The Role of Hypoxia-Inducible Signaling Pathway in Nickel Carcinogenesis

Konstantin Salnikow, Todd Davidson, and Max Costa

Nelson Institute of Environmental Medicine, NIEHS Center and Kaplan Comprehensive Cancer Center, New York University School of Medicine, New York, New York, USA

Using human and rodent cells *in vitro,* we characterized a hypoxia-inducible signaling pathway as one of the pathways affected by carcinogenic nickel compounds. Acute exposure to nickel activates hypoxia-inducible transcription factor-1 (HIF-1), which strongly induces hypoxia-inducible genes, including the recently discovered tumor marker *Cap43*. This gene has been cloned based on its nickel inducibility and was found to be highly inducible by hypoxia. To identify other HIF-1–dependent/independent nickel-inducible genes, we used cells obtained from HIF-1α null mouse embryos and analyzed gene expression changes using the microarray technique. We found that genes coding for glycolytic enzymes, known to be regulated by HIF-1, were also induced in nickel-exposed cells. In addition, we identified a number of new genes highly induced by nickel in an HIF-dependent manner. Elevated HIF-1 activity after acute nickel exposure might be selectively advantageous because nickel-transformed rodent and human cells possess increased HIF-1 transcriptional activity. Hypoxia plays an important role in tumor progression. It selects for cells with enhanced glycolytic activity, causing production of large amounts of lactic acid, one of the most common features of tumor cells (Warburg effect). Here, we hypothesize that exposure to nickel activates the hypoxia-inducible pathway and facilitates selection of cells with increased transcriptional activity of hypoxia-inducible genes, which may be important in the nickel-induced carcinogenic process. *Key words:* gene chip, HIF knockout, hypoxia, transcription factors. *Environ Health Perspect* 110(suppl 5):831–834 (2002).
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The high use of nickel-containing products in modern industry leads to environmental pollution by nickel in both soluble and insoluble forms. Additionally, combustion of fossil fuel, particularly oil, contributes significantly to environmental burdens of nickel compounds (*1*). Epidemiologic studies have clearly implicated nickel compounds as human carcinogens based upon a higher incidence of lung and nasal cancer among nickel mining, smelting, and refinery workers (*2*). In various animal models, nickel compounds induce tumors at virtually any site of administration. Additionally, nickel compounds efficiently transform rodent and human cells *in vitro*. Based on these observations, the International Agency for Research on Cancer evaluated the carcinogenicity of nickel in 1990 (*3*). All nickel compounds except metallic nickel were classified as carcinogenic to humans.

The molecular basis of nickel carcinogenesis has proved elusive because carcinogenic nickel compounds are weakly mutagenic in most assay systems even though they are able to produce oxidative DNA damage and inhibit DNA repair activity (*4–8*). Nickel induces rather weak oxidative stress that depletes glutathione and activates nuclear factor kappa B (NF-κB) and other oxidatively sensitive transcription factors (*9–12*).

Recently, new data related to the activation of hypoxia-inducible signaling pathways by nickel have emerged. It is suggested that nickel might substitute for iron in a hypothetical oxygen sensor, thus switching a cell's metabolism to a state that mimics permanent hypoxia (*13,14*). Using differential display, we have cloned a nickel-inducible gene, *Cap43* (*15*) that, in addition to its induction by nickel compounds, was also found to be induced by hypoxia through hypoxia-inducible transcription factor-1 (HIF-1) (*16*). Moreover, we have shown that acute exposure to nickel activates HIF-1 and that the transcriptional activity of HIF-1 is significantly elevated in nickel-transformed cells (*17*). Because hypoxia is common in solid tumors and selects for more malignant phenotypes, this information provides new prospects for understanding the molecular mechanisms of nickel carcinogenesis. Using Affymetrix GeneChip analysis, we show here that the exposure of cells to nickel triggers the expression of genes involved in glucose transport and glycolysis. Cellular responses to hypoxic stress usually include inhibition of cell proliferation and, when cell damage is irreversible, apoptosis (*18*). A pattern of gene induction/suppression similar to that observed in hypoxic cells was found here in nickel-exposed cells. For example, the induction of the heat-shock protein 70 (HSP 70) and the proapoptotic protein Nip3 was observed in nickel-exposed cells using both GeneChip and Northern blot analysis or polymerase chain reaction (PCR) analyses. We suggest that by mimicking a state of hypoxia, nickel may provide the necessary conditions for the selection of cells that have changed their energy metabolism or growth control requirements or have become resistant to apoptosis.

Materials and Methods

Materials

Nickel chloride and cobalt chloride were obtained from Alfa Aesar (Ward Hill, MA, USA). Cell culture media, fetal calf serum, glutamine, and antibiotics were obtained from Gibco-BRL (Rockville, MD, USA). The most commonly used chemicals were purchased from Sigma (St. Louis, MO, USA). The murine Genome U74A Array and Test3 Array were obtained from Affymetrix (Santa Clara, CA).

Cell Culture

Mouse embryo fibroblasts and cells with HIF-1α knockout (HIF-1$\alpha^{-/-}$) were obtained from R. Johnson (University of California at San Diego) and were described previously (*16*). All cells were maintained at 37°C as monolayers in a humidified atmosphere containing 5% CO_2. Cell exposure to hypoxia has been previously described (*14*). HIF-1$\alpha^{+/+}$ and HIF-1$\alpha^{-/-}$ cells were exposed to 1 mM $NiCl_2$ for various times (as indicated in figure legends). The survival rate measured by trypan blue assay was around 70%.

Isolation of RNA, cDNA Synthesis, and GeneChip Hybridization

Total RNA was isolated from nickel-exposed and nonexposed cells using TRIzol reagent (Gibco-BRL) and was used for Northern blot analysis or to prepare poly(A) mRNA. For GeneChip analysis double-stranded cDNA was synthesized with a cDNA synthesis kit (Superscript cDNA Synthesis System; Gibco-BRL) by using an oligo(dT)24 primer with a T7 RNA polymerase promoter site added to its 3′ end. The isolated cDNA was used for

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Address correspondence to K. Salnikow, Dept. of Environmental Medicine, NIEHS and Kaplan Comprehensive Cancer Centers, New York University, 550 First Ave, New York, NY 10016 USA. Telephone: (845) 731-3516. Fax: (845) 351-2118. E-mail: salnikow@env.med.nyu.edu

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in vitro transcription (Ambion T7 Megascript system; Austin, TX, USA) in the presence of biotin-11-CTP and biotin-16-UTP (Enzo Diagnostics, Farmingdale, NY, USA). A total of 25–50 µg of the cRNA product in buffer (40 mM Tris acetate, pH 8.1; 100 mM potassium acetate; 30 mM magnesium acetate) was fragmented at 94°C for 35 min. This probe was used for hybridization and mixed with herring sperm DNA (0.1 mg/mL; Sigma). The Test3 chip served for evaluation of the probe quality as directed by the manufacturer (Affymetrix).

Aliquots of the cRNA hybridization mixtures (10 µg cRNA in 200 µL hybridization mix) were hybridized to a mouse GeneChip array, washed, and scanned (GeneArray scanner G2500A; Palo Alto, CA, USA) according to procedures developed by the manufacturer (Affymetrix).

Analysis of Gene Expression Data

Scanned output files were visually inspected for hybridization artifacts and then analyzed with GENECHIP 3.1 software (Affymetrix). Arrays were scaled to an average intensity of 125 and analyzed independently. Genes showing no changes or changes less than 1.5-fold were excluded from the analysis. The intensity of the signal of the remaining 5,310 genes was then normalized based on the signal of four independent actin genes. The median value of each actin gene across the four chips was divided by the specific signal value for each chip. The resulting values were then averaged for each chip. This average value was used as a signal value normalization factor for the correction of all genes. The expression value (average difference) for each gene was determined by calculating the average of differences of intensity (perfect match intensity minus mismatch intensity) between its probe pairs.

The expression analysis files were created by GENECHIP 3.1 software, transferred to a database (Microsoft Access), and linked to Internet genome databases (e.g., GenBank at National Center for Biotechnology Information; *http://www.ncbi.nim.nih.gov*). Fold changes in gene expression were determined by dividing the mean intensity of each nickel-exposed condition by the mean intensity of the control cells.

Northern Blot Analysis

Total RNA was extracted from cells immediately after exposure using a TRIzol reagent (Gibco-BRL) and was electrophoresed (15 µg total RNA/lane) in 1.0% agarose/formaldehyde gels. Probes were labeled with [^{32}P]-α-dCTP using a random primed DNA labeling kit (Promega, Madison, WI, USA). The Nip3 probe was a kind gift from R. Bruick (University of Texas Southwestern Medical Center).

PCR Amplification

cDNA and 5′-CGCTGAGCTTGCCCTTGAGA-3′ and 5′-ACGGGCGCGACCTGAACAAG-3′ primers were used for HSP 70 amplification. The PCR parameters (18 cycles) were 3 min initial denaturation at 95°C followed by denaturation at 95°C for 50 sec, annealing of primers at 58°C for 50 sec and extension of the product at 72°C for 50 sec. The reaction was performed in a 50-µL volume with 1 µg of cDNA, 0.25 µM primers, 500 µM dNTP, and 4 U of Taq polymerase (Indianapolis, IN, USA). Actin was amplified using mouse specific primers from Stratagene (La Jolla, CA, USA).The PCR products were analyzed in 1% agarose gel containing 0.5 µg/mL ethidium bromide. Gels were photographed using a Kodak digital camera (Kodak, Rochester, NY, USA).

Results

HIF-Dependent Induction of Glycolytic Enzymes and Genes Involved in Glucose Transport by Nickel

It was previously shown that most of the genes involved in glucose metabolism and glycolysis were inducible by hypoxia and were HIF-1 dependent (*19*). Previous studies using HIF-1–proficient and HIF-1–deficient fibroblasts have shown that exposure of cells to nickel induces genes involved in hypoxic stress only in HIF-1–proficient fibroblasts (*16*). These data suggest that nickel activates an HIF-1–dependent pathway. Here, using GeneChip analysis on HIF-1–proficient and HIF-1–deficient fibroblasts, we further investigated the induction of these genes by nickel. Table 1 shows the induction of genes involved in glucose transport and glycolysis in HIF-1–proficient and HIF–1–deficient mouse embryo fibroblasts exposed to 1 mM $NiCl_2$ for 20 hr. Of 12 candidate genes involved in glucose metabolism, 10 were induced by nickel exposure in the HIF-1–proficient cells but not in the HIF-1–deficient cells. Glucose 6-phosphate dehydrogenase and hexokinase I were the only genes that were little changed in nickel-exposed cells. It is interesting to note that these data are in agreement with the literature data because, for example, glucose 6-phosphate dehydrogenase is the only gene involved in glucose metabolism not affected by hypoxia (*20*). Figure 1 shows the results of Northern blot analysis of one representative gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), confirming the changes in the expression of GAPDH in HIF-1–proficient cells but not in HIF-1–deficient cells.

HIF-Dependent Induction of Proapoptotic Genes by Nickel

Hypoxic stress can cause the induction of p53 followed by apoptosis (*21*). Recently, hypoxia was reported to induce another proapoptotic gene, Nip3 (*22*). In our experiments we

Table 1. Changes in the expression of glycolytic enzymes in $NiCl_2$-exposed HIF-1α–proficient and HIF-1–deficient fibroblasts.[a]

Glycolytic enzyme (accession number)	Fold increase in fibroblasts	
	HIF-1α–proficient	HIF-1α–deficient
Glucose transporter I (M22998)	8.27	0.38
Hexokinase I (J05277)	1.95	1.56
Hexokinase II (Y11666)	10.42	0.46
Glucose 6-phosphate dehydrogenase (Z11911)	1.09	0.56
Glucose phosphate isomerase I (L09104)	2.23	0.72
Phosphofructo kinase B (J03928)	5.80	0.33
Aldolase A (Y00516)	3.99	1.36
GAPDH (M32599)	4.53	0.75
Pyruvate kinase 3 (X97047)	2.86	1.59
Phosphoglycerate kinase I (M15668)	4.24	0.99
Lactate dehydrogenase A (M17516)	6.44	0.81
Triosephosphate isomerase (L31777)	2.48	0.64

[a]Cells were exposed to 1 mM $NiCl_2$ for 20 hr.

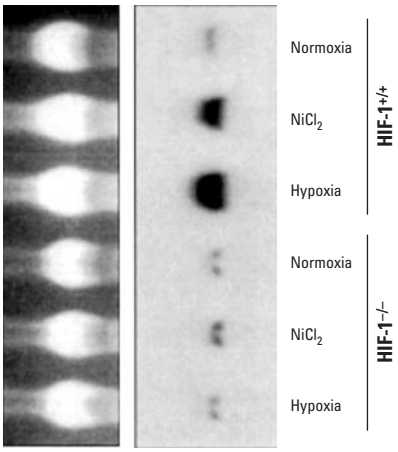

Figure 1. Induction of GAPDH by nickel in HIF-1–proficient cells or HIF-1–deficient mouse fibroblasts. Cells were exposed to hypoxia (1.0% O_2) or to 1 mM $NiCl_2$ for 20 hr. Fifteen micrograms of total RNA were subjected to a Northern blot analysis. The blot was hybridized with the GAPDH probe (top); the ethidium bromide staining of small ribosomal subunit is shown to assure loading (bottom).

found that nickel significantly induced the expression of Nip3 only in HIF-1–proficient fibroblasts (Table 2). The data obtained with the GeneChip were confirmed using Northern blot analysis. Figure 2 shows that the exposure of HIF-proficient cells to nickel induced the expression of Nip3 in a dose-dependent manner.

HIF-Independent Induction of Gene Expression by Nickel

In addition to demonstrating the activation of an HIF-dependent pathway, we were able to show that nickel induces some genes in an HIF-independent manner (Table 2). *HSP 70*, *GADD45*, *p21*, and *p53* were induced by nickel in both HIF-1–proficient and HIF-1–deficient mouse fibroblasts. Some other genes, including *ATM*, *GADD153*, *Jun B*, and *MDR-1*, were induced in both HIF-1–proficient and HIF-1–deficient mouse fibroblasts, but the induction in HIF-1–proficient cells was more pronounced, suggesting a mixed regulation. Figure 3 shows the induction of HSP 70 in both HIF-1–proficient and HIF-1–deficient mouse fibroblasts.

Table 2. HIF-dependent and HIF-independent activation of gene expression by $NiCl_2$.[a]

Gene regulation	Gene name (accession number)	Fold increase in fibroblasts: HIF-1α proficient	Fold increase in fibroblasts: HIF-1α deficient
HIF-dependent	*Nip3* (AF041054)	21.3	0.98
HIF-dependent	*Prolyl-4-hydroxylase* (U16163)	10.0	0.96
HIF-independent	*HSP 70* (M12571)	2.3	93.3
HIF-independent	*GADD45* (U00937)	9.4	12.6
HIF-independent	*p21* (U09507)	7.8	7.8
HIF-independent	*p53* (AB021961)	2.2	2.0
Mixed	*ATM* (U43678)	6.0	2.6
Mixed	*GADD*153 (X67083)	4.3	2.8
Mixed	*Jun B* (U20735)	9.6	2.4
Mixed	*MDR-1* (M60348)	4.0	1.2

[a]Cells were exposed to 1 mM $NiCl_2$ for 20 hr.

Discussion

Nickel is a modern environmental contaminant that is toxic and carcinogenic. One possible pathway for nickel-induced carcinogenesis may involve changes in DNA methylation (*23*). Epigenetic mechanisms probably play a significant role in the carcinogenicity of nickel, although alternative mechanisms involving modulation of gene expression followed by selection of favorable phenotypes are also very likely. Previously we have shown that the expression of a number of genes was altered in nickel-exposed cells (*15–17*). The changes in gene expression in the exposed cells resulted from the activation of a number of transcription factors, including ATF-1, p53, and HIF-1 (*17,24*). The accumulation of HIF-1 transcription factor, the master regulator of the hypoxic response, suggested that the hypoxia-inducible signaling pathway is affected by nickel. Indeed, we found that a set of hypoxia-inducible genes was activated by nickel in HIF-1–proficient cells but not in HIF-1–deficient cells (*16*).

In this study we used the GeneChip technology to further identify genes induced by nickel in HIF-1–proficient cells but not in HIF-1–deficient cells. The GeneChip technology allowed us to analyze simultaneously the expression of approximately 11,000 genes. One interesting finding was that all genes involved in glucose transport and glycolysis were induced by nickel. These genes are also known to be induced by hypoxia (*25*). The expression of hexokinase I and glucose 6-phosphate dehydrogenase was not changed by nickel, and these genes were also not changed by hypoxia. Therefore, the effects on the expression of genes regulating glucose transport and glycolysis in nickel-exposed or hypoxic cells were identical.

Hypoxia plays an important role in tumor progression. It selects for cells with enhanced glycolytic activity, causing production of large amounts of lactic acid, one of the most common features of tumor cells known as the Warburg effect (*26*). It is possible that exposure to nickel activates the hypoxia-inducible pathway and the induction of this pathway facilitates selection of cells with increased glycolytic activity. In addition to genes involved in glucose metabolism and glycolysis, a number of other hypoxia-inducible genes were elevated by nickel in an HIF-dependent manner. For example, Nip3 is a proapoptotic gene significantly induced by hypoxia (*22*). The microarray data showed more than 20-fold induction of this gene in nickel-exposed HIF-1–proficient cells compared with HIF-1–deficient cells. The Northern blot analysis confirmed the induction of this gene by nickel in a dose-dependent manner only in HIF-1–proficient cells. In conclusion, these data again demonstrate similarity in the pattern of gene induction by nickel and hypoxia.

Other genes induced by nickel displayed HIF-independent regulation. Among these genes were *p21*, *p53*, and *GADD45*. Both genes *p21* and *GADD45* were regulated by *p53* (*27,28*). It is likely, therefore, that the induction of p53 by nickel stimulated the expression of these genes. These data are in good agreement with our previous finding of stimulation of p53-dependent transcription in nickel-exposed cells (*17*). The induction of *HSP 70* is under the control of heat-shock factor, which is activated by misfolded proteins (*29*). It is conceivable that nickel binds to cellular proteins, producing misfolded protein stress. *ATM*, *GADD153*, *Jun B*, and *MDR-1* showed mixed regulation. They were induced more by nickel in HIF-1–proficient cells; however, some induction in HIF-1–deficient cells was also observed, indicating that factors other than HIF-1 transcription factors were involved in the upregulation of these genes.

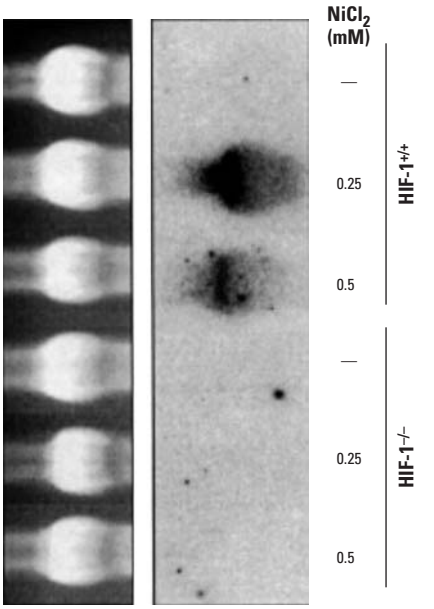

Figure 2. Induction of Nip3 by nickel in HIF-1–proficient cells or HIF-1–deficient mouse fibroblasts. Cells were exposed to $NiCl_2$ for 20 hr. Fifteen micrograms of total RNA were subjected to a Northern blot analysis. The blot was hybridized with the Nip3 probe (top); the ethidium bromide staining of small ribosomal subunit is shown to assure loading (bottom).

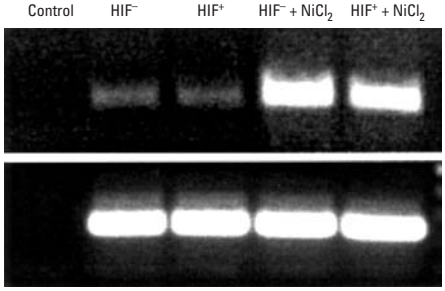

Figure 3. Induction of HSP 70 by nickel in HIF-1–proficient cells or HIF-1–deficient mouse fibroblasts. Cells were exposed to $NiCl_2$ (1 mM) for 20 hr. One microgram of cDNA was subjected to a PCR amplification. The HSP 70 amplification is shown on top; the actin amplification of the same samples is shown to assure cDNA quantity (bottom). Control, PCR mix with primers, no DNA.

Conclusions

Using GeneChip technology, we analyzed changes in gene expression in nickel-exposed cells. The induction of both HIF-dependent and HIF-independent genes was observed; HIF-dependent genes included the proapoptotic Nip3 as well as genes involved in glucose transport and metabolism. The induction of these genes was similar to that observed during hypoxic exposure. We hypothesize that exposure to nickel activates the hypoxia-inducible pathway and facilitates the selection of cells with increased transcriptional activity of hypoxia-inducible genes. These effects may be important in nickel-induced carcinogenic processes.

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