No quantitative difference in HIF-1(H9251) protein expression was observed (Fig. 3C).

The major step in determining the HIF-1(H9251) protein expression is oxygen-dependent prolyl hydroxylation by the family of prolyl hydroxylase domain-containing proteins (PHD), which is followed by pVHL recruitment and E3 ubiquitination. Therefore, we monitored the degree of prolyl hydroxylation by constructing a luciferase vector in which the N terminus of the luciferase gene was fused to the oxygen-dependent degradation domain (ODD) of rat HIF-3(H9251) (ODDluc) (13) and subjected it to transient transfection. In both the DXR(H11002) and DXR(H11001) groups, hypoxia led to a parallel increase in ODDluc activity (Fig. 3D), indicative of a similar degree of PHD inhibition. These results suggest that DXR did not alter PHD activity under these conditions.

The canonical function of HIF-1(H9251) is mediated through binding of the HIF-1(H9251)/ARNT heterodimer to the HRE, and the absence of this interaction completely abolishes induction of its target genes (14). Therefore, we tested whether DXR influenced recruitment of HIF-1(H9251), using the ChIP assay (Fig. 4, A, and quantified in B). In the hypoxic DXR(−) group, immunoprecipitation using the anti-HIF-1(H9251) antibody effectively isolated DNA fragments, including the VEGF and CA9 promoters. DXR treatment decreased the quantity recovered, indicating that DXR impaired binding of the HIF-1(H9251) protein to its target gene promoters.

FIGURE 1. Impaired hypoxia response of HIF-1 by anthracycline agents. A, DXR dose-dependently reduced the hypoxic induction of the synthetic HRE-driven luciferase (HREluc) reporter in HK-2 cells. In contrast, the reporter activity of the backbone luciferase vector (pGL3) remained unchanged by DXR treatment. B, DXR reduced the hypoxic induction of the CA9 promoter, an authentic HIF-1 target gene. C, DNR impaired the hypoxic induction of HREluc. D, etoposide, a topoisomerase II inhibitor, did not influence the reporter activity of HREluc. E, DXR reduced the hypoxic induction of HREluc in HeLa cells. Note that the concentration range of DXR is 1 order of magnitude lower in comparison with that used on HK-2 cells. F, DXR reduced the HREluc activity induced by the overexpression of HIF-1α (left panel). Expression of the vector plasmid was confirmed by immunoblotting (right panel). G, DXR did not influence xenobiologic response, as measured by the activity of the XREluc, which also contains a core -RCGTG- motif. H, DXR did not influence the activity of XREluc induced by the overexpression of the constitutively active aryl hydrocarbon receptor. Luciferase reporter assays: ##, p < 0.01 versus anthracycline-free counterparts. n = 3. DP, 2,2′-Dipyridyl.

FIGURE 2. DXR decreases the expression of endogenous HIF-1 target genes. A, DXR reduced the hypoxic induction of endogenous HIF target gene mRNA in HK-2 cells. Left panel, HIG-2; right panel, GLUT1. Real time PCR: #, p < 0.05; ##, p < 0.01 versus DXR-free counterparts. n = 4. B, (left) DXR reduced the hypoxic induction of endogenous HIF target protein, BNIP3 and CA9, in HeLa cells. Note that expression levels of HIF-1α and ARNT remained unaffected by DXR. Right, DXR decreased CA9 protein expression in a VHL-defective cell line, KMRC-3. Immunoblotting: n = 3. DP, 2,2′-Dipyridyl.
A decrease in transcription factor binding may be caused either directly or indirectly through insufficient formation of the transcription factor complex; hence, we tested whether DXR influenced the formation of HIF-1α/ARNT heterodimers. HA-tagged HIF-1α and FLAG-tagged ARNT were co-expressed in HepG2 cells. Cell lysates were incubated with an anti-HA antibody, and the immunoprecipitated protein was probed with an anti-FLAG antibody (Fig. 4C). The amount of HIF-1α/ARNT complex was unaffected by DXR treatment, suggesting the former possibility.

The transcription of HIF-1 is also determined by recruitment of the cofactor to the transactivator domain (TAD) in the C terminus of the HIF-1α chain, which alters the function of HIF-1αTAD. The function of HIF-1αTAD was evaluated by constructing a Gal4-fused HIF-1αTAD protein and co-expressing it with the Gal4-response element-driven luciferase vector (GREluc). DXR treatment did not change GREluc activity, regardless of normoxia or hypoxia, indicating that the PHD activity was similarly inhibited irrespective of DXR treatment. Parallel transfection with a P487A mutant, which escapes recognition by PHD, hydroxylation, and proteasomal degradation, serves as a control. Luciferase assay: n = 3. DP, 2,2’-Dipyridyl.

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**DXR Retards HIF-dependent Migration of RCC Cells**—Invasion and metastasis of solid tumors is characterized by a series of pathological changes, such as loss of cell adhesion, efface-
ment of epithelial markers, migration into the surrounding tissues, and acquisition of mesenchymal phenotypes, generically named epithelial-mesenchymal transdifferentiation. In experimental models of tumor growth using VHL-defective RCC cells, there is evidence that inhibition of HIF-2 is sufficient to suppress growth (15). Therefore, we asked whether DXR similarly inhibits HIF-2 activity and, if so, whether DXR treatment suppresses HIF-2-dependent migration of VHL-defective RCC cells.

FIGURE 4. DXR impairs recruitment of the HIF heterodimer to the HRE. A, chromatin immunoprecipitation (IP) analysis revealed impaired HIF-HRE interaction following DXR treatment. Cross-linked DNA-protein complexes in the DXR(−) and DXR(+) groups were captured by an anti-HIF-1α antibody, and enriched nucleotide fragments were amplified by PCR, using primers specific to the VEGF (upper panel) and CA9 (lower panel) promoters. In the DXR(+) group, the intensity of the specific PCR-amplified band was significantly lower than that in the DXR(−) group, indicating impaired recruitment of HIF-1α to the promoters. ChIP assay: n = 4. B, enrichment of target gene fragments (VEGF [upper panel] and CA9 [lower panel]) was quantitatively analyzed. Real time PCR: #, p < 0.01; n = 4. C, influence of DXR on the HIF heterodimer formation was assessed by immunoprecipitation. Cells were co-transfected with HA-tagged HIF-1α and FLAG-tagged ARNT plasmids, and the protein lysates were immunoprecipitated with anti-HA antibody and probed with anti-FLAG antibody. Similar amounts of the FLAG-ARNT protein were captured by an anti-HA antibody in both DXR(−) and DXR(+) groups, indicating that DXR did not influence binding of HIF-1α to ARNT. Immunoprecipitation: n = 3. D, activity of the HIF-1α transactivator domain (TAD) was measured based on the Gal4 reporter assay. Cells were co-transfected with the Gal4 responsive element-driven luciferase reporter (GRELuc) and the Gal4-fused HIF-1α TAD expression vector and stimulated with DXR. The reporter activity, which was hypoxia-inducible, remained unaffected by DXR treatment. Gal4 reporter assay: n = 3. IB, immunoblot. DP, 2,2′-Dipyridyl.
RCC cells in vitro, independently of its primary action to damage DNA replication and repair.

The effect of DXR on HIF-2-mediated transactivation was investigated by simultaneous transfection of HREluc and the HIF-2α expression vector. As in the case of HIF-1, DXR also decreased the hypoxic induction of HREluc (Fig. 5A).

A498 is a VHL-defective clear cell RCC with high expression of HIF-2α but no expression of other HIF-α isoforms, which...
shows resistance against DXR treatment in terms of cell viability (0.1–10 μg/ml, data not shown). Using this cell line, we tested whether HIF-2 was responsible for the facilitation of cell migration. Cells treated with siRNAs for pGL3 luciferase (3luc), HIF-1α, or HIF-2α were subjected to the scratch test and allowed to migrate. Untreated cells and those treated with 3luc siRNA or HIF-1α siRNA showed significant migration within 10 h. In contrast, the distance between edges did not narrow in the HIF-2α siRNA group, indicating that A498 migration under these experimental conditions was dependent on HIF-2 (Fig. 5B).

Based on these results, we tested whether DXR retarded HIF-2-dependent A498 migration (Fig. 5, C and D). Gap closure in the untreated group began 5 h after the scratch and progressed until the gap was no longer visible at 24 h. In contrast, DXR treatment significantly retarded this process, and ~70% of the initial gap remained after 24 h. Additionally, the use of etoposide, a topoisomerase II inhibitor that does not suppress HIF activity, did not influence migration of the A498 cells (Fig. 5E), raising the possibility that the effect of DXR on A498 migration was mediated by the inhibition of HIF-2 activity. Inhibition of cell migration was also observed in an additional VHL-defective RCC cell line, KMRC-3 (Fig. 5F), although to a lesser extent than that observed in A498 cells. This is probably a reflection of distinct susceptibility to the drug and different cellular oncogenic backgrounds.

Although the majority of clear cell RCCs harbor mutations in the VHL gene, several others do not, and tumor suppression by VHL is experimentally overridden by the forced expression of HIF-2α that is engineered to escape degradation (16). In addition, there is evidence that overexpression of HIF-1α in the renal proximal tubules is sufficient to induce tumors (17), collectively suggesting that the aggressive phenotype associated with HIF-2 may be acquired irrespective of the VHL status and that HIF-1 also serves a tumorigenic role depending on the cellular background. Therefore, we repeated experiments using A498 cells reconstituted with the mouse VHL homologue (A498/Vhlh) and the Caki-1 RCC cell line harboring normal
VHL status and a dominant pattern of HIF-1α expression. In both cases, cells migrated modestly in normoxia irrespective of DXR treatment. However, cells in hypoxia migrated more aggressively than in normoxia, and DXR treatment counteracted the hypoxia-induced migratory profile (Fig. 5, G and H).

**Suppression of RCC Migration Correlates with Decreases in the Expression of the LOX Family Genes**—Among a number of HIF target genes, the LOX family plays an important role in the acquisition of invasive tumor properties (18). Therefore, we investigated whether DXR-mediated inhibition of cell migration was associated with changes in the expression of the LOX family genes. First, we checked basal expression levels of LOX and its related genes, LOXL1 through LOXL4 in A498 cells (Fig. 6A). Among these genes, LOXL2 mRNA was determined to be abundantly expressed by 100-fold, whereas the expression of LOXL1 and LOXL3 was barely detectable, if at all. LOX, LOXL2, and LOXL4 have been proposed as HIF target genes (19, 20); hence, we speculated that
their expression may be reduced by DXR. As expected, DXR significantly reduced the expression of LOX, LOXL2, and LOXL4 mRNA (Fig. 6B). The hypoxic induction of LOX and LOXL2 and their suppression by DXR was also observed in HK-2 cells (Fig. 6C). To test whether LOX and its family members may be involved in RCC migration, we treated cells with a pharmacological inhibitor of LOX, H9252-aminopropionitrile. H9252-aminopropionitrile significantly inhibited the migration of A498 cells (Fig. 6D). Conversely, stable overexpression of LOX and LOXL2 facilitated migration of A498 cells (Fig. 6E–G). A modest increase in cell motility by genetic LOX overexpression, in contrast to marked suppression by pharmacological LOX inhibition, is consistent with a previous study using Matrigel-based in vitro invasion analysis (18). Results of these studies confirm that these LOX members, which are hypoxia-inducible and inhibitable by DXR, play critical roles in RCC migration.

**DXR Reduces the Hypoxic Induction of HIF Target Genes in Vivo**—Based on the inhibitory effect of DXR on the induction of HIF target genes in vitro, we next asked whether this pleiotropic effect applies in vivo using tumor xenografts (Fig. 7). Growing tumors are generally in a hypoxic milieu due to increasing metabolic demand and incommensurate tumor vasculature, which we confirmed by positive staining for a chemical hypoxia marker, pimonidazole. These tumors expressed HIF-1α protein, and serial staining demonstrated that these HIF-1α-positive cells also expressed CA9, a representative HIF target gene, indicating the functional operation of HIF-1 (Fig. 7A). In this model, tumor-bearing mice were either treated with DXR or vehicle, and the expression of HIF target genes was quantitatively evaluated in the tumor xenograft and the host heart, the most susceptible organ against DXR. In tumors, the degree of hypoxia and HIF-1α expression was similar in both groups (Fig. 7B). However, the expression levels of GLUT1 and HIG2 were significantly lower in the DXR treatment group (Fig. 7C). In contrast, hearts of the tumor-bearing mice, which did not stain positive for pimonidazole and were therefore nonhypoxic (Fig. 7D), displayed no recognizable inhibition of HIF target genes by DXR (Fig. 7E). These results indicate that DXR suppresses HIF-mediated hypoxia response in hypoxic tumors in vivo, but it fails to do so in nonhypoxic host hearts.

Nevertheless, the pleiotropic effect of DXR on HIF signaling possibly applies in organs of the tumor host, once they are exposed to hypoxia. In this regard, we tested whether DXR dampens the acute hypoxia response in rats with acute anemia (Fig. 8A). Hemolytic anemia was induced in rats using three consecutive injections of PHZ. The average hemoglobin value prior to the third injection was 6.9 ± 0.6 g/dl, at which time DXR or vehicle was administered. Following challenge with acute anemia, Vegf mRNA was significantly increased in the kidney, liver, gut, and heart. Remarkably, however, DXR impaired the hypoxic induction of Vegf only in the heart. Consistent with the Vegf data, the hypoxic induction of Epo mRNA in the kidney and the liver was also unaffected by DXR treatment, even when the amount of DXR was increased by 5-fold (10 mg/kg), and the hypoxic induction of Bnp mRNA in the heart was effectively blunted by DXR. The difference in DXR response among organs could be due to distinct delivery and excretion rates of the drug in the heart versus other organs and...
to the intrinsic susceptibility of cells \textit{per se}, which remains canonically proven. Nevertheless, our results clearly demonstrated that the systemic administration of DXR impairs hypoxic induction of HIF target genes in the heart.

An insufficient angiogenic response by HIF-1 has been suggested to serve as a critical switch toward decompensated heart failure in a mouse model of cardiac hypertrophy (transverse aortic constriction) (21). Therefore, we speculated that DXR may diminish the angiogenic response and adversely affect the outcome in the pathogenesis of experimental heart failure (Fig. 8B). Heart failure was induced by administration of a single dose of 150 mg/kg ISP in rats. Within 2 weeks, the histological changes of heart failure, such as cardiac hypertrophy and accumulation of extracellular matrix proteins like fibronectin,
became apparent (data not shown), in keeping with a previous report (9). The induction of angiogenic HIF target genes was evaluated on day 3. mRNA expression of several angiogenic factors, such as Vegf and angiopoietin 1 (Ang1), was not increased in this model, whereas that of several others such as angiopoietin 2 (Ang2) and Ptgs2, was significantly induced. The induction by ISP was blunted by DXR treatment. In addition, ISP induced the expression of several other HIF target genes such as Ho-1 and Bnp mRNA, which was again inhibited by DXR.

FIGURE 8. Effect of DXR on hypoxic gene induction in rats in vivo. A, to investigate the role of DXR on hypoxic gene induction in vivo, rats were challenged with acute hemolytic anemia by PHZ. Prior to the final PHZ administration, rats were injected with either DXR or vehicle. Six hours later, rats were euthanized; organs were removed, and the induction of HIF target genes was quantitatively evaluated. Vegf mRNA was significantly increased in all organs tested, and DXR treatment (2 mg/kg) reduced the hypoxic induction in the heart, but not in the liver, gut, or kidney (upper panel). Consistently, the renal and hepatic increase in Epo mRNA was not inhibited by DXR, even after increasing the concentration of DXR to 10 mg/kg (lower left panel, marked with + +). In contrast, the hypoxic induction of Bnp mRNA in the heart was significantly suppressed by DXR treatment (lower right panel). Real time PCR: #, p < 0.05; ##, p < 0.01; n = 8. B, effect of DXR on the induction of HIF target genes in a heart failure model. Heart failure in rats was induced by ISP injection, and the induction of genes related to angiogenesis and hypoxia response was evaluated by real time PCR. Although several HIF targets, such as Vegf and Ang1, were not induced in this model, many of the hypoxia-inducible genes were up-regulated, and DXR significantly blunted the induction of Ang2, Ho-1, Ptgs2, and Bnp gene mRNA. Real time PCR: #, p < 0.05; ##, p < 0.01; n = 6.
The expression profiles of several HIF targets were characterized by immunohistochemistry (Fig. 9). The induction of immunoreactive HIF-1α protein confirmed that HIF-1 was functional during cardiac pathogenesis (Fig. 9D). HO-1 protein expression at baseline was below immunodetection levels. However, a significant increase was observed in the interstitial...
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compartment on day 3, which was suppressed by DXR treatment (Fig. 9A). Consecutive staining of HO-1 and ED-1, a marker for macrophages, using semi-serial sections confirmed that a significant number of HO-1-positive cells were infiltrating macrophages (Fig. 9C). The PTGS2 protein was expressed weakly at base line and remained unaffected by DXR treatment alone. However, ISP administration led to abundant expression of PTGS2 protein in myocytes, which was suppressed by DXR treatment (Fig. 9B). We further tested whether the negative impact of DXR on the overall induction of angiogenic HIF target genes reflected changes in cardiac capillary structures. On day 3, DXR treatment led to marked narrowing and distortion of microvascular capillaries, as well as a significant decrease in the number of JG12-positive capillary lumens (Fig. 9, E and F). Collectively, these data indicate that DXR inhibited the hypoxia response in a model of heart failure and mediated an inadequate angiogenic response, which could be a critical turning point toward irreversible heart failure.

**DISCUSSION**

In this study, we demonstrated that anthracycline chemotherapeutic agents inhibit binding of the HIF-α/ARNT heterodimer to the target gene enhancer and reduce the cellular hypoxia response. Suppression of HIF with this method retarded migration of RCC cells in vitro and impaired the hypoxic and angiogenic responses in tumor xenografts and the host heart in vivo.

The majority of the cellular hypoxia response by HIF-1 and HIF-2 is mediated through binding of the HIF-α/ARNT heterodimer to the canonical enhancer, called a hypoxia-responsive element (HRE). A recent genome-wide ChIP sequence analysis identified a common -RCGTG- motif for HIF-1 in ~70% of promoters of target genes involved in angiogenesis, erythropoiesis, and metabolic adaptation (22). In this regard, interference with the HIF-HRE interaction would be an ideal target for pharmacological manipulation of HIF activity, and identification of a pleiotropic effect of DXR on HIF would provide an opportunity to develop HIF inhibitors using DXR as a chemical template. In contrast, however, the remaining 30% of HIF-1 and 15% of HIF-2 binding regions did not contain an -RCGTG- motif, and recent studies have identified additional roles for HIF-1 that are not mediated through the canonical HIF-HRE binding. These include regulation of the proto-oncoprotein c-Myc (23), signal transducers and activators of transcription-3 (STAT-3) (24), and T-helper 17 (Th17) differentiation through RORγt activation (25). By analogy, these properties of HIF will not be influenced by the pleiotropic action of DXR that we have characterized in this study.

Inhibition of the HIF-HRE interface by DXR resulted in suppression of HIF-dependent migration of RCC cells. The role of HIF on aggressive tumor phenotypes is most comprehensively characterized in clear cell carcinoma in the kidney, which accounts for 70–80% of all RCC. In many cases, the VHL gene is inactivated due to genetic mutation or hypermethylation of the promoter, and VHL-defective RCCs show a predominant expression of HIF-2α rather than HIF-1α (26). In such tumors, inhibition of HIF-2 is sufficient to suppress tumor growth (15), whereas reconstitution of HIF-1α is not sufficient to reproduce an aggressive tumor phenotype but is capable of mimicking some aspects of VHL loss (27). In contrast, HIF-1 is also involved in the acquisition of the aggressive phenotype in breast and colon cancers (28, 29), collectively indicating that the impact of HIF-1 and HIF-2 on tumor growth may vary depending on the type of tumor and their oncogenic background. In this regard, blockade of both the HIF-1α/ARNT and HIF-2α/ARNT interface by DXR would serve as an ideal therapeutic target for the suppression of tumor growth of not only VHL-defective RCC but of a variety of tumors with accelerated HIF signaling.

Suppression of RCC migration by DXR was associated with coordinated down-regulation of a group of LOXs, in particular, LOX, LOXL2, and LOXL4. LOX has been implicated in hypoxic tumor invasion (18, 19) and significantly correlates with clinical outcomes in breast, head, and neck tumors. Furthermore, LOXL2 promotes migration of breast cancer cells (30), and LOXL4 provides a metastatic niche for breast cancer (20). Importantly, they are all HIF targets, and it seems likely that DXR decreased their expression by inhibiting HIF.

In addition to the intrinsic invasive properties of tumor epithelial cells, the microvasculature in the tumor environment is a critical issue for its sustained growth in vivo. It has been reported that DXR inhibits tumor xenograft growth through antiangiogenic properties (31), but the molecular mechanisms remain largely uncharacterized. A recent screening of a library of drugs identified doxorubicin and daunorubicin as potent inhibitors of hypoxia-inducible gene induction. Daily administration of DXR to tumor-bearing mice reduced the expression of HIF target, angiogenic genes in tumor xenografts, and the recruitment of circulating bone marrow-derived angiogenic cells (32). These results are consistent with ours and lend support to our proposal that DXR dampens HIF response in vivo.

Of additional importance in this study is that DXR inhibited the acute hypoxia response in the heart of the tumor-bearing host and suppressed the angiogenic response in a model of heart failure. Remarkably, the heart was the only organ in which DXR impaired the acute hypoxia response by HIF. Although we have been unable to explain this organ-specific action of DXR on HIF, it coincides with the clinical observation that DXR toxicity manifests predominantly in the heart. To date, the molecular mechanisms of DXR cardiac toxicity remain incompletely understood, but a recent study attributes a protective role of dexrazoxane, a produg that is enzymatically hydrolyzed inside cardiomyocytes to its active metal-chelating metabolite, to HIF (33). In this regard, dysregulation of HIF may be a candidate mechanism underlying cardiac toxicity by DXR.

In addition, our results also emphasize increased susceptibility of the DXR-exposed heart to an additional ischemic insult, in that the angiogenic response by HIF was significantly blunted in the first quartile of an experimental heart failure model. Angiogenesis is critically involved in the adaptive mechanism of cardiac hypertrophy; in one study, inhibition of HIF by p53 was associated with suppression of cardiac angiogenesis, which played an essential role in the transition from sustained cardiac hypertrophy to cardiac dysfunction (21). Therefore, there is a possibility that inhibition of HIF by DXR accelerates transition from the initial compensation to the irreversible car-
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diabetic dysfunction in a similar manner, which may have relevance in human clinical settings.

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