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The carcinogenicity of nickel compounds has been shown in numerous epidemiological and animal studies. Carcinogenesis is generally considered as a multistep accumulation of genetic alterations. Nickel, however, being highly carcinogenic is only a weak mutagen. We hypothesize that nickel may act by modulating signaling pathways, and subsequently by reprogramming transcription factors. Insoluble nickel is considered to be more carcinogenic than soluble. In this study using GeneChip technology we compared changes in gene expression caused by soluble and insoluble nickel compounds. We found that both soluble and insoluble nickel compounds induce similar signaling pathways following 20 h of *in vitro* exposure. For example, both nickel compounds activated a number of transcription factors including hypoxia-inducible factor 1 (HIF-1) and p53. The induction of these important transcription factors exerts potent selective pressure leading to cell transformation. The obtained data are in agreement with our previous observations that acute nickel exposure activates HIF-1 and p53 transcription factors and in nickel-transformed cells, the ratio of HIF-1 activity to p53 activity was shifted towards high HIF-1 activity. The activation of the same signaling pathways by soluble and insoluble nickel compounds suggested that both nickel compounds have similar carcinogenic potential *in vitro*.

Aim of investigation

Nickel (Ni) and several of its compounds are used in various industrial applications. Workers in the nickel-related industry are exposed to this metal mainly by inhalation, and varying toxic manifestations affecting the respiratory system have been noted (for recent reviews see refs. 1 and 2). The high carcinogenic activity of nickel (Ni) compounds in epidemiological studies, as well as, in numerous animal studies has been reported and reviewed.³⁻⁵ A high incidence of lung and nasal cancer in humans was attributed to exposure of workers to dust containing Ni particles and to exposure to different soluble Ni compounds.⁶ In cell transformation assays, most of the soluble and insoluble nickel compounds were found to be potent carcinogens, producing morphological foci of transformation and colonies in soft agar.⁷ Nickel compounds are not strong mutagens in many test systems from bacteria to human cells.⁸⁻¹⁰ It is therefore conceivable that Ni exerts its carcinogenic activity *via* modulation of activity of several transcription factors.^{1,2} Change in the activity of two important transcription factors, p53 and HIF-1, have been recently reported in human and rodent Ni-transformed cells.¹¹ The tumor suppressor p53 was up-regulated in Ni-exposed cells but its transcriptional activity was progressively lost during Ni-induced transformation *in vitro*. Others reported that p53 was mutated in human epithelial cells *in vitro* transformed by Ni compounds.¹² The level and transcriptional activity of another transcription factor, HIF-1, was elevated by acute exposure of human HOS cells to Ni. In Ni-transformed human or rodent cells, HIF-1 activity was significantly higher than in parental non-transformed cells.¹¹

HIF-1 is involved in coordinating the up-regulation of numerous genes involved in glucose transport and glycolysis.¹³ The induction of HIF-1 by Ni is responsible for the upregulation of glycolytic genes even in the presence of oxygen.¹⁴

During chronic exposure to Ni, cells maintain a high glycolytic rate and thereby acquire a phenotype similar to that of cancer cells. This cancer cell phenotype, namely, the high glycolytic rate in the presence of oxygen, was first described by Warburg.¹⁵

In this paper we utilized GeneChip microarray technology and compared the effect of soluble (NiCl₂) and insoluble (Ni₃S₂) Ni compounds on two types of mouse fibroblastic cells. The results presented numerous examples of activation of HIF-1- and p53-dependent genes. Moreover similar genes were up- or down-regulated in mouse PW cells and mouse embryo fibroblasts in response to Ni₃S₂ or NiCl₂. These findings support the view that soluble Ni exhibits carcinogenic potential similar to insoluble Ni (see also ref. 16).

Description of the experimental procedures

Materials

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

Cell culture

Mouse embryo fibroblasts (MEF) and cells with HIF-1 α knockout (MEF HIF-1 α ^{-/-}) were obtained from Dr R. Johnson (University of California, San Diego) and were described previously.¹⁴ Another cell line, mouse embryo fibroblasts (PW) were obtained from Dr C. Huang (New York University). All mouse cells were maintained at 37 °C as monolayers in DMEM media supplemented with 10% FBS in a humidified atmosphere containing 5% CO₂. Human lung bronchoepithelial A549 cells were purchased from the American Type Culture Collection (ATCC) (Rockville, MD). Cells were grown in Ham's F-12K

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medium with 10% FBS. Human lung cells were exposed to different concentrations of soluble and insoluble Ni. Mouse cells were exposed to 1 mM NiCl₂ or 0.3 μg sm⁻² Ni₃S₂ for 20 h. The survival rate of mouse cell exposed to soluble and insoluble Ni as measured by the trypan blue assay was found to be in both cases approximately 70%.

Isolation of RNA, cDNA synthesis and GeneChip hybridization

Total RNA was isolated from Ni-exposed and non-exposed cells using the TRIzol reagent (Gibco BRL, Rockville, MD) and was used for Northern blot analysis or to prepare poly(A) mRNA. For the GeneChip analysis, double-stranded cDNA was synthesized with a Superscript cDNA Synthesis kit (Gibco BRL, Rockville, MD) 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 *in vitro* transcription using the Ambion T7 Megascript system (Austin, TX) in the presence of biotin-11-CTP and biotin-16-UTP (Enzo Diagnostics, Farmingdale, NY). 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⁻¹; Sigma). The test 3 chip served for the evaluation of the probe quality as directed by the manufacturer (Affymetrix, Santa Clara, CA).

Aliquots of the cRNA hybridization mixtures (15 μg cRNA in 200 μl hybridization mix) were hybridized to a mouse U74A GeneChip array, were washed and scanned (Hewlett Packard, GeneArray Scanner G2500A, Palo Alto, CA) 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 less than 1.5-fold were excluded from the analysis. The intensity of the signal of the remaining genes was normalized based on the signal of four independent actin genes. The expression analysis files were created using the GENECHIP 3.1 software and were transferred to a database (Microsoft Access). The extent of changes in gene expression was determined by dividing the mean intensity of each Ni-exposed condition by the mean intensity of the control cells.

Results and discussion

Workers worldwide are exposed to toxic and carcinogenic airborne fumes, dusts and mists containing nickel and its compounds.⁶ Since inhalation has been shown to be the major route of occupational and environmental exposure to Ni compounds, disease of the respiratory tract is of primary concern in these workers. In animal models insoluble Ni compounds were found to be more carcinogenic than the soluble ones.^{5,17} One likely explanation why insoluble compounds including NiO and Ni₃S₂, are better carcinogens is because, as a rule, they are not easily cleared from lung tissues. After phagocytosis Ni containing particles are delivered to vacuoles where Ni is solubilized under acidic pH. This leads to release of high amounts of Ni into cytoplasm and nucleus. In contrast soluble Ni is easily cleared from lungs thus diminishing the exposure level. *In vitro*, however, it is possible to achieve chronic exposure of cells to rather high levels of soluble Ni. Since insoluble Ni is delivered to cells through phagocytosis and soluble Ni is delivered possibly through transport systems used by other metals the important question arises, whether soluble and insoluble nickel compounds have different effects on cells at the molecular and cellular levels. Previous studies

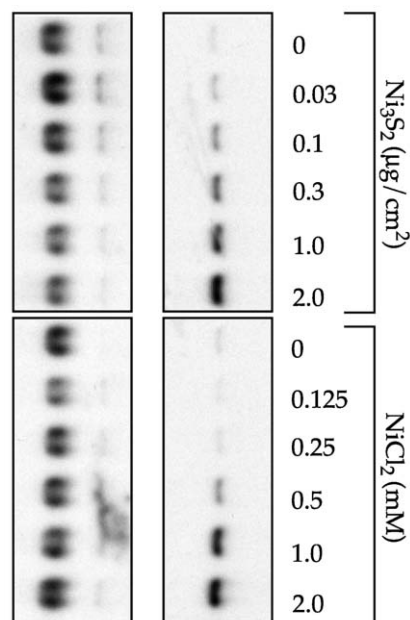


Fig. 1 Induction of NDRG1/Cap43 expression in response to insoluble Ni₃S₂ or soluble NiCl₂. A549 cells were exposed to different doses of both soluble and insoluble nickel compounds for 20 h. Total RNA was isolated and 20 μg of RNA was subjected to a Northern blot analysis. Upper panel represents NDRG1/Cap43 hybridization. Bottom panel represents actin hybridization of the same blot.

have shown that soluble Ni displayed high potency in producing foci of morphological transformation and soft agar growth suggesting that soluble Ni in *in vitro* system could be carcinogenic as well as insoluble one.^{7,18,19} Earlier we have shown that both HIF-1-dependent and p53-dependent pathways are induced by soluble Ni compounds.¹¹ Additionally, we found that in human lung cells both soluble and insoluble Ni can similarly induce a hypoxia-inducible gene, NDRG1/Cap43.²⁰ These data were confirmed here. Fig. 1 shows Northern blot analysis of dose-dependent induction of NDRG1/Cap43 gene expression in human lung cells by soluble and insoluble Ni.

Further, we used the microarray GeneChip technique in order to compare the effects of soluble and insoluble Ni compounds on gene expression. PW mouse fibroblastic cells were exposed for 20 h to two Ni compounds, insoluble nickel subsulfide (Ni₃S₂) and soluble nickel chloride (NiCl₂). The particle size of insoluble Ni₃S₂ was 5 μm or less. Should a particle of 4 μm size be completely dissolved inside the cell, that should result in intracellular Ni concentration 4.75 M.²¹ With the dissolution of 1.45 μm particles, Ni intracellular concentration should be 0.25 M. These data suggested that exposure to insoluble Ni resulted in higher accumulation of Ni inside cells. Therefore, in order to reach similar toxicity, rather high concentration of soluble Ni has been used. The concentrations of Ni 0.3 μg sm⁻² Ni₃S₂ and 1 mM NiCl₂ were selected based upon similar toxicity. In order to identify HIF-1-dependent genes induced by Ni we also used HIF-1 proficient and deficient mouse cells. Microarray data showed that in response to both Ni compounds, cells increase the expression of genes that are involved in glucose metabolism including glucose transporter I and glycolytic enzymes, such as hexokinase II, phosphofructokinase, pyruvate kinase, triosephosphate and glucose-phosphate isomerases, as well as, lactate dehydrogenase (Table 1). These genes are shown in Table 1 under Metabolism category. Since all these genes are induced by hypoxia,²² these data suggested that both Ni compounds similarly induce HIF-1 transcription factor, which regulates the expression of glycolytic enzymes and genes involved in glucose metabolism. HIF-1 α induction by soluble and insoluble Ni has been

Table 1 Modulation of gene expression by soluble and insoluble nickel in two mouse cell lines^a

	PW/Ni ₃ S ₂	PW/NiCl ₂	HIF+/NiCl ₂	HIF ⁻ /NiCl ₂	GB accession #
HIF-1-dependent genes					
Metabolism					
Glucose transporter	18.7	6.6	8.3	0.4	M22998
Phosphofructokinase	13.6	7.6	5.8	0.3	J03928
Hexokinase II	7.1	4.2	10.4	0.5	Y11666
Adenylate kinase 3	5.3	4.3	16.7	1.9	AB020239
Pyruvate kinase	5.0	4.5	2.9	1.5	X97047
Prolyl(4)hydroxylase	4.2	4.7	10.0	1.0	U16163
Triosephosphate isomerase	3.5	3.8	2.5	0.6	L31777
Glucose phosphate isomerase	2.8	3.0	2.2	0.7	L09104
Lactate dehydrogenase	2.6	5.6	6.4	0.8	M17516
Other HIF-1-dependent genes					
Tdd5	14.3	6.3	7.2	3.3	U52073
Egln I	11.6	6.7	14.3	0.3	A1850202
Nip3	11.6	10.9	21.3	1.0	AF041054
Est	10.8	4.0	16.5	2.6	AW120614
Gly96 (glycosylated protein)	9.5	5.5	8.2	3.8	X67644
Mnk 1 kinase	2.4	1.5	10.1	1.3	Y11091
Cyclin G2	2.3	2.2	4.4	1.0	U95826
Focal adhesion kinase	1.6	1.2	5.8	0.9	M95408
p53-dependent genes					
P21	7.0	1.3	7.8	7.8	U09507
GADD45	1.6	1.3	9.5	12.6	U00937
Other genes					
Elongation factor 2	3.8	2.4	2.8	2.2	M76131
Seven pass transmembrane G-protein coupled receptor	3.4	2.1	18.7	16.9	AA656014
Metallothionein I	3.5	4.8	6.3	5.6	V00835
Glutathione S-transferase theta	0.4	0.2	0.4	1.2	X98055
Aldehyde dehydrogenase II	0.4	0.3	0.2	0.1	M74570
Gas1	0.7	0.6	0.1	0.02	X65128
Transcription factors					
Arnt	1.9	1.0	3.5	1.1	U10325
EGR1	1.4	0.5	2.6	7.9	M28845
E2f1	0.5	2.2	3.7	1.8	L21973
Junb	2.0	1.1	9.6	2.4	U20735
Lrg-21	3.6	1.9	7.6	7.9	U19118
Wilms' tumor	0.4	0.5	0.4	0.6	M55512

^aNumbers more than one represent fold of induction over control, numbers less than one represent fold of suppression over control.

described earlier.^{11,14,23} Numerous genes are induced under hypoxic conditions, including glucose transporters, glycolytic enzymes, and VEGF, and the same genes are also induced when cells are exposed to Ni compounds.^{24,25} The gene coding for prolyl-4hydroxylase is highly inducible by hypoxia.²⁶ We found that this gene is also induced by both soluble and insoluble Ni in a HIF-1-dependent manner (Table 1).

In addition to HIF-1-dependent genes, Ni induced a number of genes in a HIF-1-independent manner, among them were p21, GADD45 and JunB (Table 1). These genes are known as p53-dependent.²⁷ Therefore, Ni in a manner similar to hypoxia is inducing another signaling pathway, the p53-dependent pathway. p53 has a dual role as a transcription factor and a tumor suppressor. p53 has been implicated in tumor progression and is absent or mutated in more than half of human cancers. The consequences of p53 induction by hypoxia in cells is the induction of programmed cell death. Hypoxia-induced apoptosis was found to be reduced in cells lacking p53, and small numbers of cells lacking p53 could outgrow similar p53 wild-type cells under hypoxic conditions *in vitro*.²⁸ Furthermore, highly apoptotic regions strongly correlated with hypoxic regions in p53 wild-type tumors, but not in tumors grown from p53-deficient cells. It therefore appears that tumor hypoxia can select for variants that have lost their apoptotic potential *in vivo*. Hypoxia selects for loss of p53. It is conceivable that Ni also selects for the loss of p53 function and p53 mutations were found in Ni-transformed cells.¹²

In addition to HIF-1 and p53, our data demonstrated that Ni exposure up- and down-regulates the expression of the Egr-1 and WT1 transcription factors (Table 1). WT1 which binds the same DNA sequences as Egr-1 displays opposite effects on the transcription of target genes. Interestingly, it was found that

hypoxia increased the expression of Egr-1; in contrast, the level of WT1 was markedly decreased during hypoxia.²⁹ Egr-1 activated by hypoxia can positively regulate the expression of the proangiogenic protein IGF-II. Egr-1 induction and the role of IGF-II as an autocrine/paracrine growth factor as well as an angiogenic factor can be significant in pathological conditions where cells are exposed to hypoxic environments. Thus, it is likely that the Egr-1 protein is an important nuclear intermediate in signal transduction processes under hypoxia and one of the crucial targets in carcinogenesis.

Conclusions

We found that both soluble and insoluble Ni compounds induce similar signaling pathways as measured by GeneChip analysis. The transcription factors modulated by Ni include HIF-1, p53, Egr-1 and WT1. Interestingly, all these transcription factors are similarly modulated by exposure to hypoxia. We hypothesize that the modulation of the expression of these important transcription factors by Ni exerts potent selective pressure leading to cell transformation.

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