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Cobalt induces hypoxia-inducible factor-1α (HIF-1α) in HeLa cells by an iron-independent, but ROS-, PI-3K- and MAPK-dependent mechanism

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Abstract

The iron-chelator desferrioxamine (DFO) and the transition metal cobalt induce hypoxia-inducible factor-1α (HIF-1α) in normoxia. DFO stabilizes HIF-1α from proteolysis by inhibiting the activity of iron-dependent prolyl hydroxylases, but the mechanism of action of cobalt is not fully elucidated. The purpose of this study was to examine the regulation of HIF-1α induction and HeLa cell proliferation by cobalt and the role of iron in these processes. Our results show that, unlike DFO, induction of transcriptionally active HIF-1α by $CoCl_2$ cannot be abrogated by the addition of excess Fe^{3+}, but involves the production of reactive oxygen species (ROS) and the operation of the phosphatidylinositol-3 kinase (PI-3K) and MAPK pathways. $CoCl_2$, as well as DFO, decreased HeLa cell proliferation, but these effects were reversed by the addition of Fe^{3+}. We conclude that the effect of cobalt on cell proliferation is iron-dependent, while its effects on HIF-1α induction are ROS- and signaling pathways-dependent, but iron-independent.

Keywords: *Hypoxia, hypoxia-inducible factor-1, cobalt, iron, desferrioxamine, HeLa cell proliferation*

Introduction

Cells subjected to hypoxia (reduced availability of oxygen) respond by transcriptional changes that promote increased anaerobic metabolism, erythropoiesis, angiogenesis and other adaptive responses [1–3]. The key mediator of these changes is the transcription factor hypoxia-inducible factor 1 (HIF-1), a heterodimer of the protein subunits HIF-1α, which is induced by hypoxia and HIF-1β or aryl hydrocarbon receptor nuclear translocator (ARNT), which is constitutively expressed [4].

Hypoxia regulates the levels of HIF-1α protein mainly post-translationally by inhibiting its ubiquitination and proteolysis [5,6]. In normoxia (21% oxygen), 2-oxoglutarate-, O_2- and Fe^{2+}-dependent prolyl hydroxylases (PHDs: proline hydroxylase domain proteins) catalyse hydroxylation of specific proline residues in HIF-1α. This modification is required for the binding of the von-Hippel–Lindau tumor suppressor protein (pVHL), a component of an E3 ubiquitin ligase, which targets HIF-1α for proteosomal degradation [7–10]. Furthermore in normoxia, hydroxylation of HIF-1α by an asparagine hydroxylase, called factor-inhibiting HIF-1 (FIH-1), leads to HIF-1α transcriptional inactivation [11]. In hypoxic conditions, hydroxylation by the PHDs and FIH is inhibited, leading to HIF-1α stabilization and activation. Full transcriptional activation of HIF-1 requires phosphorylation and nuclear translocation of HIF-1α, heterodimerisation with ARNT, binding to the hypoxia-response-elements (HREs) of target genes and interaction with the coactivator p300/CBP and other proteins [2,3,12,13].

HIF-1α can also be induced in normoxic conditions by certain chemicals, called "hypoxia mimetics", such as the iron-chelator desferrioxamine (DFO) [14] and

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the carcinogenic transition metal cobalt [15]. It has been suggested that both DFO and cobalt stabilize HIF-1α from degradation by inhibiting the activity of the PHDs, through Fe^{2+} depletion or substitution, respectively [16]. However, other studies have proposed alternative ways for the action of cobalt. It has been shown that cobalt can prevent the binding of HIF-1α to pVHL [17], inhibit HIF-1α degradation [18], or deplete ascorbate [19], which is essential for maintaining the PHDs in the active (Fe^{2+}) state [20]. Finally, the cobalt-induced increase in HIF-1α levels has been reported to depend on ROS formation in Hep3B [21] and Mum2B cells [22] and our previous results have implicated ROS formation, as well as the operation of the phosphatidylinositol-3 kinase (PI-3K) signaling pathway, in the effects of cobalt in tracheal smooth muscle cells [23].

HIF-1α targets include both pro- and anti-apoptotic genes [3] and depending on the cell type and severity of hypoxic stress, both increased and decreased survival, proliferation and apoptosis have been reported [24–27]. However, the question of whether the induction of HIF-1α by "hypoxia mimetics" has identical effects on cell survival and proliferation to that produced by the reduced availability of oxygen has not been systematically addressed. This is of considerable biological relevance, given the diverse actions that many of these chemicals exert. More specifically, the relative importance for cell survival and proliferation of HIF-1 expression versus other responses elicited by DFO and cobalt is unknown.

In order to address these issues, we studied the regulation of HIF-1α expression and HeLa cell proliferation and cycle progression by cobalt chloride and examined the role of iron in these processes. For comparison, we also examined the involvement of iron in HIF-1α induction and cell proliferation in DFO- or hypoxia-treated HeLa cells. Our results show differences between hypoxia and its chemical mimetics and demonstrate that the biological effects of cobalt on HIF-1α expression are ROS- and kinase signaling-dependent, but iron-independent, whereas its effects on cell proliferation are iron-dependent.

Materials and methods

Reagents

Ferric citrate, cycloheximide, DFO, cobalt chloride, *N*-acetyl-L-cysteine (NAC) and other reagents were purchased from Sigma Chemicals, unless otherwise stated. Trolox was from FLUKA. The kinase inhibitors, LY294002 and PD98059, were from Cell Signaling.

Cell cultures

The human cervical epithelial cell line HeLa was maintained in Dulbecco's modified Eagle medium (DMEM, High Glucose) (Gibco BRL), supplemented with 10% fetal bovine serum (Biochrom KG Seromed), antibiotic–antimycotic solution (Gibco BRL) and 2 mM L-glutamine (Biochrom KG Seromed). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Hypoxic conditions were created by maintaining cells in a sealed chamber (Billups-Rothenberg) flooded with 94% N_2, 5% CO_2, 1–1.5% O_2 and oxygen tension was monitored continuously with a Clark-type oxygen electrode. DFO or cobalt chloride was added at a final concentration of 150 μM. Where present, ferric citrate was added 1 h before, NAC 2 h before and Trolox simultaneously with the induction of hypoxic stress. All other reagents were added at the times and concentrations stated in the legends to figures. All experiments were performed before cells reached confluence and were repeated at least three times.

Western blot analysis of cellular proteins

After incubating HeLa cells for various times with the different effectors, cells were lysed in 20 mM Tris–Cl, 50 mM NaCl, 10% Glycerol, 1% Triton-X100, 0.5% β-mercaptoethanol, 6 mM $MgCl_2$ in the presence of a cocktail of protease inhibitors (pefabloc 1 mM, leupeptin 1 μM and pepstatin 1 μM). Protein extracts (40 μg) were resolved by 8% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membranes according to standard protocols. Western blots were analysed with anti-HIF-1α mouse monoclonal antibody (BD Transduction Laboratories), or anti-ARNT mouse monoclonal antibody (BD Transduction Laboratories), or anti-actin mouse monoclonal antibody (Serotec), or anti-p42/44 MAPK rabbit polyclonal antibody (Cell Signaling). Membranes were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (BioRad) or goat anti-rabbit IgG (Cell Signaling) and proteins were detected by enhanced chemiluminescence (ECL, Amersham). Experiments were repeated at least three times and representative blots are shown. For quantitation, image scans of Western blots from independent experiments were used with a Photoshop program and the ratio of the intensity of the HIF-1α band to the actin band was calculated and expressed as a percent of the control samples.

DNA synthesis assays

DNA synthesis was assessed in triplicate wells by the incorporation of [^{3}H]-thymidine. HeLa cells were seeded in 22 mm wells at a density of 10^5 cells/well and were incubated with the various effectors for the indicated periods of time. Four hours before the end of the incubation period, 0.25 μCi [^{3}H]-thymidine (44 Ci/mmol, Amersham) was added to each well. Radioactivity incorporated into trichloracetic

acid-insoluble material was measured in a β liquid scintillation counter.

Cell numbers were determined in triplicate 22 mm wells. After incubation in various conditions, non-confluent cells were recovered with trypsin-EDTA (Biochrom KG) and counted in a Neubauer plate after trypan blue coloration.

ROS measurements in cells

ROS levels were measured using 2,7-dichlorodihydrofluorescein diacetate (H2DCF-DA; Molecular Probes, Invitrogen detection technologies). H2DCF-DA is a nonfluorescent compound that is loaded into cells as a cell-permeant ester. On cleavage by intracellular esterases, it can be oxidized by ROS to a fluorescent product. HeLa cells, cultured for 4 h with various effectors, were loaded with 10 μM H2DCF-DA for 30 min at 37°C. Cells were then trypsinized, resuspended in PBS and submitted to flow cytometric analysis. Fluorescence was measured at 520 nm following excitation with 488 nm light from an argon laser with a FAC scan. Detection was based on the mean fluorescence intensity of 20,000 cells.

Flow cytometric analysis of cellular DNA content

Cells were exposed to hypoxia (1% O_2), 150 μM DFO or 150 μM $CoCl_2$ for 24 h and were then detached by trypsinization and fixed overnight in 70% ice-cold ethanol. After washing with PBS, cells were resuspended at a density of 10^6 cells/ml in staining solution (10 pg/ml propidium iodide, 100 pg/ml RNase A) for 45 min in the dark and then immediately analyzed on an EPICS flow cytometer (Beckman Coulter).

Immunofluorescence microscopy

Cells growing on glass slides were fixed with 3% formaldehyde and permeabilized with 1% Triton X-100. After blocking nonspecific binding with 3% bovine serum albumin (BSA) in PBS-0.1% Tween 20 overnight, the cells were incubated with an anti-HIF-1 antibody (BD Transduction Laboratories, 1: 200 dilution). Primary antibody was detected by incubation with a fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse antibody (BioRad, 1: 50 dilution). The cells were visualized and photographed utilizing a fluorescence Optiphot-2 microscope and UFX-DX camera system (Nikon).

Reporter gene assays

HeLa cells were transiently co-transfected with a hypoxia-responsive luciferase reporter gene and a constitutive β-gal expression vector, using the Trans-Pass D2 DNA transfection reagent according to the manufacturers instructions (New England Biolabs). Cells were transfected with 1 μg pGL3–5HRE–VEGF plasmid (containing the luciferase reporter gene under the control of five tandemly repeated HRE motifs from the VEGF gene, driven by the SV40 promoter), kindly provided by Dr A. Giaccia, or 1 μg pGL3-promoter plasmid (same as the previous plasmid, but without the HREs) and 1 μg CMV-lacZ plasmid, both kindly provided by Dr A. Kretsovali. The transfected cells were cultured for 24 h and were subsequently incubated for 4 h under different conditions of hypoxic stress, in the presence or absence of 0.5 mM ferric citrate. Luciferase activity was determined by a chemiluminescence assay kit (Promega) with a luminometer (TD20/20, Turner Designs) and was normalized to β-galactosidase activity.

Statistical analysis

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

Results

Kinetics of induction of HIF-1α by $CoCl_2$, DFO or hypoxia

In order to compare the characteristics of induction of HIF-1α by $CoCl_2$ with that produced by hypoxia or iron chelation, we performed the kinetic analysis shown in Figure 1. The concentrations of cobalt

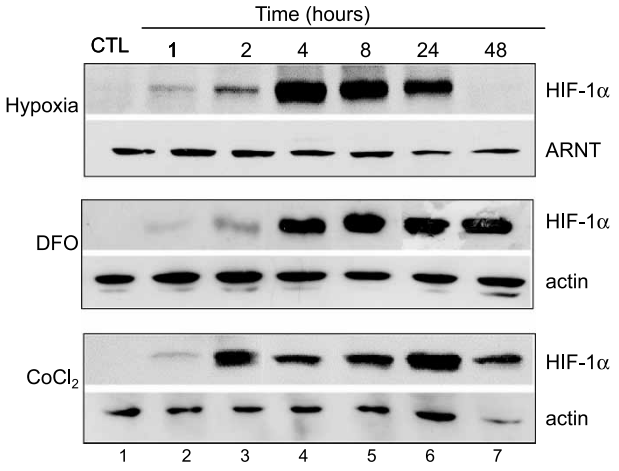

Figure 1. Western blot analysis of the kinetics of HIF-1α expression in hypoxically stressed HeLa cells. Lanes 2–7: Cells were exposed to hypoxia (1.5% O_2), DFO, or $CoCl_2$ for the indicated times and cell lysates were prepared and analysed as described in Materials and Methods. Lane 1: CTL, control untreated cells, cultured at normoxic conditions. Equal protein loading was confirmed by reprobing the membranes for ARNT or actin expression, as indicated.

or DFO employed (150 μM) were determined in preliminary dose-response experiments (using 50–200 μM of each chemical) to produce maximal induction of HIF-1α at 4–24 h, without being toxic to cells (results not shown). In subconfluent cultures, HeLa cells do not constitutively express HIF-1α, as shown by Western blotting (Figure 1, lane 1). Under hypoxic conditions, HIF-1α accumulation was detectable by 1 h, was maximal at 4–8 h, but decreased to control, non-detectable levels by 48 h, although the cells continued to grow normally and had not become confluent. Moreover, use of an anti-ARNT antibody confirmed that the observed changes were specific to HIF-1α. In contrast, in cells cultured with DFO or cobalt chloride, HIF-1α protein levels remained high up to 48 h. These results show that the expression of HIF-1α is differentially affected by prolonged exposure to hypoxia or its chemical mimetics.

Reactive oxygen species are involved in the induction of HIF-1α by $CoCl_2$, but not by hypoxia or DFO

There are conflicting reports in the literature concerning the involvement of ROS in the induction of HIF-1α [21–23,28–30]. To clarify the involvement of ROS in HIF-1α induction in HeLa cells, we investigated how the anti-oxidant NAC, a precursor of glutathione and free radical scavenger, affected the expression of HIF-1α. We found that 5 mM NAC did not influence the expression of HIF-1α by hypoxia or DFO, but led to a marked decrease of its induction by $CoCl_2$ (Figure 2A, lane 2), a compound known to generate ROS [31]. NAC had no effect on cell viability

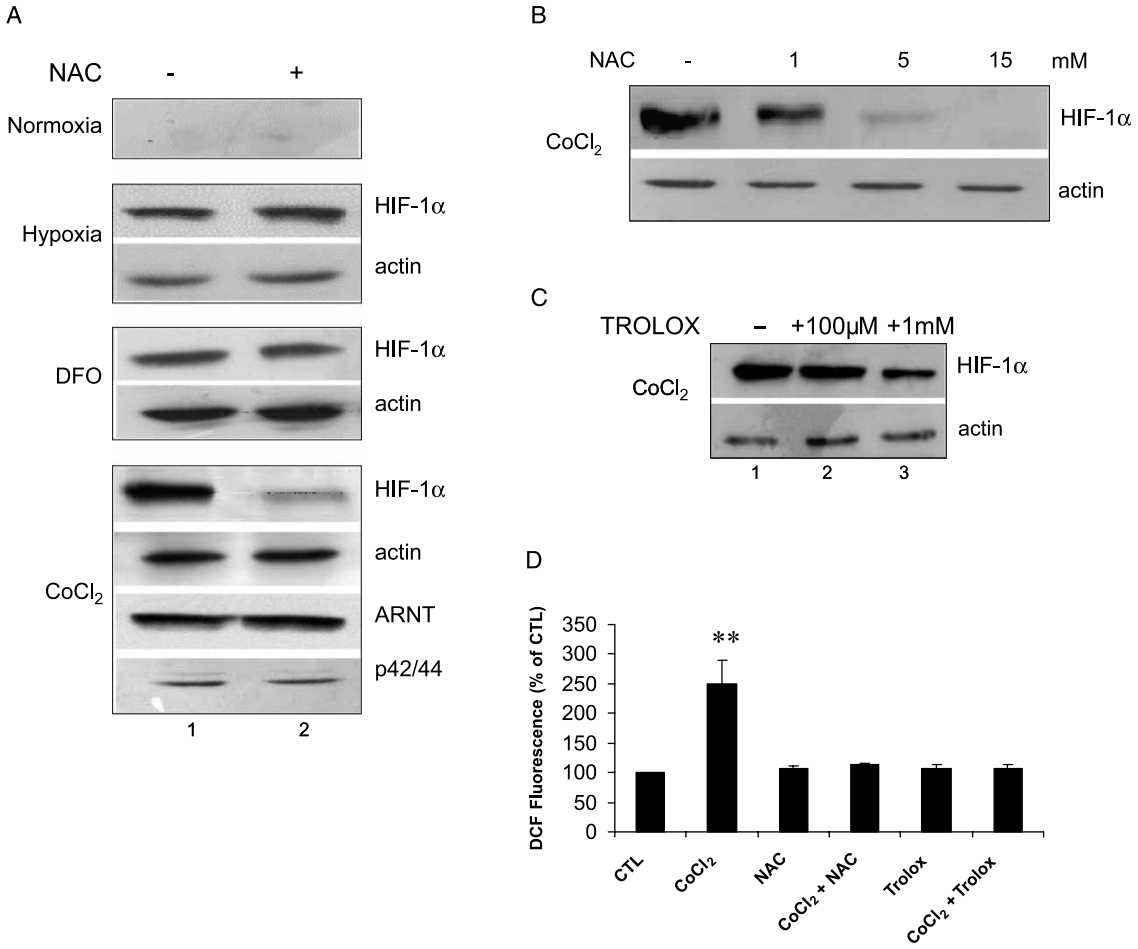

Figure 2. Involvement of ROS in HIF-1α expression in hypoxically stressed HeLa cells. A. Effect of NAC. Cells were cultured for 4 h, under the conditions indicated, in the presence (lane 2) or absence (lane 1) of 5 mM NAC and lysates were prepared and analysed for HIF-1α expression. Besides actin, the $CoCl_2$ samples were tested for the expression of ARNT and p42/44. B, C. Concentration-dependent effect of antioxidants. Cells were cultured for 4 h with $CoCl_2$ in the presence of the indicated concentrations of NAC (B) or Trolox (C) and cell lysates were analyzed for HIF-1α expression. Data in A, B and C are representative of three independent experiments. D. Measurement of ROS. After culturing for 4 h with $CoCl_2$, 5 mM NAC, 1 mM Trolox, or the combinations indicated, cells were incubated with 10 μM H2DCF-DA and processed as described in Materials and Methods. CTL, control untreated cells. The relative change in mean fluorescence was calculated as the ratio of mean fluorescence channels for the treated to untreated (control) cells. The results are the mean ± SD of three independent experiments. **, $P < 0.01$ versus control.

after 4 h of culture under conditions of hypoxic stress (results not shown). Furthermore, its effect was specific for HIF-1α, as it did not influence the expression of actin, ARNT, or p42/44 MAPK, a short-lived signaling protein. Similar results were obtained when these experiments were performed in the presence of 10 mM glutathione (results not shown).

The effect of NAC was concentration-dependent: cobalt-induced HIF-1α protein levels were slightly decreased with 1 mM NAC, but were completely abolished with 15 mM NAC (Figure 2B). Because NAC and possibly glutathione, may chelate cobalt (II), we repeated the cobalt experiment in the presence of Trolox, a water-soluble analogue of vitamin E, which is not expected to bind metals and found that 1 mM Trolox also reduced the cobalt-induced expression of HIF-1α (Figure 2C, lane 3). Furthermore, we independently confirmed, by flow cytometric analysis using the redox-sensitive dye H2DCF-DA, that under our culturing conditions, $CoCl_2$ caused a 2.5-fold increase in intracellular ROS levels and that this effect was reversed by addition of the ROS scavengers NAC or Trolox (Figure 2D). These data (Figure 2A–D) provide strong evidence that ROS are involved in the induction of HIF-1α by cobalt in HeLa cells.

The PI-3K and ERK1/2 pathways are involved in the induction of HIF-1α by $CoCl_2$, but not by hypoxia or DFO

The expression and activity of HIF-1α can be affected by the MAPK or PI-3K signaling pathways, depending on the cell type and inducer employed [2,32]. We found that when the inhibitor of the PI-3K, LY 294002, or the inhibitor of the extracellular regulated kinase ERK1/2, PD98059, were present in the cell cultures, the induction of expression of HIF-1α by hypoxia or DFO was not affected (Figure 3, left panels—compare lanes 2 and 3 with 1). However, HIF-1α expression was decreased in the presence of either inhibitor in the cobalt-treated cells. Quantitation and statistical analysis of the results of three independent experiments revealed that, in the cobalt-treated cells, this decrease in HIF-1α was significant (Figure 3, right panels). These data indicate that the action of cobalt chloride is mediated, at least partly, by PI-3K and ERK1/2 signaling.

Ferric citrate abrogates HIF-1α induction by DFO, but not by $CoCl_2$ or hypoxia

It has been shown that the induction of HIF-1α by DFO can be reversed by excess iron [14].

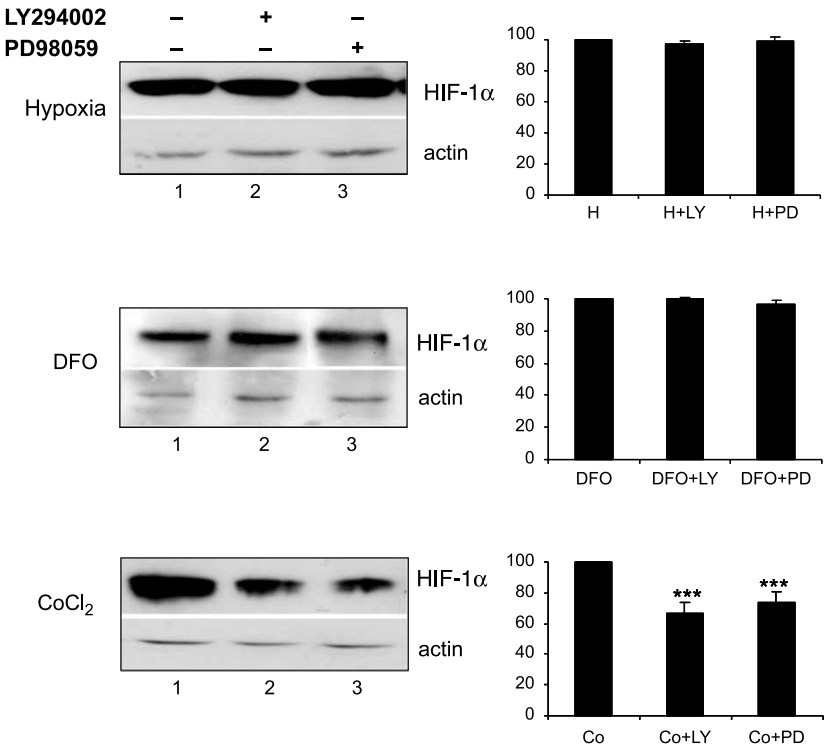

Figure 3. The effect of PI-3K and ERK1/2 kinase inhibitors on the expression of HIF-1α in hypoxically stressed HeLa cells. A. Western blot analysis. LY294002 (lane 2) or PD98059 (lane 3) was added or not (lane 1) at a concentration of 50 μM, 15 min before culturing cells for 4 h under hypoxic (1% O_2) conditions, or with DFO or $CoCl_2$ and lysates were prepared and analyzed as previously described. B. Blot quantitation and statistical analysis. These were performed as described in Material and Methods, using results from three independent experiments. *** = $p < 0.001$.

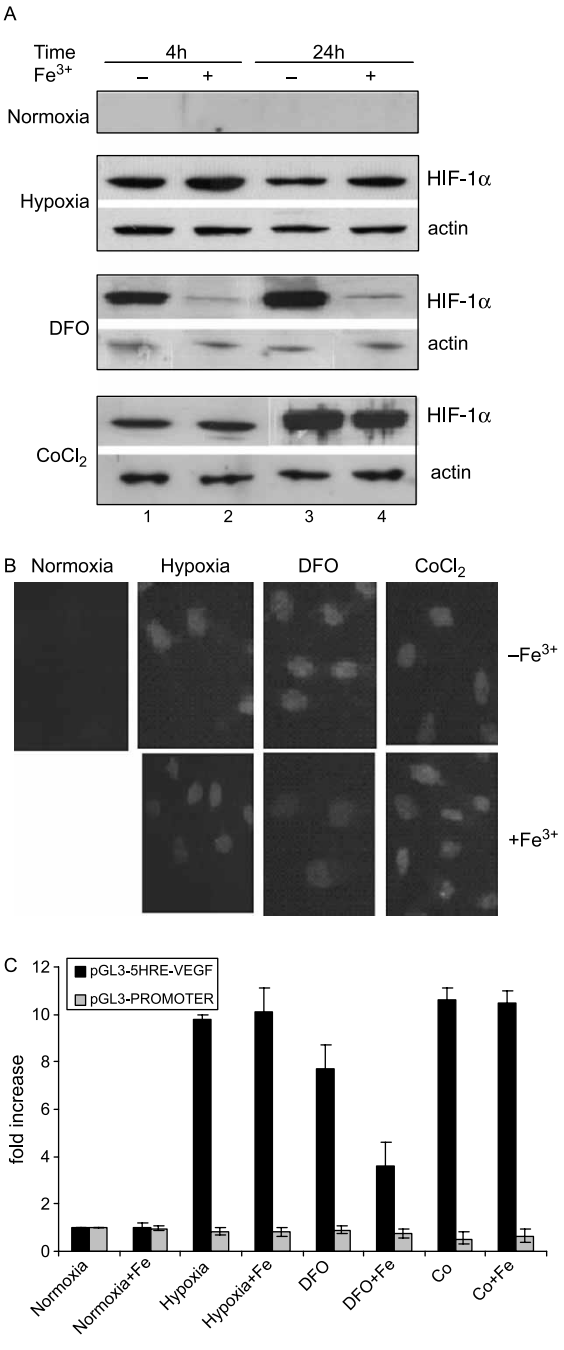

Figure 4. The effect of ferric citrate on HIF-1α expression in HeLa cells cultured under different conditions of hypoxic stress. A. Western blot analysis of HIF-1α. Cells were cultured for 4 or 24 h as indicated, in the absence (lanes 1, 3) or presence (lanes 2, 4) of 0.5 mM ferric citrate (Fe^{3+}). B. Immunofluorescence analysis of HIF-1α. HeLa cells were cultured as shown for 4 h without (upper panel) or with (lower panel) 0.5 mM ferric citrate (Fe^{3+}). Data in A and B are representative of three independent experiments. C. Reporter gene assays of the transcriptional activity of HIF-1. Transient transfections with pGL3–5HRE–VEGF (filled bars) or pGL3-promoter (open bars) were performed as described in Materials and Methods. The luciferase chemiluminescence values are the means of three separate experiments performed in triplicate for each condition, normalized to the β-galactosidase values and are expressed as fold increase compared to the control non-hypoxically stressed cells. Each value is the mean ± SD of the normalized data.

We confirmed that addition of 0.5 mM ferric citrate to HeLa cells 1 h before addition of 150 μM DFO and culturing for 4 or 24 h, abrogated the expression of HIF-1α (Figure 4A, compare lanes 2 and 4 with 1 and 3). A similar, but smaller, decrease in HIF-1α expression was obtained when equimolar amounts of iron and iron-chelator were added (results not shown). However, addition of excess iron to cells before culturing with cobalt chloride, did not decrease HIF-1α expression (Figure 4A). Similar results were obtained with ferrous ammonium sulphate (Fe^{2+}), indicating that these effects did not depend on the oxidation state of the iron salts added (results not shown). Furthermore, iron had no affect on HIF-1α induction in hypoxic cultures.

The effect of ferric citrate was also confirmed by immunofluorescence experiments. In HeLa cells, no HIF-1α could be detected in normoxia (Figure 4B). In the cobalt-treated cells, there was a strong signal for HIF-1α, which was localized mainly in the nucleus. In the presence of ferric citrate, the intensity of fluorescence was not affected (if anything, it was slightly increased), in agreement with our western blot findings. A similar effect of iron was observed in the hypoxic cultures, whereas, as expected, addition of ferric citrate decreased the overall expression of HIF-1α in the DFO-treated cells.

Next, we investigated whether the transcriptional activity of HIF-1 induced by cobalt is influenced by the addition of iron. Transfection experiments showed that hypoxia, or addition of DFO or $CoCl_2$, led to approximately a 10-fold increase in HIF-1 transcriptional activity, compared to the control untreated cells, as measured by a luciferase reporter gene assay (Figure 4C). Addition of Fe^{3+} had no effect on the control cells and did not influence the transcriptional activity of HIF-1 in the hypoxic or cobalt-treated cells, but significantly reduced it in the DFO-treated cells.

Thus, our results of Figure 4 demonstrate that an excess of exogenously added ferric ions does not affect the protein levels, subcellular localization or transcriptional activity of HIF-1α induced by cobalt. Furthermore, iron does not decrease HIF-1α protein levels induced by hypoxia, suggesting that HeLa cell PHD activity is not limited by iron availability at low-oxygen tension.

$CoCl_2$ or DFO, but not hypoxia, inhibit HeLa cell proliferation and cell cycle progression by an iron-dependent mechanism

In earlier experiments, we found that, in contrast to hypoxia, addition of 150 μM of DFO or cobalt chloride decreased HeLa cell growth, compared to control cultures. However, these agents caused no irreversible cell damage, at least during the first 24 h of incubation, since if cells were subsequently placed in fresh medium without these hypoxia mimetics,

cell growth, as assesed by measuring the number of viable cells, was restored to control levels (results not shown). Because DFO and cobalt, besides inducing HIF-1α, are known (DFO) [33] or suspected (cobalt) [19,34] to interfere with iron availability and the growing evidence that oxygen and iron homeostasis are closely linked [3,35], we investigated whether the anti-proliferative effect of these two chemicals was influenced by the presence of excess iron.

At normoxic conditions, subconfluent HeLa cells grow with a doubling time of 18 h (results not shown) and are active in DNA synthesis, as assessed by the incorporation of [^{3}H]-thymidine (Figure 5A). Flow cytometric analysis showed that approximately 63% of the cells were present at the G1 phase of the cycle, 15% at the S phase and 22% at the G2/M phase (Figure 6). After 24 h culture at 1% O_2, the [^{3}H]-thymidine incorporation (Figure 5B), as well as the cell cycle distribution (Figure 6), did not differ from the control. Addition of ferric citrate to the control or hypoxic cells resulted in a small increase in DNA synthesis and percentage of cells present in the S phase. After culturing for 24 h with DFO, the tritiated thymidine incorporation was approximately 50% of the control (Figure 5C), while the percentage of cells present at the G1 phase was significantly higher than the control (approximately 90% compared with 63%), suggesting that DFO causes an arrest of the cell cycle at this phase (Figure 6). Addition of ferric citrate to the DFO-treated cells, restored cell numbers (results not shown) as well as DNA synthesis to near control levels and resulted in a significant increase in the percentage of cells present in the S phase, leading to a normalization of the cell cycle. In cells cultured with cobalt, there was a pronounced inhibition of [^{3}H]-thymidine incorporation (Figure 5D) and a reduction in the percentage of cells present in the G1 and S phases, while the percentage of cells present in the G2/M phase was significantly higher than the control (approximately 40% compared with 22%), suggesting that cobalt tends to delay the cell cycle by accumulating cells at this phase transition (Figure 6). When excess Fe^{3+} was added to the cobalt-treated cultures, cell numbers (results not shown) and [^{3}H]-thymidine incorporation (Figure 5D) were restored to almost control levels and there was an increase in the percentage of cells present in the S phase (Figure 6).

These data demonstrate that in HeLa cells, the inhibition of proliferation produced by both DFO and cobalt is due to interference with iron availability or metabolism, since it can be reversed by excess iron, but is mediated by different mechanisms, since it results in different distribution of cells in the cell cycle. Moreover, comparison of the data of Figures 1 and 4 with Figures 5 and 6 reveal that, in the case of the control, hypoxic or cobalt-treated cells, there is no correlation between HIF-1α protein levels and HeLa cell proliferation and cycle progression, while a negative correlation exists in the case of DFO.

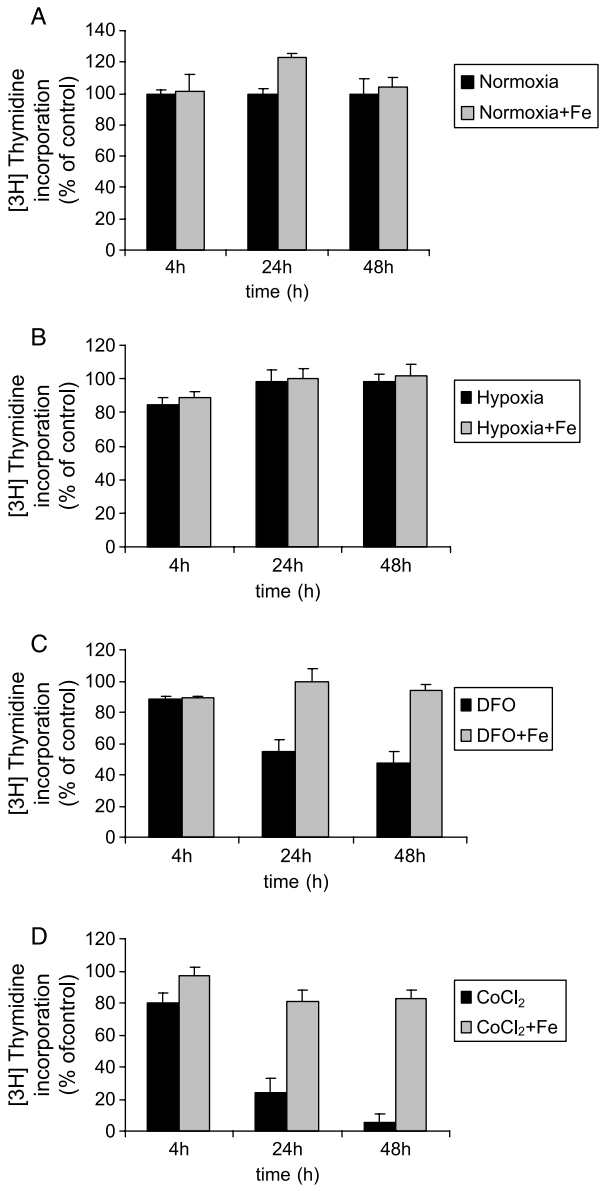

Figure 5. Effect of hypoxic stress and ferric citrate on HeLa cell proliferation. Cells were cultured as indicated, in the absence or presence of 0.5 mM ferric citrate (Fe^{3+}). For each condition, [^{3}H]-thymidine incorporation was assayed at 4, 24 and 48 h of culture, as described in Materials and Methods. All results are the mean ± SD of three independent experiments.

Discussion

In this study, we demonstrate that the mechanism of HIF-1α induction by cobalt in HeLa cells involves ROS and the operation of signaling pathways and is not, as generally assumed, simply through iron substitution. Furthermore, our results highlight the differences in the cellular response to hypoxia or its chemical mimetics, DFO and cobalt, with regards to HIF-1α induction and cell proliferation and show that both these chemicals interfere with iron metabolism,

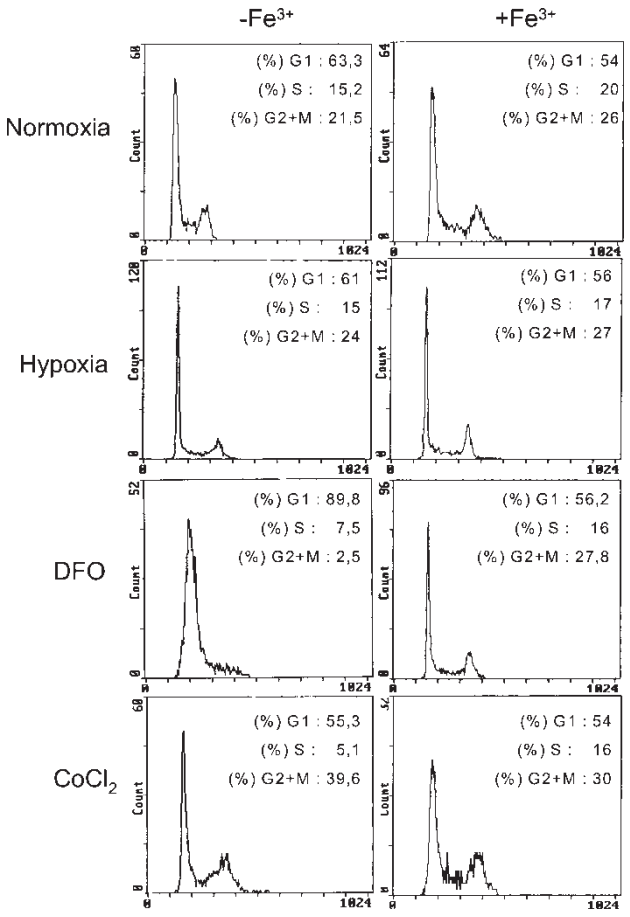

Figure 6. Flow cytometric analysis of the effect of iron on the cell cycle in HeLa cells cultured under different conditions of hypoxic stress. Cells were cultured for 24 h at normoxic or hypoxic (1% O_2) conditions, or with DFO or $CoCl_2$, in the absence or presence of 0.5 mM ferric citrate (Fe^{3+}) and were processed and analyzed, as described in Materials and Methods. The results are representative of four independent experiments.

albeit through different mechanisms. Finally, our data indicate that in the cervical adenocarcinoma cell line HeLa, a rapidly growing cell line, cell proliferation and cycle progression are not related to HIF-1α expression, but depend on iron.

Our studies show that only hypoxia, as opposed to its chemical mimetics, led to HIF-1α down regulation after prolonged induction. This probably represents a homeostatic adaptation to the continuing reduced availability of oxygen, which no longer depends on HIF-1α expression. The decrease in HIF-1α protein levels could be mediated by the increase in the levels of the prolyl hydroxylases [36–38], which are products of HIF-1α target genes, or by the induction of antisense HIF-1α [39] or other HIF-1α isoforms [40], which can act in a dominant negative manner. Apparently, this negative feedback loop is not established when HIF-1α expression is induced by DFO or cobalt, agents that affect severely the proliferative capacity of the cells (see below).

Our experiments revealed major differences in the mechanism of HIF-1α induction by DFO and cobalt chloride and the effect of iron on this process. Although the details of iron transport are not fully elucidated, it has been shown that extracellular iron is rapidly taken up by cells, via transferrin-dependent and -independent mechanisms, increasing initially the labile iron pool, the metabolically active iron compartment of the cell [41]. DFO is taken into cells by fluid phase endocytosis more slowly, leading to a decrease in the labile iron pool present in the endosomal/lysosomal compartments and to overall iron availability [42]. Cobalt, which enters cells via the divalent metal ion transporter (DMT-1), is reported both not to affect [19] and to affect [34] the levels of intracellular iron, depending on cell type. Nevertheless, it has been previously suggested that cobalt induces HIF-1α by substituting the Fe^{2+} in the active center of the prolyl hydroxylases, thereby inactivating them [16]. Our finding that prior addition of excess iron to HeLa cells cultured with cobalt chloride does not, as in the case of the DFO-treated cells, decrease the expression of HIF-1α, is contrary to this notion. Our data are, however, in agreement with reports from *in vitro* experiments [43], as well as from intact cells [17–19,21–23], that suggest alternative and more complex mechanisms of cobalt activity. More specifically, we show that, in contrast to hypoxia or DFO, induction of HIF-1α by cobalt involves ROS and depends, at least partly, on the operation of the ERK1/ERK2 and PI-3K signaling pathways. Cobalt--generated ROS may contribute to HIF-1α induction in two ways. They may oxidize Fe^{2+} to Fe^{3+} in the active site of prolyl hydroxylases, rendering them inactive [44]. Alternatively, they may activate the ERK1/ERK2 and PI-3K signaling pathways, which are generally involved in cell proliferation and survival, leading to transcriptional and translational upregulation of HIF-1α [32]. Indeed, cobalt has been previously shown to activate the ERK1/ERK2 (p42 and p44) MAP kinase pathway in HeLa cells [45] and to act via the PI-3K pathway in Hep3B cells [21] and in airway smooth muscle cells [23]. We therefore propose that cobalt increases HIF-1α expression in HeLa cells predominantly by an iron-independent, but ROS, MAPK and PI-3K-dependent mechanism. This contrasts with the mode of action of DFO, which by depleting iron, mimics hypoxia and inhibits the activity of the PHDs. It is possible that the contradictory reports in the literature concerning the action of cobalt in different cell systems, may be due to differences in the redox state, iron load or activation status of signaling pathways of the cells, which, as suggested by our work, appear to be major factors in determining its mode of action.

In HeLa cells, induction of HIF-1α by hypoxia, did not affect cell survival, DNA synthesis or the cell cycle, at least for the 48 h period we tested. On the

other hand, the iron-chelator DFO, while producing a similar induction of HIF-1α at 24 h, caused a 50% inhibition of DNA synthesis and cell cycle arrest at the G1 phase, which could be reversed by the addition of Fe^{3+}, demonstrating that the anti-proliferative effect of DFO is due to iron depletion and is not related to HIF-1α induction. Indeed, it is known that iron is essential for DNA synthesis, that rapidly growing cancer cells are often iron deficient [46] and that iron chelators can lead to arrest at the G1/S phase [33] and cell cycle dysregulation [47]. The addition of cobalt resulted in a profound inhibition of DNA synthesis, due to the accumulation of cells at the G2/M phase, which could be largely prevented by the addition of iron. However, the levels of HIF-1α were the same under both conditions of culture, demonstrating that the cobalt-induced inhibition of DNA synthesis is not related to HIF-1α induction. Indeed, with the exception of DFO, we did not observe any correlation between HIF-1α induction and HeLa cell proliferation under all conditions examined.

Although metal-induced carcinogenesis is frequently attributed to oxidative stress [48], there is no consensus in the literature about the mechanism of action of cobalt with regard to cell proliferation. To our knowledge, we are the first to report that the cobalt-induced inhibition of cell proliferation can be reversed by iron. On the basis of our data on DNA synthesis and cell cycle analysis, we conclude that the inhibition of proliferation produced by cobalt in HeLa cells, since it is different from DFO, cannot be solely due to its capacity to deplete or substitute iron and may involve, as in the case of HIF-1α induction, distinct cellular signaling pathways. Indeed, it is known that cobalt, besides activating the hypoxic pathway, also activates the Nrf2-MafG transcription factors/stress response element and the transcription factor 1/metal-responsive element pathways [49]. The precise nature of the pathway involved in the cobalt-induced inhibition of cell proliferation that can be reversed by iron requires further study.

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References

[1] Semenza GL. Regulation of mammalian O_2 homeostasis by hypoxia-inducible factor 1. Annu Rev Cell Dev Biol 1999; 15:551–578.

[2] Wenger RH. Cellular adaptation to hypoxia: O_2-sensing protein hydroxylases, hypoxia-inducible transcription factors and O_2-regulated gene expression. FASEB J 2002; 16: 1151–1162.

[3] Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer 2003;3:721–732.

[4] Wang GL, Jiang B-H, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. Proc Natl Acad Sci USA 1995;92:5510–5514.

[5] Huang LE, Gu J, Schau M, Bunn HE. Regulation of hypoxia-inducible factor 1α is mediated by an O_2 -dependent degradation domain via the ubiquitin-proteasome pathway. Proc Natl Acad Sci USA 1998;95:7987–7992.

[6] Kallio PJ, Wilson WJ, O'Brien S, Makino Y, Pollinger L. Regulation of the hypoxia-inducible transcription factor 1α by the ubiquitin-proteasome pathway. J Biol Chem 1999; 274: 6519–6525.

[7] Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, von Kriegsheim A, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PW, Pugh CW, Ratcliffe PJ. Targetting of HIFα to the von Hippel Lindau ubiquitylation complex by O_2-regulated hydroxylation. Science 2001;292:468–472.

[8] Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, Kaelin Jr, WG. HIFα targeted for VHL-mediated destruction by proline hydroxylation: Implications for O_2 sensing. Science 2001;292:464–468.

[9] Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, Mukherji M, Metzen E, Wilson MI, Dhanda A, Tian YM, Masson N, Hamilton DL, Jaakkola P, Barstead R, Hodgkin J, Maxwell PH, Pugh CW, Schofield CJ, Ratcliffe PJ. C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell 2001;107:43–54.

[10] Bruick R, McNight S. A conserved family of prolyl-4-hydroxylases that modify HIF. Science 2001;294:1337–1340.

[11] Lando D, Peet DJ, Gorman JJ, Whelan DA, Whitelaw ML, Bruick R. FIH-1 is an asparaginyl hydroxylase that regulates the transcriptional activity of hypoxia inducible factor. Genes Dev 2002;16:1466–1471.

[12] Kallio PJ, Okamoto K, O'Brien S, Carrero P, Makino Y, Tanaka H, Pollinger L. Signal transduction in hypoxic cells: Inducible nuclear translocation and recruitment of the CBP/p300 coactivator by the hypoxia-inducible factor-1-alpha. EMBO J 1998;17:6573–6586.

[13] Richard DE, Berra E, Gothie E, Roux D, Pouyssegur J. p42/p44 mitogen-activated protein kinases phosphorylate hypoxia-inducible factor 1α (HIF-1α) and enhance the transcriptional activity of HIF-1. J Biol Chem 1999; 274: 32631–32637.

[14] 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;82:3610–3615.

[15] Vincent TO, Bunn HF. Effects of transition metals on the expression of the erythropoietin gene: Further evidence that the oxygen sensor is a heme protein. Biochem Biophys Res Comm 1996;223:175–180.

[16] Schofield CJ, Ratcliffe PJ. Oxygen sensing by HIF hydroxylases. Nat Rev Mol Cell Biol 2004;5:343–354.

[17] Yuan Y, Hilliard G, Ferguson T, Millhorn DE:. Cobalt inhibits the interaction between hypoxia-inducible factor-alpha and von Hippel–Lindau protein by direct binding to hypoxia-inducible factor-alpha. J Biol Chem 2003;278:15911–15916.

[18] Kanaya K, Kamitani T. pVHL-independent ubiquitination of HIF-1α and its stabilization by cobalt ion. Biochem Biophys Res Comm 2003;306:750–755.
[19] Salnikow K, Donald SP, Bruick RK, Zhitkovich A, Phang JM, Kasprzak KS. Depletion of intracellular ascorbate by the carcinogenic metals nickel and cobalt results in the induction of hypoxic stress. J Biol Chem 2004;279:40337–40344.
[20] Knowles HJ, Raval RR, Harris AL, Ratcliffe PJ. Effect of ascorbate on the activity of hypoxia-inducible factor 1α in cancer cells. Cancer Res 2003;63:1764–1768.
[21] Chandel NS, McClintock DS, Feliciano CE, Wood JA, Melendez A, Rodriguez AM, Schumacker PT. Reactive oxygen species generated at mitochondial complex III stabilize hypoxia-inducible factor-1α during hypoxia: A mechanism for O_2-sensing. J Biol Chem 2000;275:25130–25138.
[22] Gong Y, Agani FH. Oligomycin inhibits HIF-1α expression in hypoxic tumor cells. Am J Physiol Cell Physiol 2005; 288: C1023–C1029.
[23] Chachami G, Simos G, Hatziefthymiou A, Bonanou S, Molyvdas PA, Paraskeva E. Cobalt induces hypoxia-inducible factor-1α expression in airway smooth muscle cells by a reactive oxygen species- and PI3K-dependent mechanism. Am J Respir Cell Mol Biol 2004;31:544–552.
[24] Carmeliet P, Dor Y, Herbert JM, Fukumura D, Brusselmans K, Dewerchin M, Neeman M, Bono F, Abramovitch R, Maxwell P, Koch CJ, Ratcliffe P, Moons L, Jain RK, Collen D, Keshert E. Role of HIF-1αlpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. Nature 1998;394:485–490.
[25] Piret J-P, Motter D, Raes M, Michiels C. Is HIF-1α a pro- or an anti-apoptotic protein? Biochem Pharmacol 2002; 64: 889–892.
[26] Greijer AE, van der Wall E. The role of hypoxia inducible factor 1 (HIF-1) in hypoxia induced apoptosis. J Clin Path 2004;57:1009–1014.
[27] Schmid T, Zhou J, Brune B. HIF-1 and p53: Communication of transcription factors under hypoxia. J Cell Mol Med 2004; 8:423–431.
[28] Hagen T, Taylor CT, Lam F, Moncada S. Redistribution of intracellular oxygen in hypoxia by nitric oxide: Effect on HIF1α. Science 2003;302:1975–1978.
[29] Goyal P, Weissmann N, Grimminger F, Hegel C, Bader L, Rose F, Fink L, Ghofrani H-A, Schermuly RT, Schmidt HH, Sieger W, Hanze J. Upregulation of NAD(P)H oxidase I in hypoxia activates hypoxia-inducible factor 1 via increase in reactive oxygen species. Free Radic Biol Med 2004; 36: 1279–1288.
[30] Mansfield KD, Guzy RD, Pan Y, Young RM, Cash TP, Schumacker PT, Simon MC. Mitochondrial dysfunction resulting loss of cytochrome c impairs cellular oxygen sensing and hypoxic HIF-1α activation. Cell Metab 2005;1:393–399.
[31] Leonard S, Gannett PM, Rojanasakul Y, Schwegler-Berry D, Castranova V, Vallyathan V, Shi X. Cobalt-mediated generation of reactive oxygen species and its possible mechanism. J Inorg Biochem 1998;70:239–244.
[32] Semenza GL. Signal transduction to hypoxia-inducible factor 1. Biochem Pharmacol 2002;64:993–998.
[33] Templeton DL, Liu Y. Genetic regulation of cell function in response to iron overload or chelation. Biochim Biophys Acta 2003;1619:113–124.
[34] Li Q, Chen H, Huang X, Costa M. Effects of 12 metal ions on iron regulatory protein 1 (IRP-1) and hypoxia-inducible factor-1 alpha (HIF-1α) and HIF-regulated genes. Toxicol Appl Pharmacol 2005; Dec 28; [Epub ahead of print].
[35] Wang J, Chen G, Muckenthaler M, Galy B, Hentze MW, Pantopoulos K. Iron-mediated degradation of IRP2, an unexpected pathway involving a 2-oxoglutarate-dependent oxygenase activity. Mol Cell Biol 2004;24:954–965.
[36] 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-1α in normoxia. EMBO J 2003;22:4082–4090.
[37] Apprelikova O, Chandramouli GVR, Wood M, Vasselli JR, Riss J, Maranchie JK, Linehan WM, Barrett JC. Regulation of HIF prolyl hydroxylases by hypoxia-inducible factors. J Cell Biochem 2004;92:491–501.
[38] Nakayama K, Frew IJ, Hagensen M, Skals M, Habelhah H, Bhoumik A, Kadoya T, Erdjument-Bromage H, Tempst P, Frappell PB, Bowtell DD, Ronai Z. Siah2 regulates stability of prolyl-hydroxylases, controls HIF-1αlpha abundance and modulates physiological responses to hypoxia. Cell 2004; 117:941–952.
[39] Rossignol F, Vache C, Clottes E. Natural antisense transcripts of hypoxia-inducible factor 1 alpha are detected in different normal and tumour human tissues. Gene 2002;299:135–142.
[40] Lee JIW, Bae S-H, Jeong J-W, Kim S-H, Kim K-W. Hypoxia-inducible factor (HIF-1)α: Its protein stability and biological functions. Exp Mol Med 2004;36:1–12.
[41] Kakhlon O, Cabantchik ZL. The labile iron pool: Characterization, measurement and participation in cellular processes. Free Radic Biol Med 2002;33:1037–1046.
[42] Doulias PT, Christoforidis S, Brunk UT, Galaris D. Endosomal and lysosomal effects of desferrioxamine: Protection of HeLa cells from hydrogen peroxide-induced DNA damage and induction of cell-cycle arrest. Free Radic Biol Med 2003;35:719–728.
[43] Hirsila M, Koivunen P, Xu L, Seey T, Kivirikko KI, Myllyharju J. Effect of desferrioxamine and metals on the hydroxylases in the oxygen sensing pathway. FASEB J 2005;19:1308–1310.
[44] Gerald D, Berra E, Frapart YM, Chan DA, Giaccia AJ, Mansuy D, Pouyssegur J, Yaniv M, Mechta-Grigoriou F. JunD reduces tumor angiogenesis by protecting cells from oxidative stress. Cell 2004;118:781–794.
[45] Kim HJ, Yang SJ, Kim YS, Kim TU. Cobalt chloride-induced apoptosis and extracellular signal-regulated protein kinase activation in human cervical cancer HeLa cells. J Biochem Mol Biol 2003;36:468–474.
[46] Le NT, Richardson DR. The role of iron in cell cycle progression and the proliferation of neoplastic cells. Biochim Biophys Acta 2002;1603:31–46.
[47] Le NTV, Richardson DR. Potent iron chelators increase the mRNA levels of the universal cyclin-dependent kinase inhibitor $p21^{CTP1/WAF1}$, but paradoxically inhibit its translation: A potential mechanism of cell cycle dysregulation. Carcinogenesis 2003;24:1045–1058.
[48] Galaris D, Evangelou A. The role of oxidative stress in mechanisms of metal-induced carcinogenesis. Crit Rev Oncol Hematol 2002;42:93–103.
[49] Gong P, Hu B, Stewart D, Ellerbe M, Figueroa YG, Blank V, Beckman BS, Alam J. Cobalt induces heme oxygenase-1 expression by a hypoxia-inducible factor-independent mechanism in Chinese hamster ovary cells: Regulation by Nrf2 and MafG transcription factors. J Biol Chem 2001; 276: 27018–27025.