

The Role of Hypoxia Inducible Factor 1 α in Cobalt Chloride Induced Cell Death in Mouse Embryonic Fibroblasts

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Cobalt has been widely used in the treatment of anemia and as a hypoxia mimic in cell culture and it is known to activate hypoxic signaling by stabilizing the hypoxia inducible transcription factor 1 α (HIF1 α). However, cobalt exposure can lead to tissue and cellular toxicity. These studies were conducted to determine the role of HIF1 α in mediating cobalt-induced toxicity. Mouse embryonic fibroblasts (MEFs) that were null for the HIF1 α protein were used to show that HIF1 α protein plays a major role in mediating cobalt-induced cytotoxicity. Previous work from our lab and others has shown that two BH3 domain containing cell death genes, BNip3 and NIX, are targets of hypoxia signaling. These experiments document that BNip3 and NIX expression is HIF1 α -dependent, and cobalt induces their expression in a time and dose dependent manner. In addition, their expression is correlated with an increase in BNIP3 and NIX protein. Characteristically, the elevated level of BNIP3 was correlated with an increased presence of chromatin condensation, one marker for cell injury. Interestingly, this increased chromosomal condensation was not coupled to caspase-3 activation as usually seen in a typical apoptotic response. These results show that HIF1 α is playing a major role in mediating cobalt-induced toxicity in mouse embryonic fibroblasts and may offer a possible mechanism for the underlying pathology of injuries seen in workers exposed to environmental contaminants that can influence the hypoxia signaling system, such as cobalt.

Key Words: cobalt; hypoxia; toxicity; BNIP3; metal.

Metals constitute a large percentage of the earth's crust and their biochemical and geochemical cycles have been drastically altered by human activities. Metals are stable and persistent environmental contaminants and tend to collect in soils and sediments. Many of these metals, such as cobalt, nickel, and manganese are used in a wide variety of industrial applications, including the production of alloys, paints, and batteries. Exposure to these metals often occurs in workers involved in these industrial processes. Environmental exposure has now become a cause for concern. Metal smelting and other industrial processes

close to agricultural land and the use of bio-solids in the production of fertilizers has increased the possibility that these metals are entering our food supply in greater quantities (Adamu *et al.*, 1989). In addition, the recent approval of the gasoline additive, methylcyclopentadienyl manganese tricarbonyl (MMT), increases the risk of environmental contamination. Accordingly, understanding the underlying molecular mechanism(s) of metal-induced toxicity is of increasing importance.

Certain metals are also essential for human health. For example, cobalt plays a critical role in the synthesis of vitamin B12. In contrast, excessive exposure to cobalt is associated with several conditions, including asthma, pneumonia, and hematological abnormalities (Lauwerys and Lison, 1994). In addition, nickel, cobalt, cadmium, and other metals are known or suspected carcinogens (Hayes, 1997). Despite numerous reports of metal-induced toxicity, the underlying mechanism remains unclear. Studies in various systems have shown that exposure to certain metals, such as cobalt, promotes a response similar to hypoxia. Hypoxia is defined as a state when oxygen tension drops below normal limits and it plays a central role in development and several pathological conditions including stroke, cardiovascular disease, and tumorigenesis (Giaccia *et al.*, 2003). Due to oxygen's critical role in energy production, organisms have developed a programmed response to hypoxia that increases glucose utilization and stimulates erythropoiesis and angiogenesis to compensate for the decrease in available oxygen (Bunn and Poyton, 1996; Li *et al.*, 1996; Maltepe and Simon, 1998; Semenza *et al.*, 1994). The hypoxia inducible factors (HIFs) are a family of transcription factors that mediate the response to hypoxia by regulating the expression of genes capable of regulating glycolysis, angiogenesis, and erythropoiesis, such as erythropoietin (EPO), vascular endothelial growth factor (VEGF), pyruvate kinase, and many others (Forsythe *et al.*, 1996; Gleadle and Ratcliffe, 1997; Krieg *et al.*, 1998; Sandner *et al.*, 1997; Wang and Semenza, 1995).

Prolonged hypoxia can also induce genes involved in cell death (Bruick, 2000; Guo *et al.*, 2001). Cobalt and nickel can activate hypoxia-mediated signaling pathways aberrantly under normoxia by stabilizing the cytosolic hypoxia inducible factor 1 α (HIF1 α) (Ho and Bunn, 1996). For example, cobalt is thought

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to stabilize HIF1 α by inhibiting the prolyl hydroxylase domain-containing enzymes (PHDs), a family of enzymes that play a key role in oxygen dependent degradation of the transcription factor (Epstein *et al.*, 2001). The characterization of the PHD family of enzymes offers a direct link between metal exposure and HIF mediated signaling. This direct link and the overlap between gene expression patterns between hypoxia and cobalt exposure have led us to hypothesize that HIFs may be necessary to the toxic effects of cobalt (Vengellur *et al.*, 2003). This hypothesis was tested using HIF1 α ^{-/-} cells and several markers of toxicity. Our results demonstrate that HIF1 α plays an important role in metal-induced toxicity. Among the HIF1 α dependent genes whose expression was altered by cobalt treatment are the pro-apoptotic factors, BNIP3 and NIX. Overall, these results suggest that cobalt-induced toxicity is dependent upon the HIF1 α protein and its ability to induce the expression of cell death promoting genes.

MATERIALS AND METHODS

Materials. Tissue culture media and supplements were obtained from Invitrogen, Inc. Cosmic calf serum was obtained from Hyclone. Oligonucleotide synthesis was performed at the Macromolecular Facility, Michigan State University (East Lansing, MI). SYBR Green real time PCR reagents were purchased from Applied Biosystems (Foster City, CA). All other chemicals were reagent grade and obtained from Sigma Chemical Company (St. Louis, MO).

Cell culture and toxicity assay. Mouse embryonic fibroblast cell lines were maintained in modified DMEM media (10% heat inactivated FBS, penicillin-streptomycin [10 U/ml], non-essential amino acid [10 μ g/ml], L-glutamine [2 mM]). The cells were treated with 0 and 100 μ M of CoCl₂ for 48 h. Cells were trypsinized, counted with a hemacytometer and 10,000 cells were plated in 6 cm cell culture dishes. Cells were counted with a hemacytometer on one, two, three, four, and five days after plating.

MTT assay. Cells were grown in 96 well plates and treated with 0, 50, 100, 150, and 200 μ M of CoCl₂ for 72 h. The MTT assay was performed by replacing the cobalt containing media with 100 μ l of media containing 0.5 mg/ml of MTT (3-(4,5-dimethylthiazolyl)-2, 5-diphenyltetrazolium bromide). The plates were then incubated for 4 h (37°C). Media was then removed by aspiration and 200 μ l of solvent (1:1 DMSO:ethanol) was added and the formazan crystals solubilized with continuous agitation. Optical density (OD) measurements were taken at 550 and 630 nm and the difference in OD relative to untreated controls was taken as a measure of cell viability (van de Loosdrecht *et al.*, 1991). For the CoCl₂ time course experiment cells were treated with 150 μ M CoCl₂ and MTT assay was performed on four consecutive days and values are represented as a percent of time matched controls within cell types.

Protein extraction and Western blotting. Wild type and HIF1 α ^{-/-} cells were grown under normoxic (20% O₂), or hypoxic (1% O₂) conditions (NAPCO 7000 incubator, NAPCO, Winchester, VA) or in the presence of 100 μ M or 150 μ M CoCl₂ and protein extracts were prepared as described previously (LaPres *et al.*, 2000). Briefly, cells were washed with cold PBS (4°C) and removed from surface by scraping on cold PBS and collected by centrifugation. Soluble proteins were extracted with cell lysis buffer (25 mM HEPES, pH = 7.6, 2 mM EDTA, 10% glycerol, 1 μ g/ml of aprotinin, leupeptin, pepstatin A, and 1 mM PMSF) and three rounds of sonication (5 s, 4°C). Insoluble material was removed by centrifugation (16,000 \times g, 1 h). Protein concentrations were determined using Bio-Rad Bradford assay kit and BSA standards (Lowry *et al.*, 1951). An equal amount of protein was

separated by SDS-PAGE, and Western blotting was performed with BNIP3 and NIX specific antibody (Sigma, St. Louis, MO and Exalpha Biologicals, Maynard, MA respectively) using ECL chemiluminescent detection kit (Amersham Pharmacia). A β -actin specific antibody (a generous gift of Dr. John Wang, MSU) was used to verify equal loading.

RNA extraction and reverse transcription. RNA extraction was performed using TriZol reagent (Invitrogen) via manufacturer's instructions. Briefly, cells were treated for the specific duration and washed in 1X PBS (4°C). Cells were removed by scraping in the presence of 1 ml of TriZol reagent. Phase separation was accomplished by addition of chloroform and centrifugation (16,000 \times g, 15 min). RNA was precipitated using isopropanol and was quantitated spectrophotometrically. One μ g total RNA was used in subsequent reverse transcription reaction using Superscript II RNase H- Reverse Transcriptase (Invitrogen) via manufacturer's instructions.

Real-time quantitative PCR analysis. The measurement of BNIP3 and NIX mRNA levels were performed using real-time PCR technology and SYBR Green as a detector. Primers were designed using the web based application, Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) biasing towards the 3' end of the transcript giving a gene-specific product (Table 1). The primers were characterized by BLAST, and the amplicon size was verified by gel electrophoresis. The primer and Mg²⁺ concentrations were optimized and the PCR was performed using 5% of the reverse transcription product described above. All standards, unknowns, and no template controls assays were performed with at least three biological replicates. All of the assays were performed on an ABI 7700 under standard thermal cycling parameters: 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 60 s. The mRNA expression for each gene was determined by comparing it with a standard curve of known quantities of the specific target. This measurement was controlled for RNA quality, quantity, and RT efficiency by normalizing it to the expression level of the murine hypoxanthine guanine phosphoribosyl transferase (HPRT) gene. HPRT was used as a control gene because it was shown to be unaffected by any treatment used.

Cell staining and caspase assay. Cells were left untreated or exposed to CoCl₂ (150 μ M, 48 h) or staurosporine (0.01 μ M, 48 h) and stained with Hoechst 33342 dye (1 μ g/ml, 15 min). Cells were viewed and photographed with fluorescence microscopy. The percentage of apoptotic nuclei were determined by counting the number of cells displaying a dense nuclear staining. At least 200 cells were counted in at least three separate fields for each treatment and values are displayed as a percent of total nuclei.

Caspase assays were performed using EnZChek caspase-3 assay kit #2 (Molecular Probes) via the manufacturer's instructions. Briefly, cells were left untreated or exposed to CoCl₂ (150 μ M, 48 h) or staurosporine (1 μ M, 4 h). Cell extracts were obtained by scraping the cells in lysis buffer and cleared by centrifugation. The caspase-3 activity in the supernatant was analyzed spectrophotometrically (caspase activity in the cell lysate leads to the cleavage of the non-fluorescent substrate into a fluorescent product). The specificity of the caspase 3 activity was determined by the addition of a caspase-3 inhibitor (Ac-DEVD-CHO Inhibitor).

Statistics. Statistical analysis was performed between treated and untreated or vehicle controls using *t*-test (two tailed, unequal variance, *p* \leq 0.05 cut-off).

TABLE 1
qRT-PCR Primers

Gene	Accession	Forward	Reverse
HPRT	NM 013556	AAGCCTAAGAT-GAGCGCAAG	TTACTAGGCAGA-TGGCCACA
BNIP3	NM 009760	GGCGTCTGACA-ACTTCCACT	AACACCCAAGGA-CCATGCTA
BNIP3(L) (NIX)	NM 009761	GGAAGAGTGGAGCCATGAAG	GTGTGCTCAGTCG-TTTTCCA

RESULTS

Growth Curve Analysis of Wild Type and HIF1 α ^{-/-} Cells under CoCl₂ Treatment

An equal number of untreated and cobalt exposed (100 μ M, 48 h) WT and HIF1 α ^{-/-} cells were plated onto 6 cm cell culture dishes. The cells in each plate were counted on each of the subsequent five days. Wild type cells showed little or no growth following CoCl₂ (100 μ M) treatment (Fig. 1). In contrast, HIF1 α ^{-/-} cells exposed to CoCl₂ were initially slow to grow compared to untreated controls, but had a much higher survival compared to wild type treated cells. These results suggest that HIF1 α plays a role in mediating cobalt-induced growth inhibition.

Cell Toxicity Assay of CoCl₂ Treated Cells Using MTT Assay

The growth curves described in Figure 1 suggest that HIF1 α plays an important role in cobalt-induced growth arrest; however, it does not prove that HIF1 α is involved in cobalt-induced toxicity. To determine the role of HIF1 α in the cytotoxicity induced by CoCl₂, wild type and HIF1 α ^{-/-} cells were treated with varying concentrations of cobalt (0, 50, 100, 150, and 200 μ M CoCl₂) when cells had reached approximately 40% confluence. After incubation of 72 h, cell viability was evaluated using the MTT assay. The cytotoxic effects of cobalt were significantly attenuated in the HIF1 α ^{-/-} cells when compared to the WT cells (Fig. 2A). To determine the time course of cell

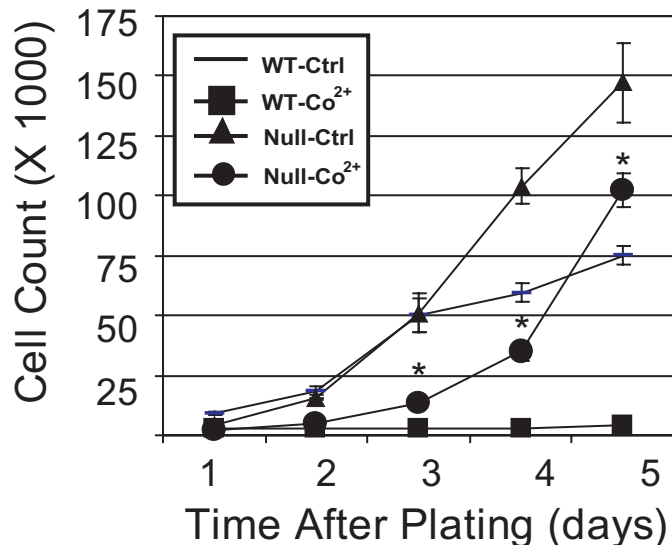


FIG. 1. Growth curve. Wild type (WT) and HIF1 α ^{-/-} cells were left untreated (Ctrl) or exposed to CoCl₂ (Co²⁺, 100 μ M) for 48 h. Following treatment, 10,000 cells were plated to 6 cm dishes in triplicate and cell counts were recorded on subsequent days. Values were normalized to Day 1 values within cell type. *Represents $p < 0.05$ when HIF1 α ^{-/-} cobalt treated cells were compared to WT cobalt treated cells.

injury, MTT assay was performed on the wild type and HIF1 α ^{-/-} cells following exposure to 150 μ M CoCl₂. As shown in Figure 2B, CoCl₂ exposure resulted in progressive increase in toxicity to wild type cells compared to HIF1 α ^{-/-} cells. These results support the growth curve analysis presented in Figure 1 and suggest that HIF1 α plays an important role in cobalt-induced cytotoxicity.

Quantitative Real-Time PCR Analysis of the BCL2 Family Genes, BNip3 and NIX Genes

Our lab and others have described the regulation by hypoxia of two members of the BCL2 family, BNip3 and NIX (Guo *et al.*, 2001; Kubasiak *et al.*, 2002; Regula *et al.*, 2002; Sowter *et al.*, 2001; Vengellur *et al.*, 2003). Global expression analysis using cDNA microarrays of cobalt-induced genes in WT and HIF1 α ^{-/-} cells has shown that this regulation is HIF1 α dependent (Vengellur *et al.*, 2003). The expression of these genes was evaluated by quantitative real-time PCR (qRT-PCR) using SYBR Green as a detector. RNA was extracted from untreated and hypoxia- or cobalt-exposed WT and HIF1 α ^{-/-} cells. BNip3 was upregulated eight-fold in a HIF1 α -dependent manner following cobalt (100 μ M) or hypoxia (1% O₂) exposure (Fig. 3A). In addition, NIX also showed a HIF1 α -dependent expression pattern and was induced more than 12 fold after cobalt exposure (Fig. 3B). These results suggest that cobalt upregulates BNip3 and NIX in a HIF1 α -dependent manner and are consistent with cobalt-induced activation of HIF1 α -regulated proapoptotic pathways.

Time Course Analysis of BNip3 mRNA Levels under CoCl₂ or Hypoxia Treatment

The time course of BNip3 upregulation by cobalt and hypoxia was also analyzed by qRT-PCR. WT cells were left untreated or exposed to CoCl₂ (100 μ M) or hypoxia (1% O₂). Total RNA was extracted at various time points (0.25 to 72 h) after treatment and analyzed for BNip3 and HPRT expression. BNip3 expression levels reached maximum expression at 8 h following exposure to CoCl₂ (Fig. 4A). This expression level remained stable for the duration of the time course. The BNip3 expression pattern following exposure to hypoxia in the WT cells gradually increased and reached a maximum after 48 h of treatment (Fig 4A). BNip3 was not regulated by cobalt or hypoxia at any time point tested in the HIF1 α ^{-/-} cells (data not shown).

Dose Response Analysis of BNip3 mRNA Levels under CoCl₂

The regulation of BNip3 mRNA expression in cells was determined by qRT-PCR following exposure to 0, 50, 100, 150, and 200 μ M CoCl₂ for 24 h. As shown in Figure 4B, wild type cells showed marked upregulation of BNip3 mRNA at all doses tested with a maximum effect at 150 μ M CoCl₂. The HIF1 α ^{-/-} cells displayed no significant changes in Bnip3 transcript levels at any dose (Fig. 4B). The Ct value and actual transcript levels of BNip3

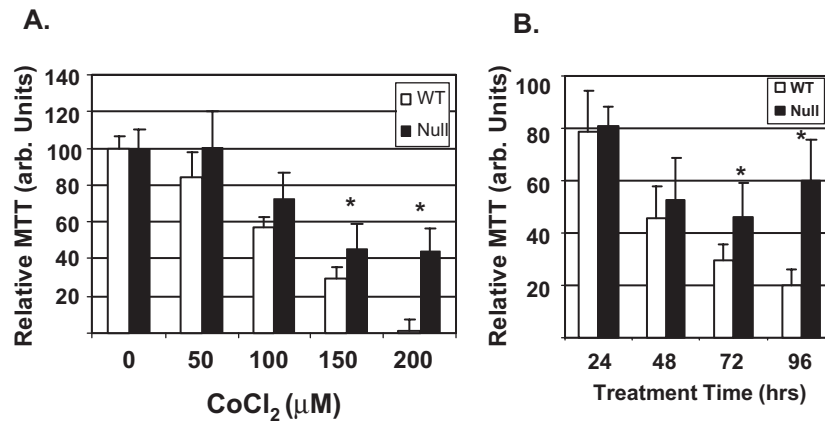


FIG. 2. Dose response and time course analysis of CoCl₂-induced cytotoxicity. (A) Wild type (WT, white bars) and HIF1α^{-/-} (black bars) cells were exposed to various concentrations of CoCl₂ (50, 100, 150, and 200 μM) or left untreated (Ctrl) for 72 h. Cell viability was assayed using a standard MTT assay. Control values within cell type were set to 100%. (B) Wild type (WT, white bars) and HIF1α^{-/-} (black bars) cells were exposed to 150 μM CoCl₂ and cell viability was assayed using MTT assay after 24–96 h. MTT values were normalized to time matched controls. *Represents *p* < 0.05 when the HIF1α^{-/-} and WT values were compared.

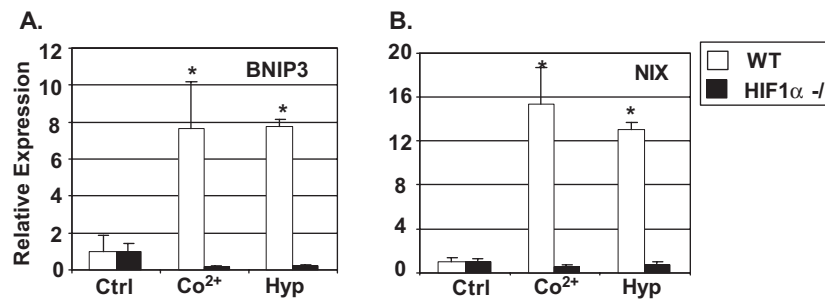


FIG. 3. qRT-PCR data for BNip3 and BNip3L. BNip3 (A) and BNip3L (NIX) (B) expression levels were analyzed by qRT-PCR in wild type (WT, white bars) and HIF1α^{-/-} cells (black bars) as described in methods section. Each value was normalized to the control level in the corresponding cell line. Ctrl = untreated, Co²⁺ = 100 μM CoCl₂, and Hyp = 1% O₂ for 24 h. *Represents *p* < 0.05 when compared to corresponding control.

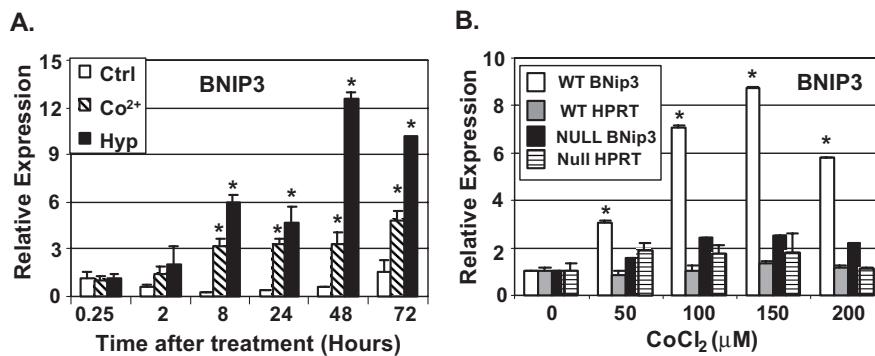


FIG. 4. qRT-PCR data for BNip3 expression under CoCl₂ and hypoxia time course in wild type cells. (A) BNip3 expression levels were analyzed by qRT-PCR in wild type cells left untreated (Ctrl, white bars) or following exposure to Co²⁺ (100 μM, hatched bars) or hypoxia (1% O₂, black bars). RNA was extracted at 0.25, 2, 8, 24, 48, and 72 h following 100 μM CoCl₂ exposure. Each value was normalized to the untreated control level at 0.25 h. (B) BNip3 and HPRT expression levels were analyzed by qRT-PCR in wild type (WT, BNip3 white bars, HPRT gray bars) and HIF1α^{-/-} cells (BNip3 black bars, HPRT hatched bars) left untreated or with 50, 100, 150, and 200 μM CoCl₂ for 72 h. BNip3 expression levels are calculated as explained in methods and normalized to respective controls. HPRT expression levels are absolute levels normalized to its respective control values. *Represents *p* < 0.05 when compared to corresponding control.

in WT cells ranged from 18.1 to 22.6 and 9.2×10^5 to 4.5×10^4 respectively (data not shown). The expression level of the control gene HPRT was also analyzed and shown not to significantly change following exposure to any concentration of CoCl_2 in either cell type (Fig. 4B).

Western Blot Analysis of BNIP3 and NIX Levels

Western blot analysis was performed to determine the levels of BNIP3 and NIX proteins in CoCl_2 treated cells. Wild type and $\text{HIF1}\alpha^{-/-}$ cells were treated with cobalt (100 and 150 μM) or hypoxia (1% O_2) for 48 h and total protein was extracted and separated by SDS-PAGE. There was a dramatic increase in BNIP3 levels following cobalt and hypoxia exposure and this increase was dependent upon the presence of $\text{HIF1}\alpha$ (Fig. 5, upper panel). The multiple bands represented in the BNIP3 Western blot are due to progressive proteolysis and has been previously reported to be indicative of cellular stress (Chen *et al.*, 1997). NIX protein levels also showed a marginal increase in CoCl_2 and hypoxia treated wild type cells (Fig. 5, middle panel). Both BNIP3 and NIX levels were unchanged in CoCl_2 treated $\text{HIF1}\alpha^{-/-}$ cells. β -actin was used to verify equal protein loading (Fig. 5, lower panel).

Cell Morphology

The results suggest that cobalt may be promoting cell death by inducing $\text{HIF1}\alpha$ -mediated upregulation of pro-apoptotic genes. Apoptosis is correlated with morphological and biochemical changes within the cell, such as nuclear condensation, DNA fragmentation, and caspase activation. However, BNIP3

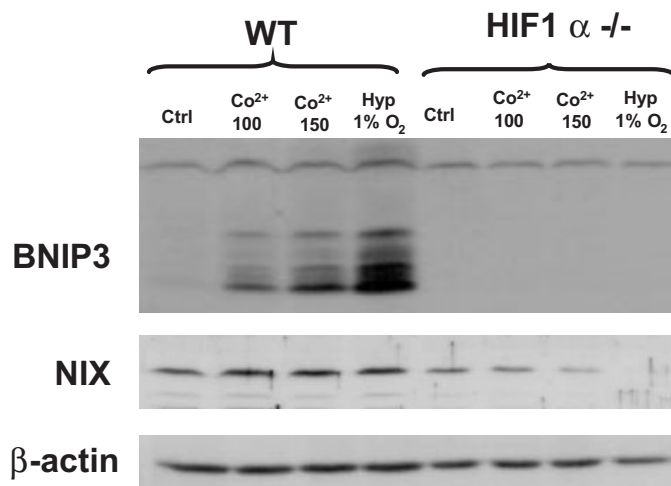


FIG. 5. BNIP3 and BNIP3L (NIX) Western blot. Wild type (WT) and $\text{HIF1}\alpha^{-/-}$ cells were left untreated (Ctrl) or exposed to 100 μM CoCl_2 , 150 μM CoCl_2 or hypoxia (1% O_2), for 48 h. Total protein was extracted and separated by SDS-PAGE, transferred to nitrocellulose membrane and probed with a BNIP3 (upper panel) and NIX (middle panel) specific antibody. Blots were stripped and reprobed with β -actin to verify equal loading (lower panel).

overexpression has been shown to induce a necrosis-like, caspase-independent cell death (Vande Velde *et al.*, 2000). To determine if cobalt treated cells were undergoing classic apoptosis or something similar to BNIP3-induced necrotic cell death, chromosomal staining and caspase-3 assay were performed in the WT and $\text{HIF1}\alpha^{-/-}$ cells.

Cells were left untreated or exposed to cobalt (150 μM), or staurosporine (0.01 μM) for 48 h and stained with Hoechst33342 nuclear dye. Staurosporine is a known inducer of apoptosis and was used as a positive control (Tamaoki *et al.*, 1986). Cobalt induced a moderate level of chromatin condensation in the WT cells, while little, if any, was observed in the $\text{HIF1}\alpha^{-/-}$ cells. In contrast, staurosporine treated WT and $\text{HIF1}\alpha^{-/-}$ cells showed marked chromatin condensation (Fig. 6A). The percentage of condensed nuclei in each treatment was also determined. Cobalt treated wild type cells induced a three-fold increase in the percentage of condensed nuclei (Fig. 6B). These experiments, taken together with the previous gene expression and Western blot results, suggest that $\text{HIF1}\alpha$ -mediated gene activation may be involved in promoting cobalt-induced cell death.

Caspase-3 Assay

As mentioned previously, caspase-3 activation is another hallmark of apoptosis and is considered one of the final steps in the process (Nicholson *et al.*, 1995). To determine whether CoCl_2 treatment causes activation of caspase-3 in a $\text{HIF1}\alpha$ -dependent manner, WT and $\text{HIF1}\alpha^{-/-}$ cells were exposed to cobalt (150 μM) or staurosporine (1 μM) and extracts were prepared (Figs. 7A and 7B). Caspase-3 activity in the extracts was assessed by fluorimetric analysis in the presence and absence of a caspase-3 inhibitor (Ac-DEVD-CHO, Molecular Probes). WT as well as $\text{HIF1}\alpha^{-/-}$ cells showed no significant increase in caspase-3 activity following CoCl_2 treatment. This was not due to a general lack of activity in the assay since the positive control, staurosporine, showed a marked activation of caspase-3 in the WT and $\text{HIF1}\alpha^{-/-}$ cells. This activity was abolished in the presence of the caspase-3 inhibitor confirming the specificity of the assay (Fig. 7). These results suggest that cobalt-induced cytotoxicity does not involve caspase-3 activation and that it involves the $\text{HIF1}\alpha$ -dependent upregulation of BNIP3 or NIX, which leads to a necrosis-like cell death.

DISCUSSION

Hypoxia is a characteristic feature of a number of pathophysiological conditions such as cancer, stroke, cardiac ischemia, etc. (Semenza *et al.*, 2000). Cobalt chloride has been widely used as a hypoxia mimic in both *in vivo* and *in vitro* studies (Wang and Semenza, 1993). However, the role of aberrant hypoxic signaling in cobalt-induced toxicity has not been addressed. Previous work has shown that on a global gene expression level, both cobalt and hypoxia regulate a similar

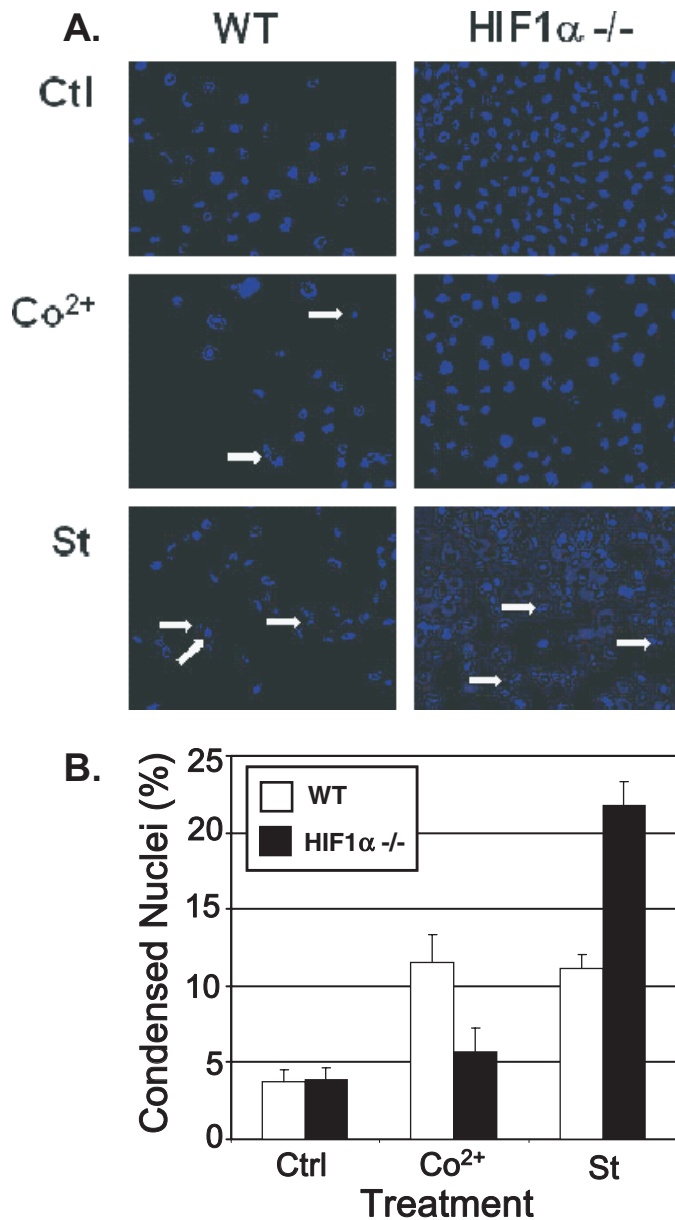


FIG. 6. Cell morphology. (A) Wild type (WT) and HIF1 α ^{-/-} cells were left untreated (CH) or exposed to 150 μ M CoCl₂ (Co²⁺) or 0.01 μ M staurosporine (St) for 48 h. Cell morphology was observed using Hoechst33342 DNA binding dye using fluorescent microscope. White arrows denote cells with morphology consistent with chromatin condensation. (B) Percentage of condensed nuclei in wild type (WT, white bars) and HIF1 α ^{-/-} cells (black bars) left untreated (Ctrl) or exposed to 150 μ M CoCl₂ (Co²⁺) or 0.01 μ M staurosporine (St) for 48 h. *Represents $p < 0.05$ when compared to corresponding control.

group of genes (Vengellur *et al.*, 2003). The observed similarity in gene expression appears to be dependent upon a functional HIF1 α protein (Salnikow *et al.*, 2000; Vengellur *et al.*, 2003). The experiments described here characterized the mechanism of action of cobalt chloride-induced cell death and determine the role of HIF1 α in this process.

Cell viability and proliferation studies show that wild type cells are more susceptible to cobalt-induced toxicity when compared to HIF1 α ^{-/-} cells. Given that HIF1 α is a transcription factor, it seemed likely that this toxicity is dependent upon gene activation. Previous genomic screens and other reports have identified BNIP3 and NIX as target genes of hypoxia signaling (Bruick, 2000; Guo *et al.*, 2001; Sowter *et al.*, 2001; Vengellur *et al.*, 2003). Expression of these pro-apoptotic factors was shown to be HIF1 α dependent and to occur at a dose and in a time frame similar to that of cobalt-induced cell damage (Figs. 2 and 4). These results offer a direct link between the cobalt exposure, hypoxia signaling, and activation of genes involved in cell injury. BNIP3 and NIX are BH3 domain-containing proteins belonging to the pro-apoptotic family of genes and their increased expression is correlated with increased cell death (Chen *et al.*, 1999). Here we show that these mitochondrial proteins are not mediating cell death through the classical caspase-3 activation pathway. There have been earlier reports that increases in BNIP3 lead to caspase activation in primary cardiac myocytes undergoing hypoxia (Regula *et al.*, 2002). However, in other cell types, BNIP3 induces a cell death similar to necrosis, which doesn't involve caspase activation (Kubasiak *et al.*, 2002). In addition, it has been shown that BNIP3 cause a necrosis-like cell death in cells through the mitochondrial permeability transition pore which involves the loss of mitochondrial potential but without caspase activation and cytochrome C release (Vande Velde *et al.*, 2000). This is not surprising, as there are alternate pathways of apoptosis that do not require caspase activation such as the AIF pathway (Lorenzo *et al.*, 1999). One important point is that caspase-dependent apoptosis is an energy requiring process. At least during hypoxia, ATP levels in the cell are low due to the inhibition of oxidative phosphorylation. Therefore, it is possible that the cells initiate an apoptotic process but resort to a necrotic pathway due to reduced ATP levels. At present it is not clear if ATP levels are reduced under cobalt treatment; however, the morphological study of CoCl₂ treated cells show moderate chromatin condensation in the absence of caspase 3 activation. Taken together, it seems likely that the HIF1 α -dependent increase in BNIP3 and NIX leads to caspase-independent, necrotic-like cell death similar to what has been demonstrated in 293T, MCF7, other MEFs and various tumors (Sowter *et al.*, 2001, 2003; Vande Velde *et al.*, 2000).

Cobalt and nickel are known to activate hypoxic signaling and nickel-induced transformation of fibroblasts requires a functional hypoxia signaling pathway (Salnikow *et al.*, 1997, 2003). The mechanism of action of CoCl₂ mediated stabilization of HIF1 α under normoxia is not completely elucidated. It is thought to inhibit the iron containing HIF prolyl hydroxylase enzyme, which plays a critical role in mediating the normal hypoxic signaling by modifying HIF1 α protein and targeting it for degradation. The chemical characteristics of cobalt also allow it to compete for iron at reactive sites of various enzymes, rendering these enzymes inactive. The first published reports of

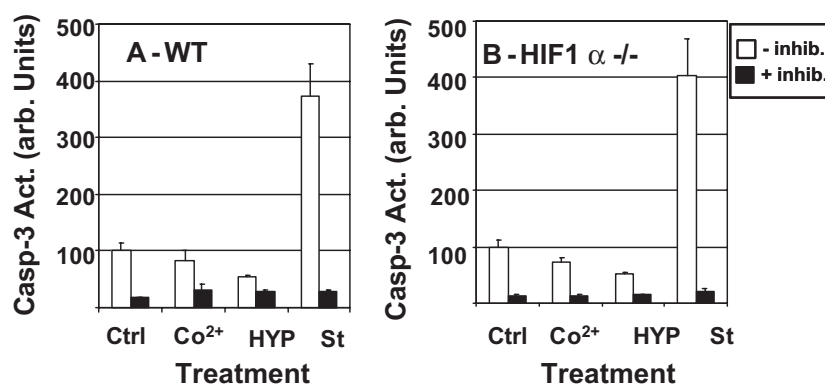


FIG. 7. Caspase-3 assay. (A) Wild type (WT) and (B) HIF1 α ^{-/-} cells were left untreated (Ctrl) or exposed to CoCl₂ (Co²⁺, 150 μ M, 48 h), Hypoxia (HYP, 1% O₂, 48 h) or staurosporine (St, 1 μ M, 4 h). Caspase3 activity was measured using EnZChek caspase-3 assay kit #2 (Molecular Probes). Assay was performed in the presence of either control (DMSO, white bars) or a caspase inhibitor (black bars).

the PHD family of enzymes characterized this inhibition and helped explain the ability of cobalt to act as a hypoxic mimic (Epstein *et al.*, 2001). Recent reports also suggest that cobalt exposure may not displace iron at the catalytic site within the hydroxylase but may sequester the available ascorbate in the cell. Since ascorbate is necessary for the transition of iron between oxidation states, this would effectively inhibit PHD activity (Salnikow *et al.*, in press).

The hypoxic signaling pathway is known to activate cell survival genes involved in glycolysis, angiogenesis, and erythropoiesis (Levy *et al.*, 1995; Semenza *et al.*, 1994; Wang *et al.*, 1995). In addition, in some cell types, cobalt and hypoxia exposure has been shown to inhibit apoptotic pathways (Piret *et al.*, 2004). Under chronic hypoxia, however, this pathway also activates genes involved in cell cycle arrest and death including proapoptotic genes (Bruick, 2000). These reports and our current results suggest a complex and at times, contradictory picture for cobalt induced damage. The protective effects of cobalt were shown using a very different experimental paradigm and this may explain the differences in results. Piret *et al.* exposed HepG2 cells to tert-butyl hydroperoxide (t-BHP) under serum-free conditions to characterize cobalt's inhibitory effects (Piret *et al.*, 2004). In contrast, we utilized MEFs in the presence of serum and absence of outside apoptotic stimuli. Treatment time may have also been a factor since the HepG2 cell's cobalt exposure was limited to 8 h in the serum containing controls. These differences highlight the complexity in metal-induced toxicity and suggest multiple pathways may be involved. For example, the observation that cobalt toxicity was only partially attenuated in the HIF1 α ^{-/-} cells suggests that cobalt-induced cell death involves HIF1 α dependent and independent mechanisms (Fig. 1). HIF1 α independent mechanisms may be dependent upon other functioning HIFs in the MEFs or the possible disruption of one or more of the essential enzymes that require iron as a cofactor, which may ultimately affect cell viability. Also, the effect of oxidative stress due to the production of reactive oxygen species

on various cell processes and integrity of cellular components such as proteins, DNA and lipid bi-layer cannot be underestimated. Consistent with this hypothesis, cobalt chloride treatment is known to induce stress responsive proteins such as metallothionein in wild type and HIF1 α ^{-/-} cells (Murphy *et al.*, 1999; Vengellur *et al.*, 2003).

In summary, WT and HIF1 α ^{-/-} cells were treated with cobalt chloride and toxicity was studied using cell count and MTT assays. Wild type cells showed a marked decrease in cell viability as well as cell proliferation compared to HIF1 α ^{-/-} cells. Wild type cells showed an increase in the expression of the cell death gene, BNip3 mRNA and protein upon CoCl₂ treatment. The cell death and expression of BNip3 mRNA overlapped in both the time course and dose response studies. This study shows that cobalt chloride exposure in mouse embryonic fibroblast leads to a necrosis-like cell death, which is dependent on the presence of functional HIF1 α protein. This indicates that the pathology of cobalt-induced toxicity might be due to the activation of aberrant hypoxic signaling leading to the increase in the cell death promoting genes such as BNIP3 and NIX levels and subsequent necrosis.

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