Published in final edited form as:

Int J Biochem Cell Biol. 2013 November ; 45(11): . doi:10.1016/j.biocel.2013.07.025.

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

Christina Befani^a, Ilias Mylonis^a, Ioanna-Maria Gkotinakou^a, Panagiotis Georgoulas^b, Cheng-Jun Hu^c, George Simos^a, and Panagiotis Liakos^{a,*}

^aLaboratory of Biochemistry, Faculty of Medicine, School of Health Sciences, University of Thessaly, Biopolis, Larissa 41110, Greece

^bDepartment of Nuclear Medicine, Faculty of Medicine, School of Health Sciences, University of Thessaly, Biopolis, Larissa 41110, Greece

^cDepartment of Craniofacial Biology, University of Colorado Anschutz Medical Campus, Aurora, CO, USA

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-1 α and HIF-2 α 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-2 α interacted poorly with USF-2, a HIF-2-specific co-activator. These data show that, despite similar induction of HIF-1 α and HIF-2 α 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.

Keywords

HIF-2 α ; EPO; SOD2; Hypoxia; Cobalt; USF2

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-1 α 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-1 α was the first isoform of HIF- α to be identified by its capacity to bind to the hypoxia-responsive element (HRE) present in the erythropoietin (EPO) enhancer (Wang et al., 1995). HIF-1 α 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-2 α), 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-1 α , HIF-2 α 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-1 α expression and whether HIF-2 α 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-1 α and HIF-2 α 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-1 α and HIF-2 α 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-2 α -deficient (knock-out) mice documenting HIF-2 α 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-1 α and HIF-2 α 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-1 α and HIF-2 α 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-2 α 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-2 α with USF2. This suggests that HIF-2 α , but not HIF-1 α , 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-2 α was constructed by inserting the full length HIF-2 α cDNA into the BamHI position of the multicloning site of the pEGFP-C1 plasmid (Clontech). pcDNAHIF-2 α , kindly provided from Dr. S. L. McKnight (Department of Biochemistry, University of Texas) (Tian et al., 1997). pEGFP-HIF-1 α 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% CO₂. When required, cells were treated for 2–48 h with 150 μ M cobalt chloride (CoCl₂). The concentration of CoCl₂ (150 μ M) was determined in preliminary dose–response experiments (using 50–200 μ M) 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% O₂, 95% N₂ and 5% CO₂ in an IN VIVO₂ 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-1 α (Lyberopoulou et al., 2007), anti-HIF-2 α 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-1 α or HIF-2 α (Qiagen) or in the presence of LipofectamineTM2000 (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 $\Delta\Delta CT$ method and presented as relative units.

2.7. Quantitation of EPO production

Medium was collected from 10⁵ cells in 6-well culture plates and centrifuged at 800 r.p.m. for 4 min at 4°C to remove cellular debris and then stored at -70°C. EPO in the medium was measured by using the EPO-TracTM RIA kit (Diasorin) according to the manufacturer's instruction.

2.8. Statistical analysis

The Graph Pad Instat Statistical package for Windows was used. Data are expressed as $\pm$ mean standard deviation (SD). The oneway analysis of variance (ANOVA) with the Bonferroni post-test was used for the comparison of data and the statistical significance limit was set at $p < 0.05$.

3. Results

3.1. Hypoxia or CoCl₂ induce both HIF-2 α and HIF-1 α protein expression in Huh7 hepatoma cells

The expression of HIF-2 α in Huh7 hepatoma cells under different “hypoxic” conditions, such as true hypoxia (1% O₂) or in the presence 150 μ M CoCl₂ (chemical hypoxia) was compared to that of HIF-1 α by western blotting analysis shown in Fig. 1A. When cells were exposed to 1% O₂, HIF-2 α as well as HIF-1 α protein levels increased rapidly and remained elevated for up to 16 h. Then, both HIF-2 α and HIF-1 α expression gradually declined and returned to control levels by 48 h. When cells were treated with CoCl₂, HIF-2 α and HIF-1 α protein levels also increased with similar kinetics. However unlike hypoxia, they both remained high up to 48 h of treatment. Therefore, expression of both HIF-2 α and HIF-1 α is induced by either hypoxia or CoCl₂ but it is differentially affected by prolonged exposure to low oxygen or cobalt, indicating dissimilar induction mechanisms. It has been previously suggested that HIF-1 α protein induction in neuroblastoma cells may be more sensitive to oxygen decline (Holmquist-Mengelbier et al., 2006). To test this in Huh7 cells, HIF- α protein levels were analyzed in cells exposed to different oxygen concentrations (1–10% O₂). HIF-2 α and HIF-1 α proteins were barely detectable at 10% O₂ but the expression of both was induced at 6% O₂ and increased with similar sensitivity at less oxygen (Fig. 2B) indicating no major differences in the hypoxic stabilization of HIF-1 α and HIF-2 α in Huh7 cells.

It is generally assumed that induction of HIF-2 α is caused by protein stabilization through inhibition of proteolysis. However, recent data indicate that control of HIF-2 α expression can also take place at the levels of transcription, translation and protein stability (Keith et al.,

2012). To investigate the mechanisms behind the induction of HIF-2 α accumulation in Huh7 cells, we performed experiments with the inhibitor of transcription actinomycin D or the translation inhibitor cycloheximide. Our data show that induction of HIF-2 α by low oxygen or CoCl₂ was not dependent on new RNA synthesis but required active protein synthesis (Supplementary Fig. S1A and B). The involvement of translation was further confirmed using LY294002, an inhibitor of the PI-3 K and its downstream AKT-mTOR pathway that has been shown before to control HIF-1 α protein synthesis (Chachami et al., 2004, Triantafyllou et al., 2006). Treatment with LY98059 indeed reduced the induction of HIF-2 α by both low oxygen and cobalt (Supplementary Fig. S1C) suggesting the involvement the PI-3K pathway under both conditions. On the other hand, treatment with PD98059, an inhibitor of the MAPK pathway leading to activation of ERK1/2, attenuated the expression of HIF-2 α only under cobalt treatment, suggesting the participation of ERK1/2 in the cobalt but not hypoxia-triggered induction (Supplementary Fig. 1SC). As the PI3K/Akt-dependent pathway can be activated by the generation of ROS (Kietzmann and Gorlach, 2005), known radical scavengers, such as NAC, glutathione and Trolox were used to treat cells simultaneously exposed to either hypoxia or cobalt. In all cases HIF-2 α expression was reduced (Supplementary Fig. S1D) suggesting the involvement of ROS in the induction of HIF-2 α in Huh7 cells irrespective of the type of “hypoxic” stimulus.

3.2. Hypoxia but not cobalt induces HIF-2 α -dependent EPO mRNA synthesis and EPO secretion in Huh7 cells

In order to define the relative contributions of HIF-1 and HIF-2 in the regulation of EPO or PGK expression by hepatoma cells, we transiently knocked-down HIF-1 α and HIF-2 α expression using siRNA-mediated silencing in Huh7 cells grown under hypoxia (Fig. 2A) Hypoxia triggered a robust increase in *EPO* mRNA in control cells and in cells with silenced HIF-1 α while up-regulation of *PGK* mRNA by hypoxia was observed also in control cells and in cells with silenced HIF-2 α (Fig. 2B and D). In contrast, knock-down of HIF-1 α decreased hypoxic PGK mRNA levels, whereas knock-down of HIF-2 α decreased hypoxic EPO mRNA levels (Fig. 2B and D). Quantification of hypoxia-induced *EPO* secretion in the culture medium by RIA analysis gave similar results as determination of *EPO* mRNA (Fig. 3A) indicating that transcriptional activation of the *EPO* gene in Huh7 cells and subsequent stimulation of EPO production and secretion by low oxygen is predominantly, if not only, mediated by HIF-2 α . On the other hand, the transcriptional activation of the *PGK* gene in Huh7 cells by hypoxia is mediated only by HIF-1 α .

Surprisingly, treatment of the cells with CoCl₂, that unregulated HIF-1 α and HIF-2 α expression (Fig. 1A), did not increase in *EPO* mRNA levels, which remained similar to that under normoxic conditions (Fig. 2C). On the other hand, CoCl₂ induced upregulation of *PGK* mRNA levels in Huh7 cells to similar extent as hypoxia (Fig. 2E). To further compare the effects of low oxygen and cobalt on EPO production, we measured EPO secretion during a time course of 8–24 h. Only true hypoxia increased EPO secretion from Huh7 cells in a time-dependent manner, whereas the presence of CoCl₂ produced no effect (Fig. 3B). We, therefore, conclude that CoCl₂, unlike hypoxia, cannot activate HIF-2-dependent EPO mRNA and protein production in Huh7 cells suggesting that HIF-2 α induced by cobalt treatment is somehow unable to activate transcription of the *EPO* gene.

3.3. Cobalt-induced HIF-2 α is nuclear but transcriptionally inactive

The inability of cobalt to stimulate production of EPO indicates that HIF-2 α that is induced by this treatment lacks transcriptional activity, preconditions for which are translocation of HIF-2 α inside the nucleus and binding to HRE-containing DNA elements. To test whether induction of HIF-2 α by CoCl₂ is followed by accumulation of the protein inside the nucleus, Huh7 cells exposed to hypoxia or CoCl₂ were analyzed by immunofluorescence

microscopy. A strong nuclear signal of HIF-2 α was readily visible under both hypoxia and CoCl₂ treatment (Supplementary Fig. S2A). These data were confirmed by biochemical fractionation, which showed the exclusive presence of HIF-2 α in the nuclear fraction under both conditions (Supplementary Fig. S2B). Therefore, cobalt treatment, like hypoxia, results in both induction and nuclear accumulation of HIF-2 α .

To test whether cobalt-induced HIF-2 α as well as HIF-1 α are active in transcription, we analyzed the effect of their induction on known HRE-containing promoters such as those of the *SOD2* and *PGK* genes, which have been previously suggested to represent specific targets of HIF-2 α and HIF-1 α , respectively (Scortegagna et al., 2003; Hu et al., 2007). To first test target specificity, *SOD2* or *PGK* promoter reporter plasmids were co-transfected with HIF-1 α or HIF-2 α expression plasmids into Huh7 cells. Over-expression of HIF-2 α , but not HIF-1 α , activated the *SOD2* promoter, whereas over-expression HIF-1 α stimulated the *PGK* promoter to a much larger extent than HIF-2 α (Fig. 4A). In the inverse experiment, knocking down of HIF-2 α , but not HIF-1 α , impaired the hypoxic activation of the *SOD2* promoter, whereas hypoxic stimulation of the *PGK* promoter was repressed by knocking down HIF-1 α but not HIF-2 α (Fig. 4B). Therefore, the *SOD2* or *PGK* promoters are reliable indicators of HIF-2 and HIF-1 unique activities, respectively, in Huh7 cells. As expected, treatment of Huh7 cells harboring the aforementioned reporter constructs with hypoxia activated both *SOD2* and *PGK* promoter-driven transcription (Fig. 4C). In contrast, treatment with cobalt activated the *PGK* promoter to the same extent as hypoxia but left the *SOD2* promoter completely inactive (Fig. 4C), suggesting that the presence of cobalt does not generally affect HRE-dependent transcription but rather specifically inhibits the activity of HIF-2 α .

To test whether this effect is specific to CoCl₂ treatment, or whether other hypoxia mimetics such as DFO or DMOG produce the same result, we analyzed their effect on the activation of the *SOD2* or *PGK* promoter in Huh7 cells. Both DFO and DMOG activated both *SOD2* and *PGK* promoters (Fig. 4D), suggesting that inactivity of HIF-2 is a phenomenon specific to CoCl₂ treatment of these cells.

3.4. CoCl₂ reduces the HIF-2 α /USF2 interaction and inhibits hypoxic stimulation of HIF-2 activity

As inhibition of HIF-2 α activity, when cells are treated with CoCl₂, cannot be attributed to its altered expression, stability or nuclear localization, the affected step in HIF-2 activation must lie downstream of its nuclear translocation and may involve its ability to interact with HIF-2-specific co-activators such as USF2 (Pawlus et al., 2012). This is also suggested by the fact that CoCl₂ affects negatively only HIF-2 but not HIF-1.

To investigate this possibility, we first examined the effect of hypoxia or CoCl₂ on the expression of USF2 in Huh7 cells. USF2 was expressed at similar levels under all conditions tested (Fig. 5A). We then tested the interaction of USF2 with HIF-2 α . Huh7 cells expressing Flag-tagged USF2 were incubated under normoxia or hypoxia or in the presence of CoCl₂, lysed and subjected to immunoprecipitation using an anti-Flag antibody. Analysis of the immunoprecipitates revealed that association of HIF-2 α with USF2 was reduced in cobalt-treated cells (by approximately 40%) in comparison to hypoxia-treated cells despite similar levels of HIF-2 α protein expression (Fig. 5B). This suggests that CoCl₂ impairs HIF-2 activity, at least partly, by weakening the HIF-2 α interaction with USF2.

To further examine the effect of CoCl₂ on HIF-2 activity, we tested combined treatment of Huh7 cells with both CoCl₂ and hypoxia. CoCl₂ reduced the hypoxic stimulation of *EPO* mRNA synthesis whereas it augmented the hypoxic induction of *PGK* mRNA synthesis (Fig. 5C). These results suggest that CoCl₂ actively impairs the hypoxic stimulation of

HIF-2 transcriptional activity probably by interfering with the formation of a competent USF2-containing transcription complex.

3.5. CoCl₂ does not induce HIF-2 transcriptional activity in HepG2 cells

To investigate whether this negative, in terms of HIF-2 activity, response to CoCl₂ is cell-line specific or a general characteristic of liver cancer cells, we tested a different hepatic cell line, HepG2. Firstly, both HIF-1 α and HIF-2 α proteins were shown to be expressed in this cell line under hypoxia or in the presence of CoCl₂ (Fig. 6A). Using HIF-1 α or HIF-2 α silencing, we also confirmed that SOD2 is a HIF-2 α specific gene and PGK a HIF-1 α specific gene (Fig. 6B). Exposure to hypoxia activated both SOD2 and PGK promoters but addition of CoCl₂ only stimulated the PGK promoter (Fig. 6C). Finally hypoxia increased EPO secretion from HepG2 cells in a time dependent manner but incubation with CoCl₂ had no effect (Fig. 6D), suggesting that the inhibitory effect of CoCl₂ on HIF-2 transcriptional activity characterizes hepatic derived cancer cell lines.

4. Discussion

Many human cancer cell types exhibit increased expression of either one or both of the main HIF- α isoforms, HIF-1 α and HIF-2 α , and in many cases this overexpression is associated with poor prognosis (Keith et al., 2012). This has made HIFs major targets of anti-cancer therapy and many HIF inhibitors are currently under clinical evaluation (Semenza, 2010). However, there are certain cases of tumors in which one of the two HIF- α isoforms, but not the other, may actually confer favorable prognosis (Lidgren et al., 2005; Noguera et al., 2009). This adds to the increasingly important and acceptable notion that HIF-1 α and HIF-2 α , despite their similarities and possibly overlapping functions, can be subject to differential regulation so as to mediate unique and even opposing cellular responses often in a cell-type specific context. It has, therefore, become important to individually study each HIF- α isoform, especially in cell types that express both. To our knowledge, this report is the first systematic study of parallel HIF-1 α and HIF-2 α induction and activation in liver cancer cells. Our analysis has revealed similarities and differences both between HIF-1 α and HIF-2 α and between the types of hypoxic stimulus, i.e. low oxygen (true hypoxia) and cobalt (chemical or mimetic hypoxia), which are physiologically important at least in the context of hepatic EPO production.

It is widely reported in the literature that cancer cell HIF-1 α and HIF-2 α exhibit diverse sensitivities to different oxygen concentrations and to acute (short-term) or prolonged hypoxia (Keith et al., 2012). This stems from studies with lung adenocarcinoma, neuroblastoma and glioma cells in which HIF-1 α was induced at lower oxygen concentrations than HIF-2 α and HIF-1 α expression was transient while HIF-2 α long-lasting (Uchida et al., 2004; Nilsson et al., 2005; Holmquist-Mengelbier et al., 2006; Dioum et al., 2009). Our results clearly show that this is not a universal but rather a cell-type specific rule as in liver cancer cells we detected no isoform-specific differences when HIF-2 α and HIF-1 α expression was examined over a range of different oxygen concentrations and hypoxic exposure times. Expression of both proteins became detectable at 6% oxygen and continued to increase with declining oxygen concentrations reaching maximum at 2%. Concerning kinetics, both proteins appeared 2 h after onset of hypoxia, reached peak levels at 16 h and gradually declined to basal levels after 48 h. The down-regulation of both HIF- α isoforms after prolonged hypoxic incubation probably represents a homeostatic adaptation to the continuing reduced availability of oxygen and could be mediated by one of several mechanisms such as HIF-driven up-regulation of prolyl hydroxylases (Berra et al., 2003; Aprelikova et al., 2004; Nakayama et al., 2004), induction of antisense HIF- α (Rossignol et al., 2002) or production of dominant negative HIF- α isoforms (Lee et al., 2004). Significantly, this negative feedback loop is not established when HIF- α is induced by

cobalt since expression of both isoforms remains high even after 48 h of treatment. The same difference between hypoxia and cobalt was also observed for HIF-1 α in HeLa cells (Triantafyllou et al., 2006) and pinpoints the “non-physiological” effects of chemical hypoxia mimetics.

It is clear that stabilization of HIF- α subunits by inhibition of PHD activity is a key and universal mechanism of HIF induction by low oxygen. However, additional, often cell-type specific, regulatory mechanisms have been identified that fine-tune HIF activity to cues from the extracellular environment and the particular cellular needs (Keith et al., 2012). As we have recently shown for HIF-1 α in prostate cancer cells (Befani et al., 2012), hypoxic induction of HIF-2 α in hepatoma cells involves the PI-3K signaling pathway, activation of which is most likely required to sustain protein synthesis that also seems to be a prerequisite for HIF- α elevated expression. Cobalt treatment also requires the PI-3K signaling pathway but in addition an active MAPK pathway to induce HIF-2 α . We have made similar observations for HIF-1 α concerning both the PI-3K (Chachami et al., 2004; Triantafyllou et al., 2006) and the MAPK (Triantafyllou et al., 2006) pathways suggesting that cobalt induces expression of the two HIF- α isoforms through similar mechanisms, which are overlapping but distinct from those utilized by cells exposed to low oxygen.

However, the most striking difference between cobalt and low oxygen that this report reveals is their effect on EPO regulation as EPO gene induction and protein secretion were not observed in liver cancer cells treated by, the widely used as “hypoxia mimetic”, cobalt. This has physiological importance, as EPO is a hematopoietic growth factor that, by regulating production of red blood cells, controls a key determinant of oxygen homeostasis, blood oxygen-carrying capacity. During fetal development, EPO is mainly produced in liver; although its primary site of production then changes to the kidney during late gestation, it continues to be produced to a lesser extent in liver. Hepatic EPO contributes about 10% of the plasma EPO in adults. Hypoxia is the primary physiological stimulus for EPO production, which, depending on the hypoxic condition increases serum EPO levels up to several hundred-fold (Haase, 2010; Jelkmann, 2011).

As already mentioned, animal studies have shown that the main regulator of hepatic EPO production is HIF-2 α and not HIF-1 α (Scortegagna et al., 2005; Rankin et al., 2007; Kapitsinou et al., 2010). We confirmed the HIF-2-dependency of human EPO gene hypoxic activation at the cellular level in Huh7 and HepG2 cells in agreement with previous studies using other cell types (Warnecke et al., 2004; Chavez et al., 2006). Therefore, the inability of cobalt to induce EPO production suggested a defect in HIF-2-dependent transcriptional activation. Indeed, we show that this was the case in both cell lines by using specific HIF targets: cobalt activated the HIF-1-dependent PGK promoter but not the HIF-2-dependent SOD2 promoter, while both promoters could be stimulated by low oxygen.

This came as a surprise, as cobalt is extensively used as a hypoxia mimetic to study HIF responsiveness. Previous reports (Vengellur et al., 2005; Borcar et al., 2013) had already indicated that cobalt can elicit unique physiological responses, influencing transcription of distinct set of genes compared to hypoxia. However, these studies did not examine the role of HIF isoforms in the cobalt-specific effects. Our study is the first to compare both HIF-1 and HIF-2 functions between hypoxia and cobalt and clearly shows that, in contrast to HIF-1 α , HIF-2 α produced in cells treated with cobalt is transcriptionally inactive. This effect is specific for cobalt and not other hypoxia mimetic chemicals such as DFO and DMOG, which apparently share with low oxygen the common ability to inhibit the activity of PHDs (Wang and Semenza, 1993; Elvidge et al., 2006).

This unanticipated observation, i.e. presence of HIF-2 α protein but lack of HIF-2 activity, is not without precedent, albeit in non-cancer cells. Two previous reports have demonstrated the expression of inactive HIF-2 α in mouse embryo fibroblasts (MEFs) and embryonic stem (mES) cells (Park et al., 2003; Aprelikova et al., 2006). In the former case, the lack of HIF-2 activity was attributed to its HIF-2 α cytoplasmic mislocalization (Park et al., 2003). This does not appear to be the case in cobalt-treated hepatic cancer cells, as HIF-2 α accumulated inside the nucleus but still remained inactive. In mES cells, inhibition of HIF-2 function occurred at the level of transcription cofactor recruitment (Aprelikova et al., 2006). A very recent report indeed identified USF2 as a factor required for hypoxic induction of HIF-2 target genes in several cell lines including mES and Hep3B cells (Pawlus et al., 2012). We tested the interaction of USF2 with HIF-2 α protein in cells treated with CoCl₂ and we could demonstrate weaker association of HIF-2 α with USF2 as compared with cells treated with hypoxia. This finding explains, at least partly, the low transcriptional activity of cobalt-induced HIF-2 α . Cobalt not only induces an inactive HIF-2 α but also inhibits the activity of hypoxia-induced HIF-2 suggesting that cobalt plays an energetic negative role and does not just fail to induce a step required for HIF-2 activation. Apart of the USF2/HIF-2 α interaction, cobalt may also interfere with additional cofactors involved in HIF-2 target gene activation. There is a growing list of transcription factors the cooperation of which is required for maximal and cell-type-specific upregulation of HIF-2-dependent transcription. HIF-2 α interacts with multiple ETS family members such as ETS-1, ETS-2, GABP and ELK that may confer target specific gene selection in numerous cell types (Elvert et al., 2003; Aprelikova et al., 2006; Hu et al., 2007). The NF- κ B essential modulator (NEMO) also physically interacts with HIF-2 α , but not with HIF-1 α , and enhances HIF-2 α transcriptional activity (Bracken et al., 2005). Finally, the stress-responsive deacetylase Sirt1 selectively augments HIF-2 signaling and EPO gene expression by deacetylating HIF-2 α during hypoxia or in the presence of other environmental stresses (Dioum et al., 2009). It is, therefore, conceivable that cobalt down-regulates the transactivation of HIF-2-specific target genes by interfering with any of these factors that may be expressed in liver cancer cells.

The lesson learnt from our study is that the widely used hypoxia mimetic cobalt is not at all equivalent to true, low oxygen hypoxia: despite the fact that both induce expression of HIF- α isoforms, their biological end-effects may be completely different. Second, the mere over-expression of a HIF- α subunit does not at all mean that a full-blown response to hypoxia has been mounted: one has to also confirm activation of target genes and corresponding biological phenotype. Finally, behavior of one HIF- α isoform tells nothing about the behavior of the second and, given their distinct functions, both should be systematically monitored in terms of expression as well as activity. These should be taken seriously into consideration by research and clinical oncologists, especially as expression of HIF- α isoforms in tumor specimens is often linked to clinical outcome and HIF-inhibitors, with poorly characterized isoform selectivity, are increasingly more often tested as targeted therapeutics against different types of cancer, including HCC, or represent promising future interventions in various other hepatic diseases (Nath and Szabo, 2012).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Drs. J. A. Garcia (University of Texas), M. C. Simon (University of Pennsylvania) and M. U. Muckenthaler (University of Heidelberg) for providing plasmids.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.biocel.2013.07.025>.

References

- Aprelikova O, Chandramouli GV, Wood M, Vasselli JR, Riss J, Maranchie JK, et al. Regulation of HIF prolyl hydroxylases by hypoxia-inducible factors. *Journal of Cellular Biochemistry*. 2004; 92:491–501. [PubMed: 15156561]
- Aprelikova O, Wood M, Tackett S, Chandramouli GV, Barrett JC. Role of ETS transcription factors in the HIF-2 target gene selection. *Cancer Research*. 2006; 66:5641–7. [PubMed: 16740701]
- Befani CD, Vlachostergios PJ, Hatzidaki E, Patrikidou A, Bonanou S, Simos G, et al. Bortezomib represses HIF-1 α protein expression and nuclear accumulation by inhibiting both PI3K/Akt/TOR and MAPK pathways in prostate cancer cells. *Journal of Molecular Medicine (Berlin)*. 2012; 90:45–54.
- Berra E, Benizri E, Ginouves A, Volmat V, Roux D, Pouyssegur J. HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1 α in normoxia. *The EMBO Journal*. 2003; 22:4082–90. [PubMed: 12912907]
- Borcar A, Menze MA, Toner M, Hand SC. Metabolic preconditioning of mammalian cells: mimetic agents for hypoxia lack fidelity in promoting phosphorylation of pyruvate dehydrogenase. *Cell and Tissue Research*. 2013; 351:99–106. [PubMed: 23138570]
- Bracken CP, Whitelaw ML, Peet DJ. Activity of HIF 2 α is regulated by association with the NF-kappaB essential modulator. *The Journal of Biological Chemistry*. 2005; 280:14240–51. [PubMed: 15653678]
- Chachami G, Simos G, Hatziefthimiou A, Bonanou S, Molyvdas PA, Paraskeva E. Cobalt induces HIF-1 α expression in airway smooth muscle cells by a reactive oxygen species- and PBK-dependent mechanism. *American Journal of Respiratory Cell and Molecular Biology*. 2004; 31:544–51. [PubMed: 15256383]
- Chavez JC, Baranova O, Lin J, Pichiule P. The transcriptional activator HIF-2/EPAS-1 regulates the oxygen-dependent expression of erythropoietin in cortical astrocytes. *Journal of Neuroscience*. 2006; 26:9471–81. [PubMed: 16971531]
- Dioum EM, Chen R, Alexander MS, Zhang Q, Hogg RT, Gerard RD, et al. Regulation of HIF 2 α signaling by the stress-responsive deacetylase sirtuin 1. *Science*. 2009; 324:1289–93. [PubMed: 19498162]
- Elvert G, Kappel A, Heidenreich R, Englmeier U, Lanz S, Acker T, et al. Cooperative interaction of hypoxia-inducible factor-2 α (HIF-2 α) and Ets-1 in the transcriptional activation of vascular endothelial growth factor receptor-2 (Flk-1). *The Journal of Biological Chemistry*. 2003; 278:7520–30. [PubMed: 12464608]
- Elvidge GP, Glenny L, Appelhoff RJ, Ratcliffe PJ, Ragoussis J, Gleadle JM. Concordant regulation of gene expression by hypoxia and 2-oxoglutarate-dependent dioxygenase inhibition: the role of HIF-1 α , HIF-2 α , and other pathways. *The Journal of Biological Chemistry*. 2006; 281:15215–26. [PubMed: 16565084]
- Haase VH. Hypoxic regulation of erythropoiesis and iron metabolism. *American Journal of Physiology – Renal Physiology*. 2010; 299:F1–13. [PubMed: 20444740]
- Holmquist-Mengelbier L, Fredlund E, Lofstedt T, Noguera R, Navarro S, Nilsson H, et al. Recruitment of HIF-1 α and HIF-2 α to common target genes is differentially regulated in neuroblastoma: HIF-2 α promotes an aggressive phenotype. *Cancer Cell*. 2006; 10:413–23. [PubMed: 17097563]
- Hu CJ, Sataur A, Wang L, Chen H, Simon MC. The N-terminal transactivation domain confers target gene specificity of HIF-1 α and HIF-2 α . *Molecular Biology of the Cell*. 2007; 18:4528–42. [PubMed: 17804822]
- Jelkmann W. Regulation of erythropoietin production. *Journal of Physiology*. 2011; 589:1251–8. [PubMed: 21078592]

- Kaelin WG Jr, Ratcliffe PJ. Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. *Molecular Cell*. 2008; 30:393–402. [PubMed: 18498744]
- Kalouisi A, Mylonis I, Politou AS, Chachami G, Paraskeva E, Simos G. Casein kinase 1 regulates human HIF-1. *Journal of Cell Science*. 2010; 123:2976–86. [PubMed: 20699359]
- Kapitsinou PP, Liu Q, Unger TL, Rha J, Davidoff O, Keith B, et al. Hepatic HIF-2 regulates erythropoietic responses to hypoxia in renal anemia. *Blood*. 2010; 116:3039–48. [PubMed: 20628150]
- Keith B, Johnson RS, Simon MC. HIF1 α and HIF2 α : sibling rivalry in hypoxic tumour growth and progression. *Nature Reviews*. 2012; 12:9–22.
- Kietzmann T, Gorkach A. Reactive oxygen species in the control of hypoxia-inducible factor-mediated gene expression. *Seminars in Cell and Developmental Biology*. 2005; 16:474–86. [PubMed: 15905109]
- Lee JS, Chu IS, Heo J, Calvisi DF, Sun Z, Roskams T, et al. Classification and prediction of survival in hepatocellular carcinoma by gene expression profiling. *Hepatology (Baltimore, MD)*. 2004; 40:667–76.
- Lidgren A, Hedberg Y, Grankvist K, Rasmuson T, Vasko J, Ljungberg B. The expression of hypoxia-inducible factor 1 α is a favorable independent prognostic factor in renal cell carcinoma. *Clinical Cancer Research*. 2005; 11:1129–35. [PubMed: 15709180]
- Lyberopoulou A, Venieris E, Mylonis I, Chachami G, Pappas I, Simos G, et al. MgcRac-GAP interacts with HIF-1 α and regulates its transcriptional activity. *Cellular Physiology and Biochemistry*. 2007; 20:995–1006. [PubMed: 17982282]
- Majmundar AJ, Wong WJ, Simon MC. Hypoxia-inducible factors and the response to hypoxic stress. *Molecular Cell*. 2010; 40:294–309. [PubMed: 20965423]
- Mylonis I, Chachami G, Paraskeva E, Simos G. Atypical CRM1-dependent nuclear export signal mediates regulation of hypoxia-inducible factor-1 α by MAPK. *The Journal of Biological Chemistry*. 2008; 283:27620–7. [PubMed: 18687685]
- Mylonis I, Chachami G, Samiotaki M, Panayotou G, Paraskeva E, Kalouisi A, et al. Identification of MAPK phosphorylation sites and their role in the localization and activity of hypoxia-inducible factor-1 α . *The Journal of Biological Chemistry*. 2006; 281:33095–106. [PubMed: 16954218]
- Mylonis I, Sembongi H, Befani C, Liakos P, Siniosoglou S, Simos G. Hypoxia causes triglyceride accumulation by HIF-1-mediated stimulation of lipin 1 expression. *Journal of Cell Science*. 2012; 125:3485–93. [PubMed: 22467849]
- Mylonis, I.; Simos, G. The involvement of the ERK-hypoxia-angiogenesis signaling axis and HIF-1 in hepatocellular carcinoma. In: Lau, W-Y., editor. *Hepatocellular carcinoma-basic research*. 2012. In: In Tech
- Nakayama K, Frew IJ, Hagensen M, Skals M, Habelhah H, Bhoumik A, et al. Siah2 regulates stability of prolyl-hydroxylases, controls HIF1 α abundance, and modulates physiological responses to hypoxia. *Cell*. 2004; 117:941–52. [PubMed: 15210114]
- Nath B, Szabo G. Hypoxia and hypoxia inducible factors: diverse roles in liver diseases. *Hepatology (Baltimore, MD)*. 2012; 55:622–33.
- Nilsson H, Jogi A, Beckman S, Harris AL, Poellinger L, Pahlman S. HIF-2 α expression in human fetal paraganglia and neuroblastoma: relation to sympathetic differentiation, glucose deficiency, and hypoxia. *Experimental Cell Research*. 2005; 303:447–56. [PubMed: 15652356]
- Noguera R, Fredlund E, Piqueras M, Pietras A, Beckman S, Navarro S, et al. HIF-1 α and HIF-2 α are differentially regulated in vivo in neuroblastoma: high HIF-1 α correlates negatively to advanced clinical stage and tumor vascularization. *Clinical Cancer Research*. 2009; 15:7130–6. [PubMed: 19903792]
- Park SK, Dadak AM, Haase VH, Fontana L, Giaccia AJ, Johnson RS. Hypoxia-induced gene expression occurs solely through the action of HIF-1 α : role of cytoplasmic trapping of HIF-2 α . *Molecular and Cellular Biology*. 2003; 23:4959–71. [PubMed: 12832481]
- Pawlus MR, Wang L, Ware K, Hu CJ. USF2 and HIF2 α cooperatively activate HIF2 target genes during hypoxia. *Molecular and Cellular Biology*. 2012

- Rankin EB, Biju MP, Liu Q, Unger TL, Rha J, Johnson RS, et al. Hypoxia-inducible factor-2 (HIF-2) regulates hepatic erythropoietin in vivo. *The Journal of Clinical Investigation*. 2007; 117:1068–77. [PubMed: 17404621]
- Rossignol F, Vache C, Clottes E. Natural antisense transcripts of hypoxia-inducible factor 1alpha are detected indifferent normal and tumour human tissues. *Gene*. 2002; 299:135–40. [PubMed: 12459261]
- Scortegagna M, Ding K, Oktay Y, Gaur A, Thurmond F, Yan LJ, et al. Multiple organ pathology, metabolic abnormalities and impaired homeostasis of reactive oxygen species in *Epas1*^{-/-} mice. *Nature Genetics*. 2003; 35:331–40. [PubMed: 14608355]
- Scortegagna M, Ding K, Zhang Q, Oktay Y, Bennett MJ, Bennett M, et al. HIF-2alpha regulates murine hematopoietic development in an erythropoietin-dependent manner. *Blood*. 2005; 105:3133–40. [PubMed: 15626745]
- Semenza GL. Defining the role of HIF 1 in cancer biology and therapeutics. *Oncogene*. 2010; 29:625–34. [PubMed: 19946328]
- Tian H, McKnight SL, Russell DW. Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. *Genes and Development*. 1997; 11:72–82. [PubMed: 9000051]
- Triantafyllou A, Liakos P, Tsakalof A, Chachami G, Paraskeva E, Molyvdas PA, et al. The flavonoid quercetin induces hypoxia-inducible factor-1alpha (HIF-1alpha) and inhibits cell proliferation by depleting intracellular iron. *Free Radical Research*. 2007; 41:342–56. [PubMed: 17364964]
- Triantafyllou A, Liakos P, Tsakalof A, Georgatsou E, Simos G, Bonanou S. Cobalt induces HIF-1α in HeLa cells by an iron-independent, but ROS-, PI-3K- and MAPK-dependent mechanism. *Free Radical Research*. 2006; 40:847–56. [PubMed: 17015263]
- Triantafyllou A, Mylonis I, Simos G, Bonanou S, Tsakalof A. Flavonoids induce HIF-1α but impair its nuclear accumulation and activity. *Free Radical Biology and Medicine*. 2008; 44:657–70. [PubMed: 18061585]
- Uchida T, Rossignol F, Matthay MA, Mounier R, Couette S, Clottes E, et al. Prolonged hypoxia differentially regulates hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha expression in lung epithelial cells: implication of natural antisense HIF-1alpha. *The Journal of Biological Chemistry*. 2004; 279:14871–8. [PubMed: 14744852]
- Vengellur A, Phillips JM, Hogenesch JB, LaPres JJ. Gene expression profiling of hypoxia signaling in human hepatocellular carcinoma cells. *Physiological Genomics*. 2005; 22:308–18. [PubMed: 15942021]
- Wang GL, Jiang BH, Rue EA, Semenza GL. HIF 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O₂ tension. *Proceedings of the National Academy of Sciences of the United States of America*. 1995; 92:5510–4. [PubMed: 7539918]
- Wang GL, Semenza GL. Desferrioxamine induces erythropoietin gene expression and hypoxia-inducible factor 1 DNA-binding activity: implications for models of hypoxia signal transduction. *Blood*. 1993; 82:3610–5. [PubMed: 8260699]
- Warnecke C, Zaborowska Z, Kurreck J, Erdmann VA, Frei U, Wiesener M, et al. Differentiating the functional role of HIF-1α and HIF-2α (EPAS-1) by the use of RNA interference: erythropoietin is a HIF-2α target gene in Hep3B and Kelly cells. *FASEB Journal*. 2004; 18:1462–4. [PubMed: 15240563]

Fig. 1. Hypoxia or CoCl₂ induce HIF-2α and HIF-1α protein expression in Huh7 cells. (A) Cells were exposed to hypoxia (1% O₂) or treated with CoCl₂, for the indicated times, and HIF-2α and HIF-1α were detected by immunoblotting. Actin served as a loading control. (B) Cells were grown at the indicated % O₂ for 2 h and analyzed as in (A).

Fig. 2. HIF-1 α -dependent PGK gene expression is induced by both hypoxia and cobalt but HIF-2 α -dependent EPO gene expression is induced only by hypoxia. (A) Western blotting analysis of HIF-1 α and HIF-2 α protein expression in Huh7 cells transfected with HIF-1 α , HIF-2 α or control siRNA for 32 h. Cells were incubated in hypoxia for 16 h before collection and lysis. (B) q-PCR analysis of *EPO* mRNA expression in Huh7 cells transfected and incubated as (A). (C) q-PCR analysis of *EPO* in Huh7 cells cultured under normoxia or hypoxia or in the presence of CoCl₂. (D) q-PCR analysis of *PGK* mRNA expression in Huh7 cells transfected and incubated as (A). (E) q-PCR analysis of *PGK* in Huh7 cells cultured under normoxia or hypoxia or in the presence of CoCl₂. Results are shown as fold increase in relation to the corresponding normoxic conditions and represent the mean of three independent experiments performed in triplicate ($\pm$ SEM).

Fig. 3.

HIF-2 α -dependent EPO secretion is induced by hypoxia but not by cobalt. (A) Huh7 cells transfected with HIF-1 α , HIF-2 α or control siRNA for 32 h were incubated in hypoxia for 16 h and EPO secretion was measured by radioimmunoassay in conditioned medium. (B) Huh7 cells were exposed to normoxia or hypoxia or CoCl₂ for the indicated times and conditioned medium was analyzed as in (A). Values are expressed as mU/ml/mg protein.

Fig. 4.

SOD2, a HIF-2α specific promoter, is activated by hypoxia, DFO and DMOG but not by cobalt in Huh7 cells. (A) Huh7 cells were transfected with *SOD2*-Luc (left) or *PGK*-Luc (right) reporter along with full length pGFP-HIF-2α or pGFP-HIF-1α or empty vector. Transfected cells cultured under normoxia were collected 24 h posttransfection and assayed for luciferase activity. Values are expressed in relation to the value obtained for the empty pGFP vector and represent the mean (±SEM) of three independent experiments performed in triplicate. (B) Luciferase activity in Huh7 cells transfected with HIF-1α (20 nM) or HIF-2α (20 nM) or control siRNA (20nM) along with *SOD2*-Luc (left) or *PGK*-Luc (right) reporter for 32 hours and incubated in normoxia or hypoxia for 16 h before collection. Values are expressed in relation to normoxia and represent the mean (±SEM) of three independent experiments performed in triplicate. (C) Luciferase activity in Huh7 cells transfected with *SOD2*-Luc (left) or *PGK*-Luc (right) reporter for 24 hours and incubated in normoxia or hypoxia or in the presence of CoCl₂ for 16 h before collection. Values are expressed as in (B). (D) Luciferase activity in Huh7 cells transfected with *SOD2*-Luc (left) or *PGK*-Luc (right) reporter for 24 h and incubated in normoxia or in the presence of DFO or DMOG for 16 h before collection. Values are expressed as in (B).

Fig. 5.

CoCl₂ weakens the HIF-2α-USF2 interaction in comparison to hypoxia and inhibits the hypoxic stimulation of HIF-2 activity. (A) Huh7 cells were exposed to hypoxia (1% O₂) or treated with CoCl₂, for 16 h, and HIF-2α and USF2 were detected by immunoblotting. Actin served as a loading control. (B) Huh7 cells expressing Flag or USF2-Flag were incubated as in (A) and were lysed and subjected to immunoprecipitation with an anti-Flag antibody. Total cell extracts (input) and precipitated proteins (IP) were analyzed by western blot using anti-Flag and anti-HIF-2α antibodies as indicated. Only the relevant parts of the blots are shown. (C) q-PCR analysis of *EPO* and *PGK* in Huh7 cells cultured under normoxia or hypoxia or in the presence of CoCl₂ or under hypoxia and CoCl₂ simultaneously for 16 h. Results are shown as fold increase in relation to the corresponding normoxic conditions and represent the mean of three independent experiments performed in triplicate (±SEM).

Fig. 6.

CoCl₂ does not induce HIF-2α transcriptional activity in HepG2 cells. (A) Cells were exposed to hypoxia (1% O₂) or treated with CoCl₂, for 4 h, and HIF-2α or HIF-1α was detected by immunoblotting. (B) Luciferase activity in HepG₂ cells transfected with HIF-1α (20 nM) or HIF-2α (20 nM) or scrambled siRNA (20 nM) along with pGL3-SOD2 promoter or PGK promoter for 32 h and incubated in normoxia or hypoxia for 16 h before collection. Values are expressed in relation to normoxia and represent the mean (±SEM) of three independent experiments performed in triplicate. (C) Luciferase activity in Huh7 cells transfected with pGL3-SOD2 promoter or PGK promoter for 24 h and incubated in normoxia or hypoxia or the presence of CoCl₂ for 16 h before collection. Values are expressed as in (B). (D) HepG₂ cells were exposed to normoxia or hypoxia or CoCl₂ for the indicated times and conditioned medium was analyzed as in Fig. 2D. Values are expressed as mU/ml/mg protein.