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Nickel-induced HIF-1α promotes growth arrest and senescence in normal human cells but lacks toxic effects in transformed cells

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Abstract

Nickel is a human carcinogen that acts as a hypoxia mimic by activating the transcription factor HIF-1α and hypoxia-like transcriptomic responses. Hypoxia and elevated HIF-1α are typically associated with drug resistance in cancer cells, which is caused by increased drug efflux and other mechanisms. Here we examined the role of HIF-1α in uptake of soluble Ni(II) and Ni(II)-induced cell fate outcomes using si/shRNA knockdowns and gene deletion models. We found that HIF-1α had no effect on accumulation of Ni(II) in two transformed (H460, A549) and two normal human cell lines (IMR90, WI38). The loss of HIF-1α also produced no significant impact on p53-dependent and p53-independent apoptotic responses or clonogenic survival of Ni(II)-treated transformed cells. In normal human cells, HIF-1α enhanced the ability of Ni(II) to inhibit cell proliferation and cause a permanent growth arrest (senescence). Consistent with its growth-suppressive effects, HIF-1α was important for upregulation of the cell cycle inhibitors p21 (CDKN1A) and p27 (CDKN1B). Irrespective of HIF-1α status, Ni(II) strongly increased levels of MYC protein but did not change protein expression of the cell cycle-promoting phosphatase CDC25A or the CDK inhibitor p16. Our findings indicate that HIF-1α limits propagation of Ni(II)-damaged normal cells, suggesting that it may act in a tumor suppressor-like manner during early stages of Ni(II) carcinogenesis.

Keywords

nickel; HIF1A; hypoxia; senescence; apoptosis

Introduction

HIF-1α is a hypoxia-inducible transcription factor that is rapidly activated in response to decreased oxygen concentrations (Wang et al., 1995). Cells continuously produce and

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translate HIF-1α mRNA but HIF-1α protein is very short-lived and its levels are barely detectable under normoxic conditions. Stability of HIF-1α protein is controlled by hydroxylation of its Pro402 and Pro564 residues, which are targets of oxygen-sensing enzymes PHD1-3 (Bruick and McKnight, 2001; Epstein et al., 2001). PHD2 plays a dominant role in Pro hydroxylation of HIF-1α in a majority of cells (Berra et al., 2003). Hydroxylated Pro402 and Pro564 are recognized by the E3 ubiquitin ligase VHL, which recruits the cullin 2/elongin B/elongin C/Rbx1-containing complex triggering polyubiquitination of HIF-1α and its subsequent degradation by the 26S proteasomes (Jaakkola et al., 2001; Ivan et al., 2001; Maxwell et al., 1999). Hypoxia causes a loss of Pro hydroxylation, which results in a rapid accumulation of HIF-1α, its association with a stable HIF-1β subunit and DNA binding (Jiang et al., 1996). Transactivation properties of HIF-1α are also enhanced in hypoxia due to the loss of Asn803 hydroxylation by FIH-1, which allows recruitment of the transcriptional coactivators p300 and CBP to hypoxia-responsive promoters (Lando et al., 2002; Mahon et al., 2001). HIF-1α upregulates expression of a large number of genes, which promote hypoxia adaptation through increased glucose uptake, activation of oxygen-independent ATP generation, angiogenesis and other processes (Semenza, 2012). Cancers with increased hypoxia transcription signature are typically more aggressive, which can be linked to their better survival in hypoxic conditions during rapid tumor growth and the role of HIF-1α–regulated genes in meeting the metabolic needs of malignant cells (Wilson and Hay, 2011). HIF-1α expression has also been linked to increased drug resistance (Samanta et al., 2011; Untruth et al., 2003). Although the hypoxia-responsive pathway is upregulated in many late cancers, a role of HIF-1α in early carcinogenesis is less clear. Inactivation of the HIF-1α ubiquitin ligase VHL is a key genetic event in the development of hereditary and spontaneous renal cell carcinoma (Shen and Kaelin, 2013). However, the majority of renal cell carcinomas lost HIF-1α expression via genetic and other mechanisms (Raval et al., 2005; Shen et al., 2011), indicating a strong counter-selection against constitutively hyperactive HIF-1α. Prolonged hypoxia also leads to downregulation of HIF-1α levels via negative feedback mechanisms (Henze and Acker, 2010; Uchida et al., 2004), indicating that cells avoid the state with the chronically active HIF-1 pathway.

Inorganic nickel compounds are recognized human respiratory carcinogens that also produce lung tumors in rodents via inhalation and malignancies at other sites through injection routes of exposure (Costa et al., 2005; Goodman et al., 2011; Salnikow and Zhitkovich, 2008). Ni has tested nonmutagenic in various biological assays, which is consistent with its inability to form DNA adducts or DNA breaks in contrast to genotoxic metals such as chromium(VI) (DeLoughery et al., 2015; Reynolds et al., 2004). Carcinogenicity of Ni has been attributed to its epigenetic effects in chromatin and chronic activation of transcription factors that are frequently altered in cancer cells (Costa et al., 2005; Salnikow and Zhitkovich, 2008; Yao and Costa, 2014). Ni(II) ions act as hypoxia mimics causing a massive accumulation of HIF-1α and inducing a hypoxia-like transcription response (Salnikow et al., 2000, 2003). Similar to hypoxia, the primary cause of HIF-1α buildup in Ni-treated cells was protein stabilization due to the loss of Pro hydroxylation. The inhibition of prolyl hydroxylases has been attributed to the depletion of their Fe(III)-reducing cofactor ascorbate (Salnikow et al., 2004) and the displacement of iron from the active center of these enzymes (Davidson et al.,

2006). Global transcriptome analyses showed that HIF-1α was responsible for upregulation of approximately 75% of induced genes and downregulation of 50% of repressed genes in Ni-treated mouse cells (Salnikow et al., 2003), raising a question about the toxicological significance of this major response.

In this work, we examined the role of HIF-1α in cell fate decisions induced by exposure to Ni(II) ions. We found that the HIF-1α pathway was not involved in Ni-induced apoptotic responses and clonogenic toxicity in transformed human cells but it enhanced growth arrest and senescence-promoting activity in normal human cells.

Materials and Methods

Chemicals

$NiCl_2 \times 6H_2O$ (N6136) and $CoCl_2 \times 6H_2O$ (C8661) were purchased from Sigma. RPMI-1640 (11875119) and DMEM (12430062) media were Gibco. F12K (30-2004) medium was purchased from ATCC. Fetal bovine serum (FBS-BBT-5XM) was acquired from RMBIO.

Cells and treatments

H460, WI38 and IMR90 human lung cells were obtained from the American Type Culture Collection. Wild-type and *HIF1*−/− A549 cells were purchased from Sigma. Cells were grown in 10% fetal bovine serum/1% PenStrep (15140, Gibco)-supplemented media (RPMI-1640 for H460, F12K for A549 and DMEM for WI38 and IMR90). Treatments with nickel chloride or cobalt chloride were performed in complete growth media. Freshly prepared metal solutions were always used.

Western blotting

Attached cells were collected by scraping and combined with floating cells for protein extraction. Whole cell lysates were prepared by boiling cells for 10 min in a 2% SDS buffer (2% SDS, 50 mM Tris-HCl pH 6.8, 10% glycerol) supplemented with protease and phosphatase inhibitors (78425, Thermo Fisher Scientific). Samples were cooled to room temperature and centrifuged at 10000×g for 10 min to obtain clear supernatants. Proteins were separated by the standard SDS-PAGE and electrotransferred overnight to PVDF membranes (162-0177, Bio-Rad). Westerns were performed with the following primary antibodies: rabbit polyclonal anti-cyclin A (sc-751, 1:1000, Santa Cruz), mouse monoclonal anti-p53 (sc-125, 1:1000, Santa Cruz), rabbit polyclonal anti-p53 phosphorylated at Ser15 (9284, 1:1000, Cell Signaling), rabbit monoclonal anti-p21 (2947, 1:1000, Cell Signaling), rabbit polyclonal anti-p27 (2552, 1:1000, Cell Signaling), rabbit polyclonal anti-caspase 9 (9502, 1:500, Cell Signaling), rabbit polyclonal anti-cleaved caspase 3 (9661, 1:500, Cell Signaling), rabbit monoclonal anti-cleaved caspase 7 (8438, 1:500, Cell Signaling), rabbit polyclonal anti-PARP (9542, 1:1000, Cell Signaling), rabbit monoclonal anti-c-MYC (13987, 1:1000, Cell Signaling), rabbit polyclonal anti-CDC25A (3652, 1:1000, Cell Signaling), rabbit anti-H2AX phosphorylated at Ser139 (2577, 1:1000, Cell Signaling), mouse monoclonal anti-HIF1α (610958, 1:500, BD Biosciences), mouse monoclonal anti-p16 (51-1325GR, 1:1000, BD Bioscience), rabbit polyclonal anti-fibrillarin (ab5821, 1:5000, Abcam), mouse monoclonal anti-γ-tubulin (T6557, 1:2000, Sigma). Horseradish

peroxidase-conjugated goat anti-mouse IgG (12-349, Millipore) and goat anti-rabbit IgG (7074, Cell Signaling) were typically used at 1:5000 and 1:2000 dilutions.

Stable knockdowns with shRNA

The pSUPER.retro.puro vector (OligoEngine) was used to produce stable knockdowns of HIF1A with two constructs. Targeting sequences for HIF1A were 5′-GAAGGAACCTGATGCTTTA-3′ (used in IMR90 and H460 cells) and 5′-GTGATGAAAGAATTACCGAAT-3′ (used in WI38 cells). Oligonucleotides containing targeting sequences and vector-compatible ends were ligated into the linearized pSUPER.retro.puro using T4 ligase (15224017, Invitrogen) overnight followed by the transformation of the plasmid products into One Shot TOPO10 Chemically Competent *E. coli* cells (C404003, Invitrogen). The viral particles were produced in 293T cells by cotransfection of pSUPER DNA with plasmids expressing MoMuLV gag-pol and VSVG. Virus-containing media was collected 24 and 48h after transfections, passed through the Millex-GV 0.2 μM filter (SLGV013SL, Millipore) and added to cells overnight. Infected cells were selected and continuously maintained in the presence of 1.5 μg/mL (H460) or 1 μg/mL puromycin (IMR90 and WI38).

siRNA knockdowns

ON-TARGETplus human HIF1A SMARTpool siRNA (L-004018-00-00200, Dharmacon) and ON-TARGETplus non-targeting pool siRNA (D-001810-10-20, Dharmacon) were used to produce transient knockdowns of HIF1A in H460 and IMR90 cells. siRNA (90 nM) was mixed with 20 μL of Lipofectamine RNAiMAX (13778150, Invitrogen) and used for transfection of H460 (10^6 cells) and IMR90 (0.5×10^6 cells) seeded onto 100-mm dishes. Cells were incubated with the transfection mixtures for 6h. The second transfection was performed 24h later and cells were seeded for Ni treatments on the following day.

Scoring of growth-arrested cells

IMR90 cells twice transfected with nonspecific and HIF1A-targeting siRNA were seeded onto 6-well plates (0.5×10^6 cells/well) and treated with Ni for 48h. Cells were reseeded onto 6-well plates containing human fibronectin-coated coverslips (354088, Corning) and grown in medium supplemented with 10 μM of 5-ethylnyl-2′-deoxyuridine (EdU) for 48h. Click-iT EdU Alexa Fluor 488 Imaging Kit (C10337, Molecular Probes) was used for the visualization of replicating cells. Coverslips were mounted onto Superfrost Microscope Slides (12-550-143, Fisher) and EdU-positive cells were scored using Nikon Eclipse E800 fluorescent microscope (Nikon) and SpotAdvanced 5.1.23 software.

Senescence assay

Cells were seeded (0.5×10^6 cells/well) onto 6-well plates, incubated for 48h with Ni followed by reseeding onto human fibronectin-coated coverslips for 72h recovery in the standard medium. β-Galactosidase Staining Set (11828673001, Roche) was used to detect senescent cells.

RT-qPCR

H460 (2.0×10^6) cells were seeded onto 100-mm dishes and treated with Ni for 24h. RNA was extracted with TRIzol Reagent (15596-026, Ambion), resuspended in RNase-tree water and quantified by NanoDrop ND-1000 UV/Vis spectrophotometer. Reverse transcription reactions were run with 1 μg RNA using RT First Strand Kit (330401, Qiagen). Serial cDNA dilutions were used to calculate reaction efficiency for each primer. PCR primers for MDM2 (PPH00193E), BTG2 (PPH01750C), PUMA (PPH02204C), NOXA (PPH02090F), BNIP3 (PPH00301C), CA9 (PPH01751A), B2M (PPH01094E), GAPDH (PPH00150F) and TBP (PPH01091G) were purchased from Qiagen, Real-Time PCR reaction was prepared using RT SYBR Green ROX qPCR Mastermix (330529, Qiagen) and performed in ViiA7 Real-Time PCR System (Applied Biosystems). PCR data were analyzed by the $\Delta\Delta C_T$ method. B2M, GAPDH and TBP were used for normalization of gene expression.

Cellular Ni

Total cellular levels of Ni were measured as described previously (Green et al., 2013) using nitric acid extracts of cells and graphite furnace atomic absorption spectroscopy (AAnalyst600 Atomic Absorption Spectrometer, Perkin-Elmer).

Cytotoxicity

Cell viability was assessed by measurements of the total metabolic activity of cell populations using the CellTiter-Glo luminescent cell viability assay (Promega). IMR90 and WI38 cells were seeded into 96-well optical cell culture plates (1000 cells/well), grown overnight and then treated with Ni. The cell viability assay was performed immediately after removal of Ni and at 48h recovery post-Ni.

Clonogenic survival

Cells were seeded onto 60-mm dishes (400 cells/dish) and treated with freshly dissolved nickel chloride for 24h. After removal of Ni-containing media, cells were grown for several days to form visible colonies that were fixed with methanol and stained with a Giemsa solution (Sigma).

Statistics

Two-tailed, unpaired *t*-test was used for the evaluation of differences between the means. Differences with $p\leq0.05$ were considered as statistically significant.

Results

Cell models and Ni uptake

We chose H460 human lung epithelial cell line and primary IMR90 human lung fibroblasts as our main biological models. H460 cells express the wild-type p53 transcription factor and displayed normal stress responses to various carcinogens, such as ionizing radiation (Zhang et al., 2006), chromium(VI) (Luczak et al., 2016) and formaldehyde (Ortega-Atienza et al., 2015; Wong et al., 2012). We have previously characterized p53 activation and mechanisms of cell death by Ni(II) ions in this cell line (Green et al., 2013; Wong et al., 2013a), making

it well-suited for dissecting potential toxicological effects of HIF-1α. Using infections with the pSUPER-retro vector and selection with puromycin, we constructed H460 and IMR90 cells with stable knockdowns of HIF-1α. Both types of cells expressing target shRNA contained almost undetectable amounts of HIF-1α after treatments with Ni or another hypoxia-mimicking metal cobalt (Fig. 1A). HIF-1α is known to confer resistance to several anticancer drugs through their enhanced efflux by upregulated MDR1 (Comeford et al., 2002; Samanta et al., 2011). Therefore, we first examined the impact of HIF-1α on Ni accumulation by cells. We found that 6 and 24h treatments with Ni resulted in very similar metal levels in H460 cells with normal and depleted HIF-1α (Fig. 1B). The amount of Ni in cells after 6h exposure was only 15–20% lower than that after 24h incubations, indicating that the equilibrium between influx and efflux processes was achieved within the first several hours. HIF-1α knockdown also had no significant effect on accumulation of Ni by primary IMR90 cells (Fig. 1C). Thus, any differences in pathophysiological responses between cells with normal and silenced HIF-1α would be unrelated to the internal doses of Ni.

Apoptotic responses in H460 cells

Ni treatments caused a robust activation of the stress-sensitive transcription factor p53 and a caspase-mediated apoptosis as the main form of death in H460 cells (Green et al., 2013; Wong et al., 2013a). The stable knockdown of HIF-1α produced no appreciable effects on the ability of Ni to upregulate p53, as evaluated by its protein and Ser15 phosphorylation levels in H460 cells treated for 48h (Fig. 2A). The disappearance of HIF-1α at the higher Ni concentration probably resulted from the activation of negative feedback mechanisms, as noted previously for chronic hypoxia in lung epithelial and other cells (Henze and Acker, 2010; Uchida et al., 2004). Consistent with the western blotting results for p53, readouts of the p53-dependent caspase cascade including the decrease in the procaspase 9 form, production of active (cleaved) executioner caspase 3 and PARP cleavage were all very similar between cells with normal and depleted HIF-1α (Fig. 2B). Activation of the second main executioner caspase, caspase 7, was also unaffected by the absence of HIF-1α (Fig. 2C). To exclude possible off-target effects of the employed shRNA, we also tested the impact of HIF-1α silencing with unrelated siRNA delivered via transfection. Measurements of p53 and caspase activation after Ni treatments again found no noticeable effects of the HIF-1α knockdown (Fig. 2D, E, F). The formation of a biochemical marker of DNA breaks, Ser139-phosphorylated histone H2AX (γ-H2AX) was also unaffected in HIF-1α-depleted cells, indicating normal apoptotic DNA fragmentation (Fig. 2G). HIF-1α has been reported to interfere with the transactivation activity of p53 (Filippi et al., 2008), allowing cells to survive in hypoxic conditions despite elevated amounts of p53 protein. We found that the ability of Ni to increase mRNA levels of three p53-inducible genes, *MDM2*, *BTG2* and *PUMA* (*BBC3*), (Wong et al., 2013a) were not significantly altered by HIF-1α depletion (Fig. 3A). Expression of the p53-independent proapoptotic gene *NOXA* (*PMAIP*) was also unaffected by the HIF-1α absence (Fig. 3B). As expected, cells expressing targeting shRNA completely lost upregulation of *BNIP3* and *CA9* genes by Ni, confirming the effectiveness of HIF-1α knockdown (Fig. 3C). Overall, these results indicate that HIF-1α does not play a significant role in activation of p53-dependent and p53-independent apoptotic responses by Ni in H460 cells. Further supporting this conclusion, we found that a long-term cell survival

measured by the colony formation assay, which is sensitive to all forms of cell death, was also unaffected by HIF-1α depletion (Fig. 3D).

Cytotoxicity in primary human cells

IMR90 normal human cells do not undergo apoptotic or necrotic cell death after moderate doses of Ni and remain attached to the dish. Therefore, Ni cytotoxicity in these cells was evaluated by analysis of their metabolic activity. We found that shRNA-mediated silencing of HIF-1α had protective effects in IMR90 cells, as evidenced by cytotoxicity measurements immediately after Ni treatments and following 48h recovery post-Ni (Fig. 4A, B). The differences in cell viability between control and HIF-1α knockdown cells were larger at the 48h post-exposure time. Similar to uptake determinations after 24h exposures (Fig. 1C), HIF-1α knockdown also did not affect Ni accumulation in IMR90 cells after 48h treatments (Fig. 4C). To further test survival effects of HIF-1α in normal human cells, we created its stable knockdown in WI38 cells using a different shRNA construct. This second shRNA was also very effective in silencing HIF-1α expression (Fig. 4D). Cytotoxicity measurements in WI38 cells confirmed a higher resistance of the HIF-1α knockdown to Ni (Fig. 4E, F). Again, the cell viability effect of HIF-1α depletion was more pronounced at 48h recovery post-Ni. As in other cells, HIF-1α depletion did not affect uptake of Ni by WI38 cells (Fig. 4G). Together, these findings indicate that a prolonged HIF-1α induction by Ni is toxic to normal human cells.

Growth arrest and senescence in normal human cells

Larger differences in viability between normal and HIF-1α knockdown cells after 48h recovery in comparison to measurements at the end of Ni exposures (Fig. 4) could be related to an impairment of growth recovery by HIF-1α. To assess the replication potential of Ni-treated populations, we labeled them for 48h with the thymidine analogue EdU and scored the percentage of positive (replicated) cells. We found that siRNA-mediated depletion of HIF-1α resulted in a significantly higher percentage of cycling IMR90 cells following Ni(II) treatments (Fig. 5A). The knockdown of HIF-1α also strongly suppressed the conversion of the growth-arrested state in Ni-treated cells into senescence (Fig. 5B), as determined by staining for a classic marker of cellular senescence, senescence-associated β-galactosidase activity (Itahana et al., 2007). The establishment of cellular senescence is typically mediated by either p21 or p16 cell cycle inhibitors, which varies with the type of the cellular stress (Itahana et al., 2004). In some cases, senescence can also be promoted by the cyclin-dependent kinase (CDK) inhibitor p27 (Lin et al., 2010). To identify potential mediators of Ni and HIF-1α-induced growth arrest, we examined protein levels of several regulators of cell cycle progression. Consistent with growth arrest-promoting properties of HIF-1α, we found that its knockdown helped preserve the expression of the S/G2-specific cyclin A at the midrange of our Ni doses and inhibited upregulation of the CDK inhibitor p27 (Fig. 5C). The cell cycle arrest by Ni did not result from the checkpoint response triggering proteolysis of CDC25A (Busino et al., 2003), as protein levels of this G1 and S-phase promoting phosphatase remained unchanged. In contrast with a reported degradation of MYC in carcinoma cells (Li et al., 2009), Ni strongly stimulated accumulation of MYC protein in both control and HIF-1α knockdown IMR90 cells (Fig. 5C). HIF-1α enhanced protein expression of the CDK inhibitor p21 but moderately diminished upregulation of p53 protein

or its Ser15 phosphorylation by Ni (Fig. 5D). Protein expression of another important CDK inhibitor, p16, was not appreciably affected by Ni treatments.

Effects of HIF-1α in A549 and WI38 cells

Knockdowns of HIF-1α in transformed H460 and normal IMR90 cells produced divergent impacts on stress signaling and cell fate decisions in response to Ni: no changes in H460 but increased growth arrest and senescence in IMR90. To further explore these cell line-specific effects, we examined the role of HIF-1α in Ni-induced cell death in another pair of human cells: transformed A549 and normal WI38. A549 lung carcinoma cells are widely used in toxicological and cancer research and similar to H460, they express wild-type p53 tumor suppressor. Examination of the long-term viability by the colony-formation assay found no appreciable differences between normal and *HIF1A* knockout A549 cells in their responses to Ni treatments (Fig. 6A). Similar to results with knockdown models in other cells, loss of *HIF1A* gene in A549 cells produced no significant effects on Ni uptake (Fig. 6B). In agreement with findings in IMR90 cells, knockdown of HIF-1α in WI38 cells resulted in a significant reduction in the number of senescent cells induced by Ni (Fig. 6C). This result is consistent with the decreased cytotoxicity of Ni in WI38 cells with depleted HIF-1α (Fig. 4F). H460 cells treated with the highest concentration of Ni for 48h (Fig. 2A, 0.5 mM Ni) showed a complete loss of HIF-1α. However, Ni treatments did not suppress HIF-1α induction in normal IMR90 cells, raising a question whether the presence of the strong negative feedback mechanism is common to other cancer cells. We found that treatments of A549 carcinoma cells with a range of Ni concentrations overlapping with those used in H460 cells produced only a modest decrease in HIF-1α at 48h relative to 24h treatment at 1 mM but not 0.5 mM Ni (Fig. 6D). Thus, the strength of the negative feedback response of HIF-1α to Ni is the cell line-specific characteristic rather than the transformation-related feature.

Discussion

The transcription factor HIF-1α regulates expression of a large number of genes that are involved in glucose metabolism, energy production, angiogenesis, migration and other cellular processes (Semenza, 2012; Wilson and Hay, 2011). In Ni-treated mouse embryonic fibroblasts, stabilization and activation of HIF-1α was responsible for upregulation of approximately 75% of induced genes and downregulation of 50% of repressed genes (Salnikow et al., 2003). Upregulation of HIF-1α in hypoxic environment frequently confers drug resistance to cancer cells (Wilson and Hay, 2011). We found that HIF-1α induction by Ni under normoxic conditions did not inhibit cytotoxicity of this metal in transformed human cells. A common mechanism of drug resistance in hypoxic cancer cells is a HIF-1α-dependent upregulation of the drug transporter MDR1, which results in the diminished doses of toxic drugs inside the cells (Comeford et al., 2002; Samanta et al., 2011). Our studies in four human cell lines showed that Ni accumulation was not changed by the loss of HIF-1α, indicating that it did not have a significant effect on efflux of this metal. HIF-1α deficiency created with different genetic approaches (siRNA, shRNA, gene knockout) produced no significant effects on Ni-induced p53-dependent and p53-independent apoptotic responses, as well as the long-term clonogenic survival of transformed human H460 and A549 cells. In

contrast, inactivation of the HIF-1α pathway in two primary human cell lines suppressed long-term cytotoxic effects and senescence by Ni treatments. Biochemically, accumulation of HIF-1α was associated with the increased protein expression of two cell cycle inhibitors, p21 (CDKN1A) and p27 (CDKN1B). Levels of another important CDK inhibitor, p16, were practically unchanged by Ni irrespective of HIF-1α status of cells. CDKN1A is an established mediator of senescence for inducers of DNA damage whereas p16 is typically responsible for the terminal growth arrest by nongenotoxic stressors (Itahana et al., 2004). Thus, although Ni is commonly described as a nongenotoxic carcinogen, its senescence-associated responses resemble those of DNA damaging-agents.

Growth arrest is a well-known cellular response to hypoxia (Hubbi and Semenza, 2015), which has been associated with the accumulation of the CDK inhibitors p21 and p27 (Gardner et al., 2001; Goda et al., 2003) and required p27 in mouse embryonic fibroblasts (Gardner et al., 2001). Although hypoxia causes cell cycle arrest, it does not progress into senescence (Leontieva et al., 2012). Upregulation of p21 by HIF-1α overexpression has been linked to MYC sequestration and the resulting derepression of p21 promoter (Koshiji et al., 2004). The same mechanism has been suggested to be responsible for the induction of p27 by HIF-1α (Hubbi and Semenza, 2015; Koshiji et al., 2004). In hypoxia, inhibition of p21 and p27 expression is further relieved due to degradation of MYC protein (Zhang et al., 2007; Wong et al., 2013b). Still relying on the downregulation of MYC, an alternative mechanism for p21 protein induction by HIF-1α involves repression of miR-17 (He et al., 2013). We found that normal human cells strongly upregulated protein levels of MYC in response to Ni, suggesting that antagonistic effects of HIF-1α on MYC may not be as effective as in hypoxia. Deactivation of MYC activity by HIF-1α also results in downregulation of the cell cycle-promoting phosphatase CDC25A (Hammer et al., 2007), which did not occur in Ni-treated normal human cells further arguing against a global inhibition of MYC activity. The determination of the exact molecular mechanisms underlying Ni-induced upregulation of p21 and p27 requires further investigation.

Cancer cells frequently overexpress HIF-1α and display upregulated expression of hypoxia-responsive genes (Wilson and Hay, 2011; Zhong et al., 1999). Growth arrest-promoting effects of HIF-1α mediated via induction of CDK inhibitors or other mechanisms are not compatible with uncontrolled proliferation of cancer cells. Therefore, it appears that transformed cells evolved to suppress the growth-inhibitory component of HIF-1α-mediated signaling while retaining beneficial properties of hypoxic metabolism (high glucose uptake, for example). This selective loss of HIF-1α functions in cancer helps explain why the absence of HIF-1α had no effect on Ni cytotoxicity in H460 and A549 carcinoma cells but suppressed Ni-induced cytotoxicity and senescence in normal human cells. Although HIF-1α is frequently described as an oncogene due to its promotion of many cancer characteristics (Wilson and Hay, 2011), this classification may be appropriate only for advanced stages of cell transformation. In renal cell carcinoma that are caused by the inactivation of the HIF-1α/HIF-2α E3 ubiquitin ligase VHL, expression of HIF-1α is almost always lost and it is viewed as a tumor suppressor (Shen and Kaelin, 2013). Our findings in normal cells suggest that in the early phase of Ni carcinogenesis, HIF-1α may also operate in a tumor suppressor-like manner acting to restrict proliferation of severely damaged cells.

Acknowledgments

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Abbreviations

CDK	cyclin-dependent kinase
EdU	5-ethynyl-2′-deoxyuridine

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Highlights

- HIF-1α enhances growth arrest and senescence by Ni(II) in normal human cells
- HIF-1α is important for upregulation of the cell cycle inhibitors p21 and p27 in Ni(II)-treated normal cells
- Apoptotic responses and clonogenic toxicity by Ni(II) in transformed human cells are HIF-1α independent
- HIF-1α does not affect Ni(II) accumulation in normal or transformed human cells

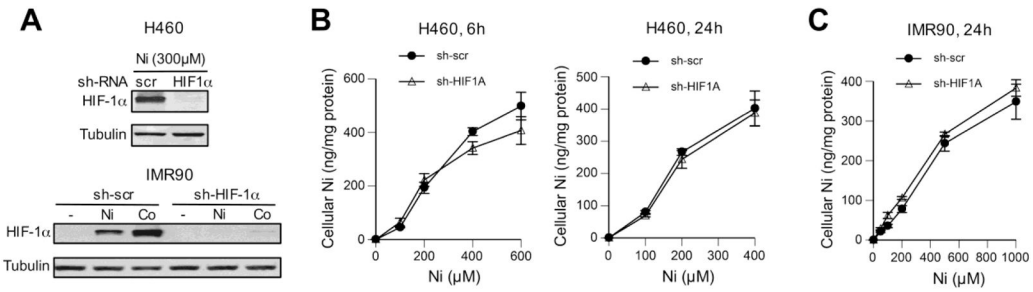

Figure 1. Ni accumulation in human cells with HIF-1α knockdown

H460 and IMR90 cells were stably expressing scrambled (sh-scr) or HIF-1α-targeting shRNA (sh-HIF1A). (**A**) Western blots demonstrating HIF-1α knockdown in H460 (300 μM Ni, 24h) and IMR90 cells (500 μM Ni or Co, 24h). (**B**) Ni levels in H460 cells treated for 6h (left panel) or 24h (right panel). Data are means±SD, n=3. (**C**) Ni accumulation in IMR90 treated for 24h (means±SD, n=3).

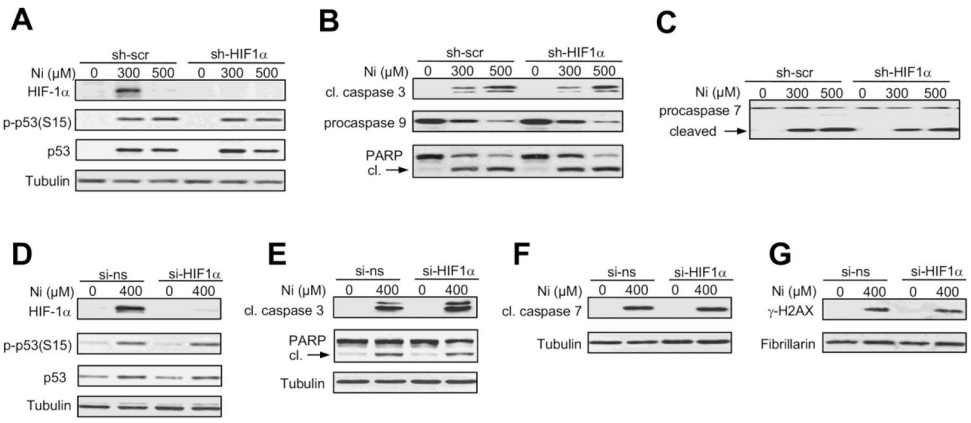

Figure 2. Apoptotic responses and p53 activation

H460 cells were treated with Ni(II) for 48h (panels A–C, shRNA-silenced HIF-1α, scr - scrambled shRNA) or 24h (panels D–G, siRNA knockdown of HIF-1α, ns - nonspecific siRNA). (**A**) Westerns for HIF-1α, p53 and Ser15-phosphorylated p53. (**B**) Westerns for activation of caspases 3 and 9 and PARP cleavage (cl.- cleaved). (**C**) Activation cleavage of caspase 7. (**D**) Westerns for HIF-1α and p53 (p-p53: Ser15-phosphorylated p53). (**E**) Formation of active caspase 3 and PARP cleavage (cl.- cleaved). (**F**) Production of active (cl.-cleaved) caspase 7. (**G**) Western for Ser139-phosphorylated histone H2AX (γ-H2AX).

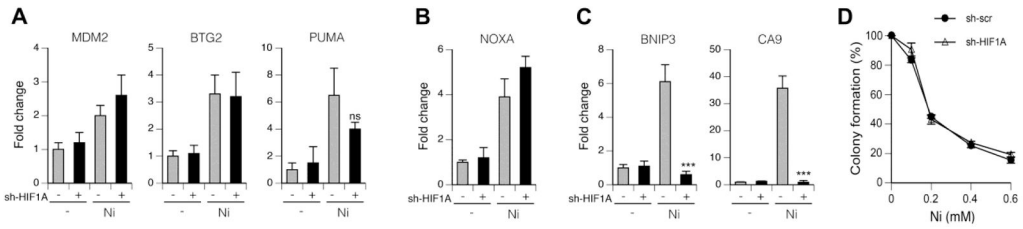

Figure 3. Expression of Ni-inducible genes and clonogenic survival

H460 cells expressing scrambled (−) or HIF1A-targeting shRNA (+) were treated with 400 μM Ni for 24h. Data are means±SD, n=3. (**A**) Expression of p53-dependent genes (ns - nonsignificant). (**B**) Levels of proapoptotic NOXA mRNA. (**C**) Expression of HIF1A-dependent genes BNIP3 and CA9 (***- $p<0.001$). (**D**) Colony formation by H460 cells treated with Ni for 24h (means±SD, n=3).

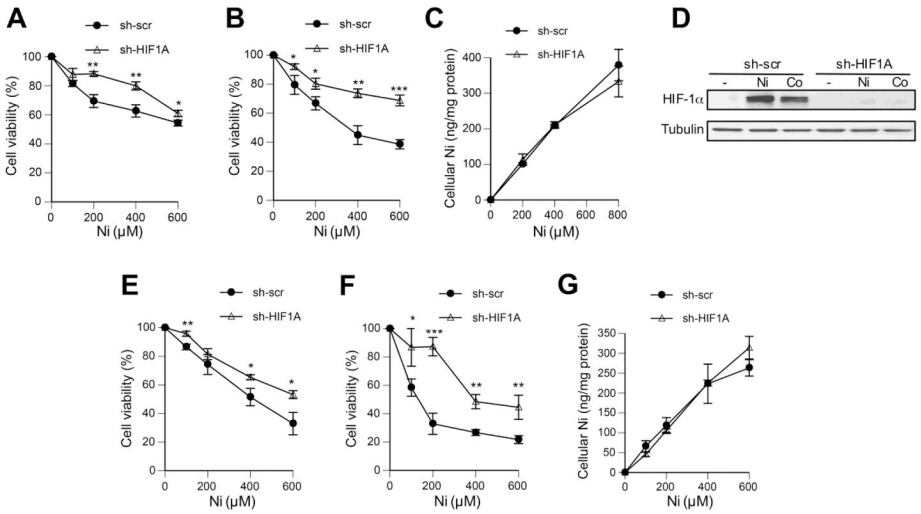

Figure 4. Cytotoxicity of Ni in normal human cells

Cells expressing scrambled (sh-scr) or HIF-1α-targeting shRNA (sh-HIF1A) were treated with Ni for 48h. Data are means±SD, n=3. Statistics: *- p<0.05, **- p<0.01, ***- p<0.001. (**A**) Viability of IMR90 cells at the end of Ni treatments and (**B**) after 48h recovery post-Ni. (**C**) Ni levels in IMR90 cells at the end of treatments. (**D**) Western blot demonstrating HIF-1α knockdown in WI38 cells (500 μM Ni or Co, 24h). (**E**) Viability of WI38 cells at the end of Ni treatments and (**G**) after 48h recovery post-Ni. (**G**) Ni accumulation in WI38 cells at the end of treatments.

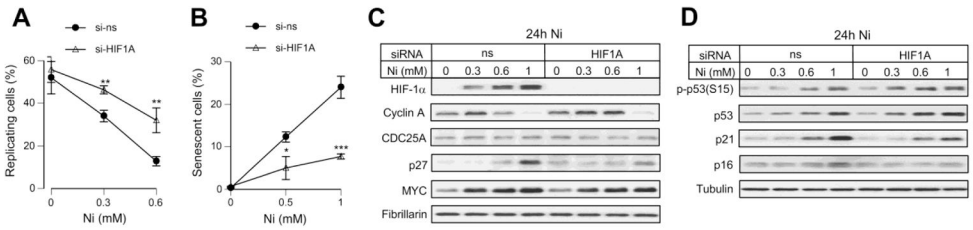

Figure 5. Ni-induced growth arrest and senescence in IMR90 normal human cells

(**A**) Percentage of replicating IMR90 cells transfected with nonspecific (si-ns) and HIF1A-targeting siRNA and treated with Ni for 48h. Replicated cells were labeled with EdU for 48h after removal of Ni. Data are means±SD, n=3, **-$p<0.01$. (**B**) Percentage of senescent IMR90 cells as determined by staining for senescence-associated β-galactosidase activity at 72h after Ni removal (48h Ni treatments). Data are means±SD, n=3, *-$p<0.05$, ***-$p<0.001$. (**C**) Protein expression of selected cell cycle regulators and (**D**) Senescence-promoting factors after 24h treatments with Ni (ns - nonspecific siRNA, HIF1A - targeting siRNA).

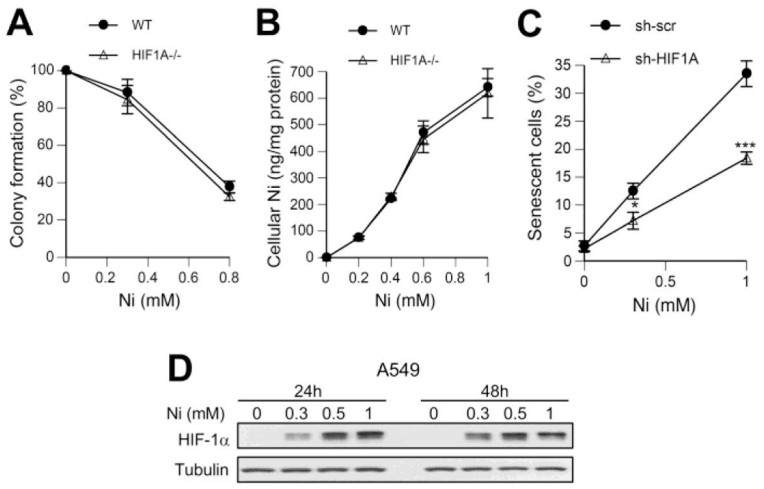

Figure 6. Long-term survival of Ni-treated A549 and WI38 cells

(A) Colony formation by wild-type and *HIF1A*−/− A549 cells treated with Ni for 24h (means±SD, n=3). **(B)** Ni accumulation by A549 cells treated for 24h (means±SD, n=3). (**C**) Frequency of senescent WI38 cells expressing control (sh-scr) and HIF1A-targeting shRNA. Cells were treated with Ni for 48h, reseeded and scored for expression of senescence-associated β-galactosidase 72h later (means±SD, n=3, *- p<0.05, ***- p<0.001 relative to sh-scr). (**D**) Protein levels of HIF-1α in A549 cells treated with Ni for 24 and 48h.