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Cobalt stimulates HIF-1-dependent but inhibits HIF-2-dependent gene expression in liver cancer cells

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ABSTRACT

Hypoxia-inducible factors (HIFs) are transcriptional regulators that mediate the cellular response to low oxygen. Although HIF-1 is usually considered as the principal mediator of hypoxic adaptation, several tissues and different cell types express both HIF-1 and HIF-2 isoforms under hypoxia or when treated with hypoxia mimetic chemicals such as cobalt. However, the similarities or differences between HIF-1 and HIF-2, in terms of their tissue- and inducer-specific activation and function, are not adequately characterized. To address this issue, we investigated the effects of true hypoxia and hypoxia mimetics on HIF-1 and HIF-2 induction and specific gene transcriptional activity in two hepatic cancer cell lines, Huh7 and HepG2. Both hypoxia and cobalt caused rapid induction of both HIF-1α and HIF-2α proteins. Hypoxia induced erythropoietin (EPO) expression and secretion in a HIF-2-dependent way. Surprisingly, however, EPO expression was not induced when cells were treated with cobalt. In agreement, both HIF-1- and HIF-2-dependent promoters (of PGK and SOD2 genes, respectively) were activated by hypoxia while cobalt only activated the HIF-1-dependent PGK promoter. Unlike cobalt, other hypoxia mimetics such as DFO and DMOG activated both types of promoters. Furthermore, cobalt impaired the hypoxic stimulation of HIF-2, but not HIF-1, activity and cobalt-induced HIF-2α interacted poorly with USF-2, a HIF-2-specific co-activator. These data show that, despite similar induction of HIF-1α and HIF-2α protein expression, HIF-1 and HIF-2 specific gene activating functions respond differently to different stimuli and suggest the operation of oxygen-independent and gene- or tissue-specific regulatory mechanisms involving additional transcription factors or co-activators.

1. Introduction

Hypoxia-inducible factors (HIFs) play a central role in hepatocellular carcinoma (HCC) (Mylonis and Simos, 2012). HIFs are frequently up-regulated in HCC and seem to control tumor progression and sensitivity to radiation therapy (Nath and Szabo, 2012). HIFs are heterodimers of the protein subunits HIF-α, which are induced by hypoxia and HIF-β or aryl hydrocarbon receptor nuclear translocator (ARNT), which is constitutively expressed (Keith et al., 2012). The HIF-α subunits are rapidly degraded in normoxia by a process involving prolyl hydroxylation, interaction with the von Hippel–Lindau protein (pVHL) E3 ubiquitin ligase complex and proteasomal degradation. Hydroxylation is catalyzed by a family of Fe(II) and 2-oxoglutarate-dependent prolyl hydroxylases (PHDs) whose absolute requirement for molecular oxygen confers sensitivity to hypoxia, under which HIF-α members rapidly accumulate, translocate inside the nucleus, heterodimerize with ARNT and bind to hypoxia-response elements (HREs) DNA elements in the promoters or enhancers of their target genes. The activity of PHDs and subsequent stabilization of HIF-1α is also affected by chemical agents ("hypoxia mimetics") such as the iron chelator desferrioxamine (DFO) (Wang and Semenza, 1993), flavonoids such as quercetin (Triantafyllou et al., 2007), 2-oxoglutarate-dependent oxygenase inhibitors such as DMOG (Elvidge et al., 2006) and transition metals such as cobalt (Kaelin and Ratcliffe, 2008). Treatment with these agents ("chemical hypoxia") is often used to simulate hypoxic conditions and induce HIF-1. However, the action of certain of these agents (e.g. cobalt and flavonoids) may be mediated by signaling pathways not necessarily shared by the "true" hypoxic response and may cause, therefore, different and oxygen-independent biological effects (Chachami et al., 2004; Triantafyllou et al., 2006, 2008).

HIF-1α was the first isoform of HIF-α to be identified by its capacity to bind to the hypoxia-responsive element (HRE) present

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in the erythropoietin (EPO) enhancer (Wang et al., 1995). HIF-1α is ubiquitously expressed under hypoxia and is responsible for the regulation of a wide range of cellular adaptation responses and more preferentially metabolic enzymes (Majmundar et al., 2010; Mylonis et al., 2012). A second isoform (HIF-2α), is encoded by the *EPAS1* gene, its expression is restricted to specific cell types, including hepatocytes, it appears to be more involved in angiogenesis and erythropoiesis and its regulation is considerably less investigated (Keith et al., 2012). Like HIF-1α, HIF-2α is degraded under normal conditions via the same PHD-VHL-proteasome-dependent system and is stabilized under hypoxia due to PHD inhibition. However, it is not yet known whether oxygen-independent mechanisms that regulate HIF-1 activity, such as for example nuclear transport and phosphorylation (Mylonis et al., 2008; Kalousi et al., 2010), also apply to HIF-2. In the same line, the effects of "hypoxia mimetics" on different cell types is usually studied by monitoring HIF-1α expression and whether HIF-2α activity is also affected remains relatively unknown. This has important biological relevance given that HIF-α stabilizers are used both experimentally and clinically as erythropoiesis-stimulating agents.

HIF-1α and HIF-2α share certain overlapping functions by regulating common hypoxia-inducible genes. However, it has recently become clear that, through independent regulation of distinct target genes or unique protein-protein interactions, HIF-1α and HIF-2α can also mediate divergent functions when expressed in the same cell type and especially in the context of cancer (Keith et al., 2012). The predominant role of HIF-2 in erythropoiesis has been established by studies in HIF-2α-deficient (knock-out) mice documenting HIF-2α as the main regulator of hepatic EPO production and essential for the maintenance of systemic EPO and iron homeostasis (Scortegagna et al., 2005; Rankin et al., 2007; Kapitsinou et al., 2010). Other prominent HIF-2-specific targets are genes for major antioxidant enzymes such as superoxide dismutase (SOD2) (Scortegagna et al., 2003). However, these animal studies have not determined the exact degree to which individual HIF-α subunits contribute to EPO production in cancer cells. Many subtle differences on the regulation of the expression and transcriptional activity between HIF-1α and HIF-2α need to be elucidated, especially in HCC as inhibition of HIFs may have an important role in targeted cancer therapy (Nath and Szabo, 2012).

In this report, we investigate the expression and transcriptional activity of HIF-1α and HIF-2α as well as expression and secretion of EPO in response to hypoxia and the "hypoxia mimetic" cobalt in hepatic cancer cell lines expressing both HIF-α isoforms. Our findings show that cobalt has opposing effects on HIF-2α expression and activity, rendering it ineffective in EPO gene activation. This specific effect of cobalt on HIF-2 signaling was accompanied by reduced interaction of the HIF-2α with USF2. This suggests that HIF-2α, but not HIF-1α, requires the assistance of a cobalt-sensitive factor in order to activate transcription of its target genes in liver cancer cells.

2. Materials and methods

2.1. Plasmids

pGL3-SOD2 promoter and pGL3-PGK promoter were kindly provided by Joseph A. Garcia (Department of Medicine, University of Texas) and M. Celeste Simon (Abramson Family Cancer Research Institute, University of Pennsylvania) respectively (Scortegagna et al., 2003; Hu et al., 2007). Plasmid pEGFP-HIF-2α was constructed by inserting the full length HIF-2α cDNA into the BamHI position of the multicloning site of the pEGFP-C1 plasmid (Clontech). pcDNA-HIF-2α, kindly provided from Dr. S. L. McKnight (Department of Biochemistry, University of Texas) (Tian et al., 1997). pEGFP-HIF-1α plasmid was previously described (Mylonis et al., 2006). Plasmid pCDNA-Flag-USF2 full-length was previously described (Pawlus et al., 2012).

2.2. Cell culture

Huh7 and HepG2 cells were cultured in DMEM (Gibco) containing 10% FCS and 100 U/ml penicillin–streptomycin (Gibco). All cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. When required, cells were treated for 2–48 h with 150 μM cobalt chloride ($CoCl_2$). The concentration of $CoCl_2$ (150 μM) was determined in preliminary dose–response experiments (using 50–200 μM) to produce maximal induction of HIF-α at 4–48 h, without being toxic to cells (results not shown). For hypoxic treatment, cells were exposed for 2–48 h to 1% O_2, 95% N_2 and 5% CO_2 in an IN VIVO$_2$ 200 hypoxia workstation (RUSKINN Life Sciences). All other incubation protocols were as stated in figure legends.

2.3. Western blot and immunoprecipitation

Antibodies used for western blotting were: affinity purified rabbit polyclonal (pAbs) antibody against HIF-1α (Lyberopoulou et al., 2007), anti-HIF-2α pAb (Abcam and Novus Biologicals for Fig. 5), anti-Flag pAb (Sigma), anti-USF2 pAb (Santa Cruz) and anti-actin monoclonal antibody (Millipore). Analysis by immunoblotting and immunoprecipitation was carried out as previously described (Lyberopoulou et al., 2007; Mylonis et al., 2008). All experiments were performed in triplicate and representative results are shown.

2.4. Cell transfection and luciferase reporter assays

To examine the transcriptional activity of HIF-1 and HIF-2, cells were co-transfected with the firefly luciferase reporter plasmid pGL3-PGK or pGL3-SOD2 and the Renilla luciferase expressing plasmid PCIRenilla, under the control of an autologous promoter (pGL3) as previously described (Lyberopoulou et al., 2007; Mylonis et al., 2008). Luciferase activity was measured using the Dual-luciferase assay system (Promega, WI, USA) with a luminometer (TD20/20, Turner Designs).

2.5. siRNA mediated silencing

Huh7 cells were incubated in serum-free DMEM for 4 h with siRNA (10 nM) against HIF-1α or HIF-2α (Qiagen) or in the presence of Lipofectamine™2000 (Invitrogen). All Stars siRNA (Qiagen) was used as negative control.

2.6. RNA extraction and real-time PCR

Total RNA from Huh7 cells was isolated using the Trizol reagent (Invitrogen) and cDNA was synthesized with the High Capacity Reverse Transcription kit (Applied Biosystems). Real-time PCR was performed with KAPA SYBR® FAST One-Step qRT-PCR Universal (Kapa Biosystems) in a MiniOpticon instrument (Bio-Rad). The mRNAs encoding EPO, PGK and actin were amplified using primers listed in Supplementary Table 1. Each sample was assayed in triplicate for both target and internal control. Relative quantitative gene expression was calculated using the ΔΔCT method and presented as relative units.

2.7. Quantitation of EPO production

Medium was collected from 10^5 cells in 6-well culture plates and centrifuged at 800 r.p.m. for 4 min at 4 °C to remove cellular