A Novel Pathway for Nickel-induced Interleukin-8 Expression

Received for publication, March 27, 2002
Published, JBC Papers in Press, April 26, 2002, DOI 10.1074/jbc.M202941200

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Inhalation of particulate nickel subsulfide (Ni₃S₂) causes chronic active inflammation and fibrosis of the lungs. However, the mechanisms for these effects are not well understood. Therefore, cell culture experiments with BEAS-2B human airway epithelial cells were conducted to test the hypothesis that exposure to noncytotoxic levels of Ni₃S₂ induces expression of inflammatory cytokines such as interleukin-8 (IL-8). Exposure to Ni₃S₂ for 48 h was required to significantly increase IL-8 protein levels. Transcriptional stimulation of IL-8 mRNA levels preceded the increase in protein. Transient exposure to soluble nickel sulfate failed to increase IL-8 mRNA. Transfection with truncated IL-8 promoter constructs linked to the luciferase gene demonstrated that nickel-induced IL-8 transcription required −272 bp of the promoter relative to the transcriptional start site. A −133-bp construct, containing cytokine and hypoxia-sensitive AP-1, NF-IL6, and NF-κB sites, was insufficient for induction by nickel. Transfection with a dominant negative AP-1 construct or mutation of the AP-1, GATA, or C/EBP sites in the −272-bp IL-8 promoter construct blocked induction by nickel. Inhibiting ERK, phosphatidylinositol 3-kinase, but not p38 kinase, diacylglycerol kinase, or hypoxia-inducible factor-1α, attenuated nickel induction of IL-8. These studies indicate that nickel induced IL-8 transcription through a novel pathway that requires both AP-1 and non-traditional transcription factors.

Environmental exposure to inhaled nickel particles has been linked to increased mortality in the United States (1), and inhalation is the primary route of occupational exposure to nickel compounds (2). In the lower airways, nickel exposure is associated with immunological sensitization, epithelial dysplasia, asthma, lung cancer, and fibrosis (2). At the cellular level, nickel stimulates signaling cascades in airway epithelium that increase expression of the profibrotic gene, plasminogen activator inhibitor-1, and genes involved in hypoxic responses (3–5). This stimulation requires stabilization of hypoxia-inducible factor (HIF)¹ and AP-1 transcriptional activity (3, 4). These genes may contribute to the fibrotic process and may be common to the variety of pulmonary diseases elicited by nickel. In addition, HIF-independent pathways for nickel-induced transcription are not well described and could contribute to pleiotropic profibrotic and inflammatory effects observed in nickel-related lung diseases.

The CXC chemokine interleukin 8 (IL-8) is markedly up-regulated in pulmonary fibrosis (6–8) and has been implicated in promoting the growth of lung tumors (9, 10). IL-8 attracts and activates neutrophils, induces transendothelial migration of neutrophils, modulates chemotaxis for T-lymphocytes, promotes angiogenesis, and induces contraction of airway smooth muscle cells (10, 11). IL-8 mRNA levels were induced in human oral carcinoma and umbilical vein cells following nickel chloride treatment (12, 13). Although this suggests that IL-8 expression is sensitive to nickel exposure, enhanced IL-8 mRNA and protein levels were not detected in human airway cells exposed to soluble nickel sulfate for 2 h (14) or dermal cells treated with nickel chloride (13). In addition to potential cell-type-specific responses to nickel, different nickel species vary greatly in their kinetics and toxicity (2, 15). The lung rapidly clears water-soluble nickel salts, but insoluble particulate forms of nickel can be retained with biological half-lives of up to 3 years (2). Particulate Ni₃S₂ is one of the most toxic nickel species with respect to fibrosis and carcinogenesis (15), yet little is known of its effects on cytokine release.

The IL-8 promoter is found between −1481 and +44 bp of the transcriptional start site and contains multiple potential regulatory transcription factor binding sites, including glucocorticoids receptor, AP-1, AP-3, C/EBP, octamer motif binding proteins, NF-IL6, and NF-κB (11, 16, 17). Cytokine and inflammatory mediators, such as TNF, IL-1, or endotoxin, require a minimal promoter region of −130 bp, which contains essential AP-1, NF-IL6, and NF-κB sites, to fully stimulate transcription (16). Glucocorticoids and octamer binding proteins suppress IL-8 by either binding to their own DNA cis elements or preventing the binding of NF-κB (18, 19). The portions of the IL-8 promoter required for induction by nickel have not been previously investigated.

Ni₃S₂, in contrast to soluble nickel, could be retained in lung cells for a sufficient time to exacerbate the development of pulmonary inflammation and fibrosis in exposed individuals (15). The current experiments were designed to examine the time course for IL-8 expression by human airway epithelial cells exposed to Ni₃S₂, to determine whether this expression required retention of the particles by the cells. The results indicate that Ni₃S₂ causes prolonged transcriptional activation of the IL-8 gene by stimulating cell signaling cascades. In contrast, short exposures to soluble nickel do not induce IL-8 expression. Activation of the IL-8 gene by nickel is unique in

extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase.

* This work was supported by the Superfund Basic Research Program at Dartmouth (Grant ES07373) and the facilities of the Norris Cotton Cancer Center. The costs of publication of this article were defrayed in part by the payment of page charges. This article must indicate this fact.

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The abbreviations used are: HIF, hypoxia-inducible factor; PAI-1, plasminogen activator inhibitor-1; IL-8, interleukin 8; C/EBP, CAAT/enhancer-binding protein; TNF, tumor necrosis factor; RT, reverse transcription; EGFP, enhanced green fluorescence protein; PMA, phorbol 12-myristate 13-acetate; PI3K, phosphatidylinositol 3-kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase.

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that it does not require elements in the proximal promoter that are stimulated by other inflammatory mediators.

MATERIALS AND METHODS

Cells—Human bronchial epithelial cells (Beas-2B, ATCC, Rockville, MD) were grown to post-confluence in 6- or 12-well plates (Corning Costar, Corning, NY) on 1 mg of fibronectin, 0.33 mg of Vitrogen 100 (Collaborative Research), and 0.1 mg of BSA in 100 ml of LHC-8 medium (Biofluids), as previously described (3-5). The cultures were maintained in LHC-9 medium (Biofluids, Inc., Rockville, MD) at 37 °C under an atmosphere of 5% CO2/95% air.

Nickel—Respirable size particulate nickel was prepared by applying NiS2 particles (Aldrich, Milwaukee, WI) to a water column and allowing the larger particles to settle out. Particle size was measured during settling using a particle counter (Coultier Corp., Miami, FL). Particles of less than 2.5 μm in diameter were decanted, concentrated by centrifugation, and sterilized by baking at 200 °C for 18 h. This preparation gives the same quantitative and qualitative responses as a standard preparation of nickel subsulfide obtained from the Nickel Producers Environmental Research Association (a kind gift from Dr. Andrea Oller). Nickel(II) sulfate hexahydrate (Aldrich, Milwaukee, WI) was used to prepare soluble nickel solutions.

Previous studies demonstrated by clonogenic survival assays that addition of 2.34 μg of NiS2/cm2 of nickel subsulfide were not toxic to this cell model (5). This amount of NiS2 is roughly equivalent to 90 μg soluble NiSO4. However, direct comparisons of concentration are not possible due to the inability of NiS2 particles to remain in suspension above the cells. Tumor necrosis factor-α (TNF-α) (10 ng/ml) was used as a positive control to induce IL-8 expression. Kinase inhibitors (Calbiochem, La Jolla, CA) and antioxidants (Sigma Chemical Co., St. Louis, MO) were added 30 min prior to adding NiS2.

Protein Levels—The effect of nickel on secreted IL-8 protein levels was determined using the Quantikine IL-8 Immunoassay (R&D Systems, Minneapolis, MN). Briefly, conditioned medium was removed from treated cells and centrifuged for 10 min at 2000 rpm to remove insoluble debris. Protease inhibitors were added, and medium was incubated for 30 min at 4 °C in microtiter plates pre-coated with antibodies specific to IL-8. Western analysis for HIS-1a protein levels was performed using a polyclonal antibody to HIS-1a (Transduction Laboratories, Lexington, KY), essentially as described previously (3). Immuno-reactive bands were detected using horseradish peroxidase-linked secondary antibody and enhanced chemiluminescence (PerkinElmer Life Sciences, Boston, MA).

mRNA Levels—Total cellular RNA was harvested using TRIzol reagent (Invitrogen, Gaithersburg, MD) according to the manufacturer’s instructions. Reverse transcription-polymerase chain reaction (RT-PCR) was performed with 0.5 μg of the resulting RNA, as described previously (5, 20, 21). Specific primers for IL-8 (forward 5’-atgacttcagaatgcttgctgcc; reverse 5’-tgctgatacttccttttgctcg) and β-actin (forward 5’-tggaatgcttctgggcccttgct; reverse 5’-tggtcttgggcccttgctcg) were synthesized in the Molecular Biology Core at Dartmouth. PCR products were either visualized in agarose gels stained with ethidium bromide or quantified using the double-strand DNA fluorescent dye Picogreen (Molecular Probes Inc., Eugene, OR) at 430-nm emission/525-nm absorption. Densitometry was performed on ethidium bromide-stained gels using IMAGE (National Institutes of Health). IL-8 mRNA expression was normalized to the housekeeping gene β-actin by determining the ratio of the IL-8 to β-actin band density or Picogreen fluorescence.

Transient Transfection—Seventy percent confluent cells were transfected with IL-8 promoter constructs, normal and dominant-negative c-Jun expression plasmids, and enhanced green fluorescence protein (EGFP) using LipofectAMINE Plus reagent (Invitrogen, Gaithersburg, MD), as previously described (3, 4). The 1481, 272, 272, 161, or 133 IL-8 promoter constructs linked to luciferase, described previously (11, 22), were a kind gift from Dr. Naomoi Mukaida (Kanazawa University, Japan). The following site-directed mutants of the −272-luc IL-8 promoter plasmid were prepared by PCR: AP-1 (bp 126 to 120) from TGAATCA to TATCATA; GATA (bp −248 to −245) from GATA to GGTATA; and CEBP (bp −246 to −233) from TAAACCTCAATTT to TAAT- TCACATTAAA. Expression constructs for c-Jun, and TAMEβ were obtained from Dr. Michael Birrer (National Institutes of Health, Rockville, MD) and EGFP-N2 plasmid was from CLONTECH (Palo Alto, CA). Transfection efficiency in each well was assessed by measuring EGFP fluorescence using a fluorescent plate reader (PE Biosystems (Foster City, CA), excitation of 485 nm, emission of 508 nm). Transfection efficiency of BEAS-2B cells was estimated to be 20-30%.

Luciferase Assay—Cells were harvested in 80 μl of lysis buffer (25 mm glycyglycine, 4 mm EDTA, 15 mm MgSO4, 1% Triton X-100, 1 mm diithiothreitol). The lysates were then centrifuged at 13,000 × g at 4 °C for 5 min. Luciferase activity was determined using 50 μl of supernatant and 150 μl of fresh assay buffer (25 mm glycyglycine, 15 mm potassium phosphate, 15 mm MgSO4, 4 mm EDTA, 2 mm ATP, 1 mm diithiothreitol) in a Dynatech Microlite ML 2250 luminometer by injecting 50 μl of 0.1% luciferin.

Anti sense Oligonucleotides—HIF-1a phosphorothioate antisense and sense oligonucleotides were synthesized in the Molecular Biology Core at Dartmouth according to sequences published by Camiglia et al. (23). Cells were incubated for 24 h with 10 μM sense or antisense oligonucleotides prior to nickel treatment. Nickel-induced HIF protein levels were suppressed for greater than 56 h after a single dose of antisense (3).

Statistical—Statistical analysis was performed on data pooled from duplicate or triplicate determinations in two to three separate experiments to yield a total n = 6. Significant differences between treatment groups and controls were determined using one-way analysis of variance. The means of groups were compared using the Newman-Keuls post-hoc test. All statistics were performed using GraphPad Prism, version 3.0 (GraphPad Software, San Diego, CA). Data are presented as means ± S.D. or as percentage of control.

RESULTS

Nickel Stimulates Prolonged IL-8 Protein Release—The time course for IL-8 release from untreated BEAS-2B cells was compared with cells treated with NiS2 to determine whether the metal can induce an inflammatory response in airway epithelial cells. Secreted protein was measured by enzyme-linked immunosorbent assay at 24-h intervals following addition of NiS2. As shown in Fig. 1, more than 24 h of nickel exposure was required before significant increases in IL-8 expression occurred. These data suggest that particulate nickel stimulates a latent, but prolonged inflammatory phenotypic change in the BEAS-2B cells.

Phenotypic Change in Response to Nickel Occurs at the Level of Transcription—Previous reports indicate that nickel induces a probiotic phenotype by stimulating transcription of specific genes (5). To determine whether the observed increases in IL-8 protein were also due to induction of mRNA levels, total RNA was isolated from control cells or cells exposed to nickel subsulfide for up to 48 h. The data in Fig. 2 demonstrate that nickel stimulates a progressive increase in IL-8 mRNA levels, which was significantly greater than control by 24 h. These levels continued to rise between 24 and 48 h and were consistent with the time course for nickel stimulation of IL-8 protein.

IL-8 can be regulated at both the transcriptional and post-transcriptional level depending on the cell type and inducing agent used (16, 17). To determine the effect of nickel IL-8 message stability, cells were exposed to nickel or PMA (10 nM) for 24 h prior to the addition of the transcriptional blocker, actinomycin D (5 μg/ml). Total RNA was isolated over a 7-h
At the end of the exposure period, total RNA was extracted and IL-8 and β-actin mRNA levels were amplified by RT-PCR. Ethidium bromide staining was used to detect PCR products. B, the mean ± S.D. of the ratio of IL-8 product relative to β-actin product band density was compared with controls. The data represent RNA collected from separate cultures and are representative of at least six replicates. **, significant difference from IL-8 mRNA levels in non-treated cells (p < 0.001).

Transcriptional Activation by NiS2 Requires a Unique Region of the IL-8 Promoter—To determine whether nickel induces transcription of IL-8, BEAS-2B cells were transiently transfected with full-length or truncated portions of the IL-8 gene promoter linked to a luciferase reporter. The data in Fig. 4A were consistent with transcriptional activation of the promoter within 48 h of exposing the cells to NiS2. The proximal -272 bp contained the minimal nickel-responsive region of the promoter that provided maximal stimulation of luciferase activity. This -272 bp construct was more responsive than the full-length construct, consistent with loss of potential repressive elements, as previously reported for cytokine induction of the promoter (11, 16, 17). The construct containing -161 bp also responded to NiS2 with a significant increase in luciferase expression. Truncation by another 28-bp eliminated significant responses. These results differ greatly from reports defining the -133 bp construct as the minimal promoter for cytokine-induced IL-8 expression (11, 16, 17). The data in Fig. 4B demonstrated that the time course for nickel-stimulated expression of the -272 bp construct was consistent with the time course for increased endogenous IL-8 mRNA. This suggested that nickel stimulated the gene at the level of transcription. It is also important to note that the luciferase activity was progressively increased following exposure to NiS2. This contrasts with induction by TNF, which required only the -133-bp portion of the -272 construct (11, 16) and declined after 24 h.

Induction of IL-8 by Nickel Requires Unique Cis Elements in the Proximal Promoter—PCR site-directed mutagenesis of the -272 IL-8 promoter construct was performed to determine which unique cis elements were required for induction by nickel. Of several putative cis elements, the adjacent GATA and C/EBP sites at -248 to -245 and -246 to -233, respectively, appeared to be the most relevant sites to lung inflammation and pathophysiology. Mutation of either of these sites abolished induction of luciferase activity by nickel but not by TNF (Fig. 5). Disrupting the C/EBP site enhanced control and TNF-induced activity indicating that this site may be somewhat repressive. As discussed below, mutating the AP-1 site at -126 to -120 bp reduced both basal and nickel-induced activity indicating that cooperativity between proteins binding at this site and the proximal elements may be needed for full activation of the promoter by nickel.
Induction of IL-8 by Ni$_3$S$_2$ Requires Uptake into the Cells—Ni$_3$S$_2$ is reported to be more toxic to the lung due to its uptake into the cells and increased persistence relative to soluble forms of nickel (2, 15). To demonstrate that uptake and sustained exposure to Ni$_3$S$_2$ were required for inducing transcription, the effects of short exposures to Ni$_3$S$_2$ and soluble nickel sulfate on HIF-1α/H929 protein and IL-8 mRNA levels were compared. In this paradigm, cells were treated with either form of the metal for 30 min before the cultures were rinsed and the medium was replaced. Despite repeated rinsing, microscopic examination demonstrated that Ni$_3$S$_2$ particles remained associated with the cells (data not shown). After a 24-h incubation period, total protein was collected for Western analysis of HIF-1α protein levels and total RNA was collected to measure IL-8 mRNA levels. As seen in Fig. 6A, short term exposure to Ni$_3$S$_2$ but not nickel sulfate effectively increased HIF-1α protein levels, relative to control. Short term Ni$_3$S$_2$, but not NiSO$_4$, also increased IL-8 mRNA levels (Fig. 6B). Continuous exposure of the cells to either form of nickel over the 24-h period increased IL-8 mRNA levels; although NiSO$_4$ was less effective (Fig. 6B). These data indicated that prolonged exposure to nickel or slow dissolution of the particles was required for full induction of IL-8.

IL-8 Induction by Nickel Is Independent of a Hypoxia-like Response—Nickel mimics hypoxia to induce profibrotic changes in BEAS-2B cell PAI-1 expression (3, 5). Hypoxia is capable of inducing IL-8 levels, although this induction required activation of the –133-bp region of the IL-8 promoter (24). Nickel-induced HIF-1α levels and PAI-1 expression are attenuated by inhibiting diacylglycerol kinase (3). Therefore, the effect of the diacylglycerol kinase inhibitor, R-59949, on nickel-induced IL-8 expression was examined to establish whether induction of PAI-1 and IL-8 share similar HIF-dependent pathways. Under conditions where nickel-induced HIF protein stabilization was completely blocked (Fig. 7A), R-59949 had little or no effect on nickel-induced IL-8 expression (Fig. 7B). The HIF independence of transcriptional activation of IL-8 by nickel was further demonstrated by the experiments presented in Fig. 7C where luciferase activity was compared in cells transfected with the –272-luciferase construct and then stimulated with nickel in the absence or presence of a HIF-1α antisense oligonucleotide. This antisense sequence is highly effective in preventing nickel from inducing PAI-1 expression (3). However, neither the antisense nor the sense control affected luciferase activity in
nickel-treated cells (Fig. 7C). These data confirm that IL-8 expression in nickel-exposed cells is independent of the hypoxia response, despite the fact that nickel is capable of mimicking hypoxia by increasing HIF-1α stability.

AP-1 Is Necessary but Not Sufficient for Induction of IL-8 by Nickel—In certain cell types, including pulmonary epithelial cell lines, AP-1 is essential for induction of the IL-8 promoter stimulated by IL-1, TNF-α, and human T-cell lymphotropic virus 1 (11, 16, 22). Nickel-induced transactivation of PAI-1 in BEAS-2B cells also requires AP-1 activity (4), and the data in Fig. 5 indicate that the AP-1 site is necessary for stimulation of the IL-8 promoter by nickel. Therefore, a dominant negative AP-1 construct, TAM67, was used to test the hypothesis that AP-1 is required for transcriptional activation of IL-8 by nickel. RT-PCR for IL-8 mRNA from cells transfected with the TAM67 construct with and without nickel treatment indicated that some basal AP-1 activity is necessary for the induction of IL-8 by nickel (Fig. 8). Overexpression of c-Jun did not induce IL-8 mRNA (Fig. 8). These data and the data in Figs. 4A and 5 indicate that the AP-1 site at −126 to −120 bp of the promoter is necessary but not sufficient for induction of IL-8 by nickel.

Nickel Stimulates Kinase Signaling Cascades to Induce IL-8 Transcription—Nickel-induced transcription of PAI-1 is dependent on the activation of upstream signaling cascades (3). The role of these pathways in mediating nickel stimulation of IL-8 is unknown. Therefore, cells were pre-treated with specific inhibitors of the ERK (U0126), PI3K (wortmannin), p38 MAPK (SB20358), or diacylglycerol kinase (R-59949) for 30 min prior to addition of NiS₂. Inhibition of ERK or PI3K significantly attenuated but did not eliminate IL-8 induction by nickel (Fig. 9). However, consistent with previous observations for PAI-1 induction (3), there was no effect of inhibiting p38 kinase on nickel-stimulated IL-8 expression (Fig. 9).

DISCUSSION

The role of IL-8 in inflammatory responses to inhaled toxicants and lung injury has been extensively studied. In contrast, much less is known of the cellular and molecular events that regulate IL-8 expression induced by specific toxicants. Particulate nickel represents an environmentally and occupationally important lung toxicant that promotes inflammation, airway cell dysplasia, and fibrosis (2). However, as with all metals, nickel is a pleiotropic cell stimulant that can affect multiple signaling pathways to alter cell phenotype. The data presented above indicate that non-toxic exposure to particulate nickel induces prolonged expression of IL-8 with latent increases in protein release. This prolonged time course contrasts with the more rapid nickel-induced effects on fibrinolytic activity and HIF-dependent expression of PAI-1 and vascular endothelial growth factor (4–5). It also contrasts with more rapid IL-8 induction caused by short exposure to high concentrations of other metals, such as vanadium, chromium, and zinc (14, 25). However, the data suggest that the uptake and durability of NiS₂ contribute to its ability to activate specific signaling cascades that culminate in increased transcription of IL-8 mRNA. Addition of a chronic inflammatory state, as indicated by prolonged increases in IL-8 expression, could compound the pathophysiological responses of the airways to particulate nickel exposures.

IL-8 induction and its recruitment of inflammatory cells into the lung may play an important role in disease development. Along with other cytokines, IL-8 may be responsible for the
chronic inflammation that is characteristic of pulmonary fibrosis. The fact that IL-8 is not induced by nickel until 24 or 48 h following the onset of exposure may indicate that other events mediate the pathogenesis of nickel-induced pulmonary fibrosis, such as inhibition of the fibrolytic cascade (5). The prolonged time course may be explained by the need for dissolution of the particles to accumulate the active Ni$^{2+}$ that reacts with proteins and DNA (15, 26). Retention of nickel sulfide particles in the lung and their slow dissolution in endosomes are reported to be the primary reasons why inhalation of this form of nickel is more toxic than inhalation of soluble nickel (2, 15). However, there is still considerable risk of injury from prolonged exposure to the soluble forms as well (27). The data in Fig. 6 are consistent with these in vivo observations, because transient exposure to soluble nickel sulfate stimulated neither a hypoxic (Fig. 6A) nor an inflammatory response (Fig. 6B). However, cells in culture cannot clear toxicants in the same manner as the intact lung, and kinetic differences between different forms of nickel are often lost during continuous exposure (15). The effects of losing this differential were seen as continuous exposure to soluble nickel increased IL-8 mRNA levels (Fig. 6B). These data contrast with previous reports that the airway epithelial cells do not respond to nickel with increased expression of IL-8 (14, 25). In these previous investigations, high levels of nickel were used for only a 20-min exposure, which was insufficient to elevate IL-8 protein levels or activate MAPK pathways.

The protracted time course of particulate nickel-stimulated IL-8 production observed in the present study contrasts with immediate IL-8 induction in response to cytokines, hypoxia (24), and many inhaled toxicants, including cigarette smoke, asbestos (28), ozone (29), particulate matter (14, 30), and viruses (22). IL-8 expression in response to many stimuli is controlled mainly at the transcriptional level (16). The lack of a nickel effect on message stability (Fig. 3) and the stimulation of the IL-8 promoter constructs indicate that the major action of nickel is transcription activation. However, the results from the promoter analysis in Figs. 4 and 5 suggest that induction by nickel diverges from the other inducers of IL-8 by acting on elements outside of the cytokine-inducible portion of the promoter that contains critical AP-1, NF-IL6, and NF-xB elements (11, 16, 22, 24). The levels of Ni$_{3}$S$_{2}$ used do not stimulate an immediate-early gene response in the BEAS-2B cells (4). Instead, they cause protracted induction of both Fos and c-Jun (4). This induction and associated AP-1 activity is necessary but not sufficient for nickel-induced PAI-1 expression (4). Similarly, AP-1 appears to be required for nickel induction of IL-8 (Figs. 5 and 8). However, the AP-1 site at -126 to -120 bp, relative to the transcriptional start site, is insufficient to provide for full induction of the promoter (Figs. 4A and 5). This implies that the AP-1 site cooperates with sites between -272 and -133 bp to respond to nickel. This region of the promoter has not been well studied but does contain putative GATA and C/EBP binding elements. Because both GATA and C/EBP families of binding proteins were implicated in regulating phenotypic change in lung development and injury (31–34), these sites were the focus of mutational analysis. The data in Fig. 5 indicate that both sites are important for induction by nickel. However, it is also possible that disrupting one of the sites influences the other because they are adjacent. The data are also significant for the large increase in basal transcription observed when the C/EBP site was mutated. TNF induction was also increased by this mutation indicating that proteins that bound to this site were repressive.

Ni$_{3}$S$_{2}$ mimics hypoxia-like responses in the BEAS-2B cells, which result in stabilization of HIF-1$\alpha$ (Fig. 5A) and increased expression of PAI-1 and vascular epidermal growth factor (3–5). Hypoxia induces IL-8 in several cell models through poorly defined sites in the proximal promoter (24, 35). It is unlikely that Ni$_{3}$S$_{2}$ induces IL-8 by mimicking hypoxia, despite its chronic elevation of HIF-1$\alpha$ protein (Figs. 6A and 7A (3)). The data in Fig. 7 demonstrate that blocking signals that increase HIF-1$\alpha$ levels or eliminating HIF-1$\alpha$ synthesis had no effect on nickel-stimulated IL-8 expression. The data in Fig. 4A indicate that the putative hypoxia-responsive element in the IL-8 promoter (24) lies in a region that is not responsive to nickel. Thus, nickel stimulates divergent signaling cascades to chronically induce inflammatory IL-8 and profibrotic PAI-1 expression.

Despite the HIF independence of nickel-induced IL-8 expression, some of the nickel-responsive signaling cascades that activate HIF or HIF-responsive genes and IL-8 expression may be similar. Nickel-stimulated PAI-1 expression is attenuated but not eliminated by inhibiting ERK and PI3K (3). As shown in the previous study and in Fig. 7A, both U0126 and R-59949 reduced the level of HIF-1$\alpha$ protein and transactivation in nickel-treated cells. A role for each of these kinases in hypoxia- and non-hypoxia-induced HIF-1$\alpha$ gene expression and transcriptional competence has been demonstrated (36–38). The data indicate that the divergence of IL-8 induction from the hypoxia-like signaling must occur downstream of ERK and above diacylglycerol kinase. Alternatively, both PAI-1 and IL-8 genes share requirements for ERK and AP-1 for full induction. Divergence of inducibility may come from the requirement for other trans-acting factors that cooperate with AP-1. Induction of PAI-1 in nickel-exposed cells requires HIF and AP-1 (3). Putative transcription factors binding to the −272 to −133 region of the IL-8 promoter, such as C/EBP or GATA family members, appear to be independent of HIF and diacylglycerol kinase signaling. U0126 blocks ERK activation and AP-1-dependent transcription (39, 40) and prevents C/EBP-dependent gene expression (41). PI3K, in turn, regulates multiple actions on C/EBP family members that result in either increased or decreased transcriptional activation (42, 43). However, as stated above, more studies are needed to identify the proteins that bind to the −272 to −133 region and to define their regulation in nickel-exposed cells. The lack of a role for p38 MAPK in nickel-induced IL-8 expression is consistent with the lack of effects of the p38 MAPK inhibitor on nickel-stimulated HIF-1$\alpha$ stability and PAI-1 expression (3). This further distinguishes the actions of nickel from hypoxia, which stimulates p38 MAPK to induce HIF-1$\alpha$ expression and transcriptional activity (44, 45) and to phosphorylate and activate xanthine oxidase (46).

In conclusion, the current studies indicate that particulate Ni$_{3}$S$_{2}$ activates specific signaling cascades following uptake by pulmonary epithelial cells. These activated cascades stimulate parallel pathways for inducing transcription of both inflammatory and profibrotic genes. The molecular switches for nickel activation remain undefined, but must lie proximal to stimulation of both hypoxia-like responses and stimulation of MAPK cascades. Further investigation will be needed to identify these molecular switches and to further define the molecular program that supports chronic gene activation by nickel. This chronic phenotypic change may play an important role in the mechanisms driving increased incidence of pulmonary diseases following environmental or occupational exposure to nickel.

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J. Biol. Chem. 2002, 277:24225-24231.
doi: 10.1074/jbc.M202941200 originally published online April 26, 2002

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