Activation of Nuclear Factor-κB and Not Activator Protein-1 in Cellular Response to Nickel Compounds

Yi Huang,[1] Gerard Davidson,[2] Jingxia Li,[2] Yan Yan,[2] Fei Chen,[3] Max Costa,[2] Lung Chi Chen,[2] and Chuanshu Huang[2]

[1]Monroe-Woodbury High School, Central Valley, New York, USA; [2]Nelson Institute of Environmental Medicine, New York University School of Medicine, Tuxedo, New York, USA; [3]Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, West Virginia, USA

The predominant exposure route for nickel compounds is by inhalation, and several studies have indicated the correlation between nickel exposure and respiratory cancers. The tumor-promoting effects of nickel compounds are thought to be associated with their transactivation of transcription factors. We have investigated the possible activation of activator protein-1 (AP-1) and nuclear factor κB (NF-κB) in mouse C141 epidermal cells and fibroblasts 3T3 and B82, and human bronchoepithelial BEAS-2B cells in response to nickel compound exposure. Our results show that NF-κB activity is induced by nickel exposure in 3T3 and BEAS-2B cells. Conversely, similar nickel treatment of these cells did not induce AP-1 activity, suggesting that nickel tumorigenesis occurs through NF-κB and not AP-1. We also investigated the role of NF-κB in the induction of *Cap43* by nickel compounds using dominant negative mutant Iκβ kinase b-KM BEAS-2B transfectants. *Key words:* AP-1, NF-κB, nickel compounds. *Environ Health Perspect* 110(suppl 5):835–839 (2002). *http://ehpnet1.niehs.nih.gov/docs/2002/suppl-5/835-839huang/abstract.html*

Nickel is one of the most abundant transition metals in the earth's crust (*1*). It is used in a variety of industrial processes, e.g., nickel refinement, nickel-cadmium batteries, and electroplating (*2*). These processes, in addition to the incineration of nickel-containing wastes and fossil fuels, are responsible for the majority of nickel aerosols found in both the workplace and the environment (*2*). It has been estimated that the average daily exposure to nickel is between 0.2 and 0.4 μg in both urban and rural environments (*2*). Workplace exposure is considerably higher.

The main route for exposure to nickel compounds is by inhalation. Indeed, a variety of epidemiologic studies have indicated a significant correlation between the number of respiratory cancers and workplace nickel exposure (*3,4*). An effect of nickel compounds in animal models, through inhalation, injection or ingestion, is to produce tumors (*5–7*).

The mechanism by which nickel toxicity is exerted has been extensively studied (*8–10*). Nickel exposure induces several types of cellular and nuclear damage (*8,11,12*). Although nickel is a potent carcinogen, it is generally not active in mutagenic assays (*13–16*). This suggests that nickel-induced toxicity/carcinogenicity may be caused by alterations in gene expression rather than by direct DNA damage. For example, transcription factors, metallothionein, and heat shock proteins can be induced by exposure to nickel (*17–19*).

Nuclear factor κB (NF-κB) was first described as a B-cell nuclear factor that binds to immunoglobulin κ enhancer and thus was implicated in immune response (*20,21*). Subsequent research revealed that NF-κB was not B-cell specific and could bind to specific sites in a variety of gene promoter/enhancers, e.g., interleukin (IL)-2, IL-6, granulocyte macrophage colony-stimulating factor, intercellular adhesion molecule-1, and class I major histocompatibility complex (*22–24*). Initially the number of inducers of NF-κB was quite small but has since grown substantially, e.g., tumor necrosis factor, IL-1, ultraviolet radiation, growth factors, free radicals, and viral infection (*22–24*). Additionally, there is an increasing body of evidence suggesting a role for NF-κB in carcinogenesis. For example, NF-κB is implicated in signaling tumor promoter-induced transformation and is activated by viral transforming proteins (*24–26*). The importance of NF-κB cannot be overstated, as failure in any of the mechanisms leading to NF-κB activation can have serious consequences for the cell. Studies involving NF-κB are frequently compared with those involving activator protein-1 (AP-1). AP-1 is a transcription factor complex composed of Jun family homodimers or Jun/Fos heterodimers (*27,28*). As with NF-κB, AP-1 is activated by a number of different stimuli, including cell stress, cytokines, growth factors, and neurotransmitters (*27–29*). Both AP-1 and some of the gene transcripts regulated by AP-1 are involved in neoplastic transformation (*30–33*). As a result we have further investigated the possible involvement of NF-κB and AP-1 in nickel-induced carcinogenesis.

Materials and Methods

Plasmids and Agents

The cytomegalovirus (CMV)-neo vector plasmid and AP-1-luciferase reporter, as well as NF-κB-luciferase reporter plasmids, were constructed as previously described (*34–36*). Anhydrous nickel chloride ($NiCl_2$) was purchased from Aldrich (Milwaukee, WI, USA); nickel subsulfide (Ni_3S_2) was obtained from INCO (Toronto, Canada). Fetal bovine serum (FBS) was obtained from Life Technologies, Inc. (Carlsbad, CA, USA). Eagle's minimal essential medium (MEM) and Dulbecco's modified Eagle's medium (DMEM) were both obtained from BioWhittaker (Walkersville, MD, USA). The luciferase assay substrate was purchased from Promega (Madison, WI, USA).

Cell Culture

Mouse fibroblasts 3T3 and B82 cells, as well as their NF-κB-luciferase reporter or AP-1–luciferase reporter stable transfectants, were cultured in DMEM with 10% FBS, 2 mM L-glutamine, and 25 μg gentamicin/mL (*34*). Human bronchial epithelial cell BEAS-2B stable transfectants IκB kinase β (IKKβ) or IKKβ dominant negative mutant (IKKβ-KM) were cultured in 10% FBS, 2 mM L-glutamine, and 25 μg gentamicin/mL as reported by Chen et al. (*37*) and Huang et al. (*38*).

Generation of Stable Transfectants with NF-κB-Luciferase Reporter or AP-1-Luciferase Reporter

3T3 cells were cultured in a 6-well plate until they reached 85–90% confluence. CMV-neo vector (1 μg) and 15 μL LipofectAMINE reagent, (Gibco BRL, Rockville, MD, USA) together with 12 μg NF-κB-luciferase reporter plasmid DNA or AP-1-luciferease reporter plasmid DNA, were used to transfect each well in DMEM in the absence of serum. After 10–12 hr, the medium was replaced with 10% FBS DMEM. Approximately 30–36 hr after the beginning of the transfection, the cells were trypsinized with 0.033% trypsin, and cell suspensions were plated onto 75-mL culture flasks and cultured for 24–28 days with G418 selection (600 μg/mL). Measuring basal level of luciferase activity

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Address correspondence to C. Huang, Nelson Institute of Environmental Medicine, New York University School of Medicine, 57 Old Forge Rd., Tuxedo, NY 10987 USA. Telephone: (845) 731-3519. Fax: (845) 351-4510. E-mail: chuanshu@env.med.nyu.edu

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identified the stable transfectants. Stable transfectants 3T3 NF-κB mass1 or 3T3 AP-1 mass1 were established and cultured in G418-free MEM for at least two passages before each experiment.

Assay for NF-κB Activation

Confluent monolayers of 3T3 NF-κB mass1 or IKKβ were trypsinized, and 8×10^3 viable cells were suspended in 100 µL culture medium in each well of a 96-well plate. Plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Twelve to 24 hr later, cells were starved by culturing them in 0.1% FBS DMEM for 24 hr. The cells were then exposed to either Ni_3S_2 or $NiCl_2$ for NF-κB induction and maintained in culture. The cells were extracted with lysis buffer at various times, and luciferase activity was measured. The results are expressed as relative NF-κB activity (*35*).

Assay for AP-1 Activity

Confluent monolayers of 3T3 AP-1 mass1 or B82 AP-1 mass2 were trypsinized, and 8×10^3 viable cells suspended in 100 µL culture medium were added to each well of a 96-well plate. Plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Twelve to 24 hr later, cells were starved by culturing them in 0.1% FBS DMEM for 24 hr. The cells were then exposed to either Ni_3S_2 or $NiCl_2$ for AP-1 induction and maintained in culture. The cells were extracted with lysis buffer, and luciferase activity was measured. The results are expressed as relative AP-1 activity (*34*).

Statistical Analysis

The significance of the difference in the NF-κB and AP-1 activities was determined with the Student *t* test. The results are expressed as mean ± SEM.

Western Blot Analysis

Human bronchial epithelial cell line BEAS-2B and its stable transfectant IKKβ-KM were cultured in each well of 6-well plates to 90% confluence. The cells were exposed to $NiCl_2$ or Ni_3S_2 and incubated for different times indicated in the figure legends. The cells were then washed once with ice-cold phosphate-buffered saline (PBS) and extracted with sodium dodecyl sulfate (SDS)-sample buffer. The cell extracts were separated on polyacrylamide–SDS gels, transferred, and probed with one of two antibodies, including rabbit specific antibody against Cap43 protein or specific antibody against protein kinase C α. The protein bands specifically bound to primary antibodies were detected using an anti-rabbit immunoglobulin G (IgG)–AP-linked (Amersham Biosciences, Piscataway, NJ, USA) as second antibody and an ECF Western blotting system (*36*).

Results

Effect on Induction of NF-κB in Mouse Fibroblast 3T3 Cells by Nickel Compounds

To determine the effects of nickel on NF-κB activation in mouse fibroblast cells, we incubated mouse fibroblast 3T3 cells with either Ni_3S_2 (2 µg/cm^2) or $NiCl_2$ (1 mM) and monitored the effect on NF-κB-dependent transcriptional activation. Shown in Figure 1A is the relative NF-κB activity in 3T3 cells after treatment with either Ni_3S_2 or $NiCl_2$. It can be seen that Ni_3S_2 is a potent activator of NF-κB and induces an approximately 12-fold increase in NF-κB relative to untreated cells. $NiCl_2$ treatment also produces an increase in NF-κB activity (~6-fold), although not quite as pronounced as with Ni_3S_2. Figure 1B shows the time course for maximal NF-κB activation upon Ni_3S_2 treatment. The results indicate a gradual increase in relative NF-κB activity over a period of 24 hr (5-fold increase). Activation increases to a maximum after 48 hr (12-fold) before decreasing again after 72 hr (3-fold). A dose–response study of the effect of Ni_3S_2 treatment indicates that induction of NF-κB activity is concentration dependent, as shown in Figure 1C. The most effective dose range of Ni_3S_2 treatment on 3T3 cells was 1.0–2.0 µg/cm^2. These results indicate that NF-κB is involved in the cellular response to nickel compounds.

Effect on Induction of NF-κB in Human Bronchial Epithelial BEAS-2B Cells by Nickel Compounds

A variety of epidemiologic studies indicated that nickel exposure is correlated with an increase in the incidence of respiratory cancers (*3–7*). To understand the involvement of NF-κB activation in the response of the respiratory system to nickel compounds, we tested the effect of Ni_3S_2 and $NiCl_2$ on NF-κB activity in human bronchial epithelial BEAS-2B cells. As shown in Figure 2A, treatment of cells with either Ni_3S_2 or $NiCl_2$ also leads to an increase in NF-κB activity. The increase in NF-κB

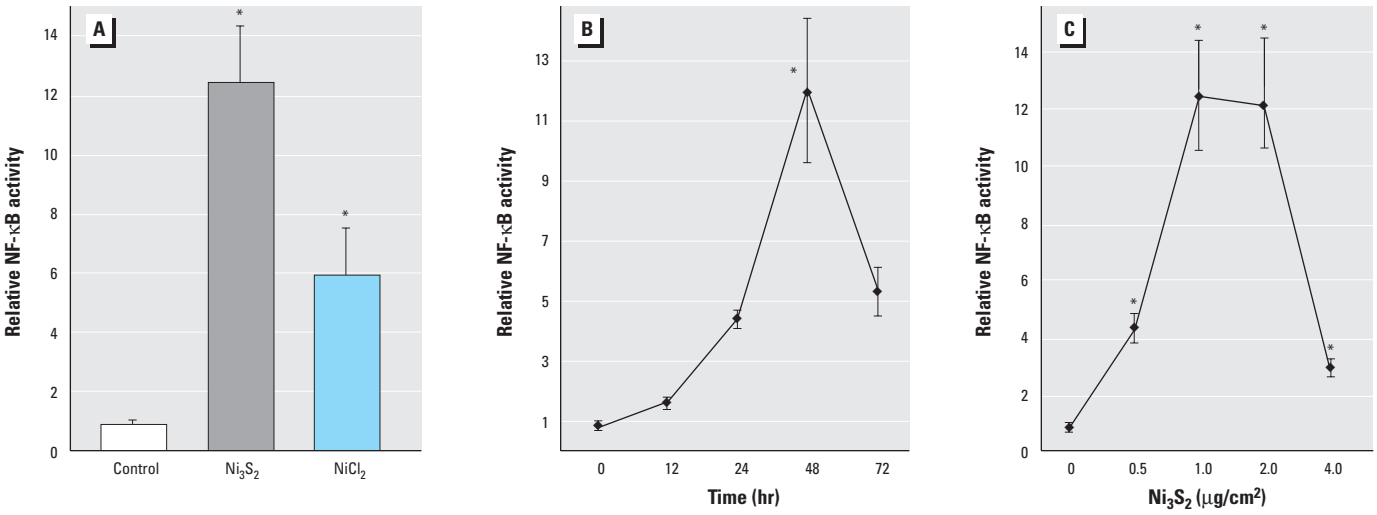

Figure 1. Induction of NF-κB activity by nickel compounds in mouse fibroblast 3T3 cells. 3T3 NF-κB mass1 cells (8×10^3) were seeded into each well of a 96-well plate. After being cultured at 37°C overnight, the cells were starved for 12 hr by replacing the medium with 0.1% FBS DMEM. The cells were then treated as follows: (*A*) 1 µg/cm^2 Ni_3S_2 or 1 µM $NiCl_2$ for 48 hr. (*B*) For a time-course study, the cells were exposed to 1 µg/cm^2 Ni_3S_2 for various times as indicated. (*C*) For a dose–response study, the cells were exposed to different concentrations of Ni_3S_2 as indicated for 48 hr. The luciferase activity was then measured and the results are presented as NF-κB–dependent transcription activity relative to control. Each bar indicates the mean and standard deviation of four repeat assay wells. Asterisk (*) indicates a significant increase from control ($p < 0.05$).

activity upon Ni_3S_2 treatment is approximately 4.5-fold relative to that in the control, whereas $NiCl_2$ treatment leads to an approximately 2.7-fold increase in activity. This induction was also observed in the dose response of NF-κB activity to Ni_3S_2 (Figure 2B). These results, taken together with the results from 3T3 cells, indicate that the induction of NF-κB is involved in the response of the cell to nickel compounds.

Absence of Induction of AP-1 Activity with Nickel Compounds

To test whether the response of cells to Ni_3S_2 and $NiCl_2$ involves AP-1, we also generated stable AP-1-luciferase 3T3 transfectants. As shown in Figure 3, treatment of cells with Ni_3S_2 or $NiCl_2$ did not show any induction of AP-1 activity in 3T3 cells, whereas NF-κB activation was observed. In contrast, ultra violet-C (UVC) radiation resulted in increases in both NF-κB and AP-1 activity (Figure 3). These results indicated that NF-κB but not AP-1 was involved in the response of cells to nickel compounds.

To further explore whether the effects of Ni_3S_2 and $NiCl_2$ on AP-1 activity are cell specific, we tested the effect of nickel compounds on AP-1 activity in fibroblast B82 cells. As with the 3T3 cells, treatment of B82 cells with either Ni_3S_2 or $NiCl_2$ did not lead to an increase in AP-1 activity (Figure 4). Again, UVC radiation treatment resulted in an increase in AP-1, indicating that the absence of induction of AP-1 transcriptional activation toward Ni_3S_2 and $NiCl_2$ treatment was not cell-type specific. This was consistent with our previous findings in C141 cells (*39*).

Induction of *Cap43* in BEAS-2B Stable Transfectants

Both Ni_3S_2 and $NiCl_2$ induce a novel gene, *Cap43*, which is also induced by hypoxia and the calcium ionophore A23187 (*40,41*). Recently it was found that *Cap43* was expressed only in cancer cells, not in normal cells (*42*). The mechanism by which nickel acts is not well understood. To determine whether NF-κB activation by nickel is involved in nickel-induced *Cap43* expression, we compared *Cap43* expression between human bronchial epithelial BEAS-2B cells and their stable transfectant IKKβ-KM cells. The results showed that an overexpression of a IKKβ-KM did not affect nickel-induced *Cap43* expression (Figure 5). This suggests that the signal transduction pathway leading to NF-κB activation by nickel compounds does not involve *Cap43* expression by nickel.

Discussion

In this study we investigated the effect of Ni_3S_2 and $NiCl_2$ on the transcription factor NF-κB and AP-1 in various cell culture models. NF-κB activation by nickel compounds was found in mouse fibroblasts (3T3) and human bronchoepithelial cells (BEAS-2B), whereas nickel treatment did not induce any activation of AP-1 in the same cells. Furthermore, NF-κB activation by nickel compounds was not required for *Cap43* expression, as overexpression of the IKKβ-KM had no effect on *Cap43* expression.

Our results indicate that both insoluble Ni_3S_2 and soluble $NiCl_2$ are effective inducers of NF-κB activation in mouse fibroblast 3T3 cells and human bronchoepithelial BEAS-2B cells. As has been shown previously, insoluble Ni_3S_2 appears to be more effective in potentiating a biochemical response than $NiCl_2$ (*43*). The effect of Ni_3S_2 is both time and dose dependent. The maximum effect on NF-κB activation by Ni_3S_2 takes place after 48-hr exposure. The most effective dose is 1.0–2.0 μg/cm^2, although the lower dose of 0.5 μg/cm^2 is still very effective in inducing an increase in NF-κB activity. Ni_3S_2 was toxic to cultured hamster lung fibroblasts at 0.5 μg/cm^2 (*15*), whereas in our system cytotoxicity does not appear to be a factor until after greater than 48-hr exposures and doses above 2.0 μg/cm^2. This observation is supported by data that NF-κB activity would increase relative to that of the control (Figure 1B). The reason for this difference may be due to cell-type specificity.

The results showing that Ni_3S_2 and $NiCl_2$ potentiate NF-κB but not AP-1 activity in different cell culture models were intriguing. NF-κB has been the focus of considerable research since its discovery in 1986 (*20, 21*). NF-κB is a member of the NF-κB/Rel family and exists in an inactive form in cells through formation of a complex with IκB (*22,44–51*). Phosphorylation of IκB leads to ubiquitination of the cytoplasmic NF-κB complex and subsequent degradation of the complex to

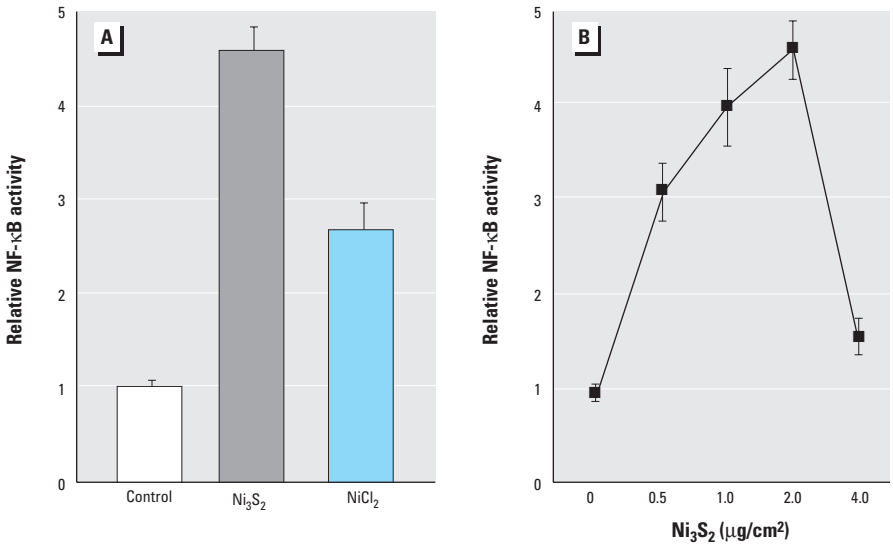

Figure 2. Induction of NF-κB activity by nickel compounds in human bronchial epithelial BEAS-2B cells. BEAS-2B IKKβ transformed cells (8×10^3) were seeded into each well of a 96-well plate. After being cultured at 37°C overnight, the cells were starved for 12 hr by replacing the medium with 0.1% FBS DMEM. The cells were then treated as follows: (*A*) 1 μg/cm^2 Ni_3S_2 or 1 mM $NiCl_2$ for 36 hr. (*B*) For a dose–response study, the cells were exposed to different concentrations of Ni_3S_2 as indicated for 36 hr. The luciferase activity was then measured and the results are presented as NF-κB–dependent transcription activity relative to control. Each bar indicates the mean and standard deviation of four repeat assay wells. Asterisk (*) indicates a significant increase from control ($p < 0.05$).

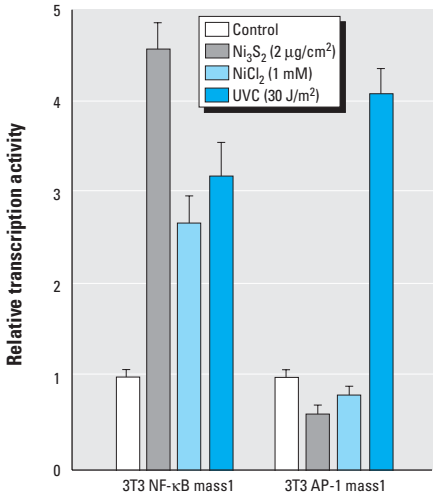

Figure 3. Nickel compounds induce activation of NF-κB, but not AP-1, in 3T3 cells. 3T3 NF-κB mass1 or AP-1 mass1 (8×10^3) were seeded into each well of a 96-well plate. After being cultured at 37°C overnight, the cells were starved for 12 hr by replacing the medium with 0.1% FBS DMEM. Then the cells were treated with Ni_3S_2 (2 μg/cm^2), $NiCl_2$ (1 mM), or UVC radiation (30 J/cm^2) for 36 hr. The luciferase activity was then measured and the results are presented as relative NF-κB activity or relative AP-1 activity. Each bar indicates the mean and standard deviation of four repeat assay wells. Asterisk (*) indicates a significant increase from control ($p < 0.05$).

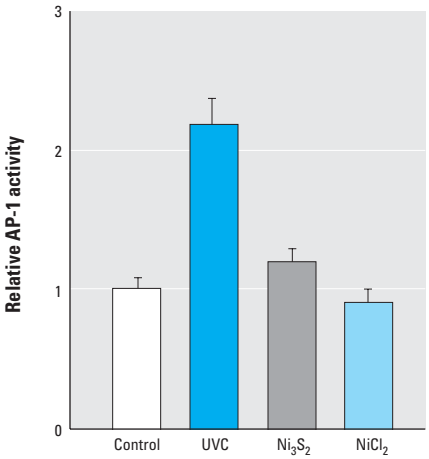

Figure 4. No induction of AP-1 activity by nickel compounds in mouse fibroblast B82 cells. B82 AP-1 mass2 (8×10^3) were seeded into each well of a 96-well plate. After being cultured at 37°C overnight, the cells were starved for 12 hr by replacing the medium with 0.1% FBS DMEM. The cells were then treated with Ni_3S_2 (2 μg/cm^2), $NiCl_2$ (1 mM), or UVC radiation (30 J/cm^2) for 36 hr. The luciferase activity was then measured and the results are presented as relative AP-1 activity. Each bar indicates the mean and standard deviation of four repeat assay wells. Asterisk (*) indicates a significant increase from control ($p < 0.05$).

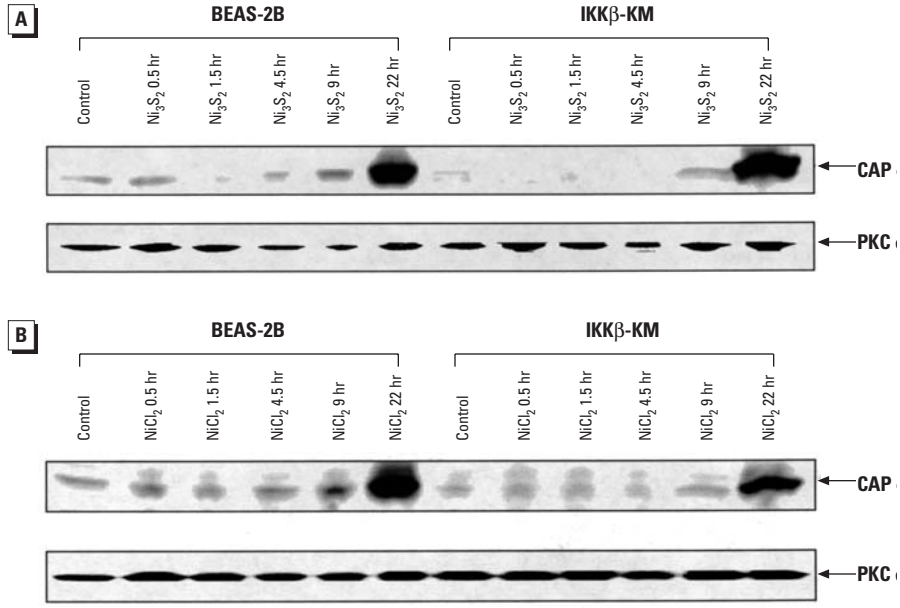

Figure 5. Induction of Cap43 protein expression by Ni_3S_2 (*A*) or $NiCl_2$ (*B*) in both BEAS-2B and IKKβ-KM cells. Subconfluent (90%) monolayers of BEAS-2B and IKKβ-KM in 6-well plates were subjected to either (*A*) Ni_3S_2 (2 μg/cm^2) or (*B*) $NiCl_2$ (1 mM) and cultured for time points as indicated. The cells were then washed once with ice-cold PBS and extracted with SDS-sample buffer. The cell extracts were separated on polyacrylamide-SDS gels, transferred, and probed with rabbit polyclone antibodies against Cap43. The Cap43 protein band specifically bound to the primary antibody was detected using an antirabbit IgG-AP-linked as second antibody and an ECF Western blotting system (*38*). PKC was used as internal control of protein loaded.

produce the active form of NF-κB (*52–54*). NF-κB is then translocated to the nucleus from the cytoplasm, where it induces gene activation. Considerable evidence has been presented to implicate NF-κB activation with tumor promotion in cell models (*23,25,55*). For example, both v-Rel and p52/Lyt-10, members of the NF-κB family, and Bcl-3, an IκB family member, are potentially oncogenic (*24*). In addition, it was shown separately that the c-*myc* oncogene promoter implicated in Burkitt lymphoma is activated by NF-κB (*56*) and that NF-κB positively regulates the expression of the translocated c-*myc* gene in Burkitt lymphoma. Additionally, overexpressed IκBα in 3T3 cells blocked the ability of *ras* alleles to induce focus formation, again suggesting a role for NF-κB (*57*). Furthermore, overexpressed IκBα crossed with v-Rel transgenic mice induced a delay in death from leukemia (*23*).

AP-1 is a transcription factor complex composed of members of the Jun and Fos families of proteins (*27,28*). Both AP-1 and NF-κB are activated by similar stimuli, including growth factors, cytokines, and UVC radiation, leading to altered gene expression (*27–29*). Like NF-κB, AP-1 has been implicated in tumor promotion in different cell models (*27,30,31,58–65*). AP-1 activity was also elevated in mouse epidermal JB6 cells, indicating various stages of tumor promotion (*59*). Furthermore, tumor promotion could be inhibited by the use of several types of AP-1 inhibitors (*38,60–63,66*).

In light of the important roles that both NF-κB and AP-1 play in tumor promotion by many chemicals, we wished to investigate the signal transduction pathways involved in the carcinogenic properties of nickel. The results indicate that Ni_3S_2 and $NiCl_2$ specifically induce NF-κB activity but not AP-1 activity in mouse fibroblast 3T3 cells. The specificity of Ni_3S_2 and $NiCl_2$ for NF-κB activity is further supported by the time-course and dose–response studies, as well as by the observation that UVC stimulates both NF-κB and AP-1 in 3T3 cells. A comparison with fibroblast B82 cells also showed that AP-1 activity was increased by UVC exposure but not by Ni_3S_2 or $NiCl_2$.

Cap43 has been reported to be specifically induced by nickel compounds in a variety of cell lines (*40,41*). Although the function of the Cap43 protein is not well understood, it does appear to be induced in response to an increase in intracellular concentration of Ca^{2+} (*41*). The complete mechanism of signal transduction leading to *Cap43* expression has yet to be elucidated, but it has been shown that nickel induces HIF-1 and that this, in turn, activates Cap43 transcription (*67*). Our current investigation using BEAS-2B and IKKβ-KM indicates that overexpression of IKKβ-KM did not block *Cap43* induction in response to both Ni_3S_2 and $NiCl_2$. Our results suggest that induction of *Cap43* does not involve signals arising from the NF-κB pathway.

To summarize the results, Ni_3S_2 and $NiCl_2$ activate NF-κB in both mouse fibroblast 3T3 cells and human bronchoepithelial BEAS-2B cells. In addition, AP-1 activity is unaffected by nickel treatment in mouse 3T3, human bronchoepithelial BEAS-2B, and mouse C141 cells, which indicates that the response to nickel must involve a signal transduction pathway that terminates with NF-κB rather than AP-1. Also, NF-κB activation by nickel compounds is not required for *Cap43* expression.

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