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Nickel Compounds Act through Phosphatidylinositol-3-kinase/Akt-Dependent, $p70^{S6k}$-Independent Pathway to Induce Hypoxia Inducible Factor Transactivation and Cap43 Expression in Mouse Epidermal Cl41 Cells

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ABSTRACT

Nickel compounds are a somewhat unique class of carcinogens. Previous studies have demonstrated that $NiCl_2$ exposure leads to marked induction of hypoxia inducible factor 1 (HIF-1) in human osteosarcoma and BALB/c 3T3 cells, a transcription factor that has been considered to play an important role in tumor promotion and progression. However, the signal transduction pathways leading to HIF-1 induction are not well understood. The present study indicated that exposure of mouse epidermal Cl41 cells to either Ni_3S_2 or $NiCl_2$ resulted in activation of phosphatidylinositol 3-kinase (PI-3K), Akt, and p70 S6 kinase ($p70^{S6k}$). Inhibition of PI-3K, Akt, and $p70^{S6k}$ by overexpression of a dominant-negative mutant of PI-3K (Δp85) impaired nickel-induced HIF-1 transactivation. Furthermore, an overexpression of the dominant-negative Akt mutant (Akt-T308A/S473A) blocked nickel-induced Akt phosphorylation and HIF-1 transactivation, whereas inhibition of $p70^{S6k}$ activation by pretreatment of cells with rapamycin did not show significant inhibitory effects on HIF-1 transactivation induced by nickel compounds. Consistent with HIF-1 transactivation, inhibition of the PI-3K/Akt pathway by either overexpression of Δp85 or Akt-T308A/S473A caused dramatic inhibition of Cap43 protein expression induced by nickel compounds, whereas pretreatment of cells with rapamycin did not exhibit inhibition of Cap43 induction. These results demonstrated that nickel compounds induce HIF-1 transactivation and Cap43 protein expression through a PI-3K/Akt-dependent and $p70^{S6k}$-independent pathway. This study should help us understand the signal transduction pathways involved in the carcinogenic effects of nickel compounds.

INTRODUCTION

The carcinogenicity of nickel compounds has been well documented by studies both *in vitro* and *in vivo* (1–6). Because carcinogenesis is a complex process (1, 7), it is likely that nickel compounds contribute to human carcinogenesis by multiple mechanisms (1). It is accepted that the carcinogenic effects of nickel compounds occur through alterations in gene expressions that may be mediated by activated transcription factors (1).

Hypoxia inducible factor 1 (HIF-1) is a transcription factor that was originally found to be activated in cells in response to hypoxia and is considered as an important factor in tumor development (8). HIF-1 is a heterodimer composed of two subunits, HIF-1α and HIF-1β. HIF-1β is constitutively expressed in cells, whereas HIF-1α is induced in response to hypoxia and various other growth factors such as insulin-like growth factor 2 (8). The consequence of HIF-1 induction is to activate transcription of a variety of genes responsible for cell survival, including vascular endothelial growth factor (VEGF) in angiogenesis, insulin-like growth factor 2 for cell survival, glucose transporter 1 and glucose transporter 3, and numerous glycolytic enzymes required for an up-regulation of metabolism (8–14).

Failure by cells to regulate HIF-1α levels is implicated in carcinogenesis because HIF-1α is overexpressed in both primary and metastatic tumors presumably to allow cells to survive low oxygen tension (9–14). HIF-1α levels are regulated by changes in the stabilization of HIF-1α protein and to a lesser extent by transcription of HIF-1α gene (8). Transcription of HIF-1α is altered by genetic alterations of tumor suppressor genes, such as p53, PTEN (phosphatase and tensin homologue deleted from chromosome 10), and von Hippel Lindau protein (pVHL), leading to a loss of function (14–17), or by mutations in proto-oncogenes that activate the mitogen-activated protein kinase or phosphatidylinositol-3-kinase (PI-3K) pathways, leading to a gain of function (18–21). Alternatively, HIF-1α protein stabilization is controlled by its state of oxidation. It is now known that HIF-1α is hydroxylated at Pro^{564} and, when it is in the hydroxylated state, binds to pVHL, which targets HIF-1α for ubiquitination and subsequent degradation by a ubiquitin E3 ligase complex (22–24). Thus, HIF-1 overexpression is a significant determinant of cancer cell survival and progression.

Previous studies indicated that nickel exposure results in an increase in HIF-1α protein accumulation (17, 25, 26). However, the signal transduction pathways leading to HIF-1α induction are not well understood. In the present study, we investigated the role of the phosphatidylinositol-3-kinase/protein-serine-threonine-kinase/p70-S6-kinase (PI-3K/Akt/$p70^{S6k}$) pathway in nickel-induced HIF-1 transactivation. Because previous studies suggested that HIF-1α activation is required for nickel-induced Cap43 gene expression (27), we also tested the possible involvement of the PI-3K/Akt/$p70^{S6k}$ pathway in nickel-induced Cap43 induction.

MATERIALS AND METHODS

Plasmids and Reagents. The cytomegalovirus-neo vector plasmid, the Akt mutant plasmid (SRα-Akt-T308A/S473A), and the dominant-negative mutant PI-3K plasmid (Δp85), were as described previously (28–30). The dominant-negative form of HIF-1 was subcloned into pCEP4 (Invitrogen) as described previously (31, 32). The VEGF promoter-driven luciferase reporter (VEGF-Luc) construct was obtained by inserting a 2.65 kb *Kpn*I-*Bss*HII fragment of the human VEGF gene promoter, which contains the VEGF promoter sequence from −2274 to +379 relative to the transcription initiation site, into the pGL2-basic vector (Promega, Madison, WI) as described previously (31, 32). The VEGF mutant reporter (Mut-VEGF-Luc) was constructed by 3 bp of substitution in the HIF-1 binding sequence of the human VEGF promoter region that abolishes HIF-1 binding as described in previous publications (31, 32). Nickel chloride ($NiCl_2$), anhydrous, was purchased from Aldrich (Milwaukee, WI); nickel subsulfide (Ni_3S_2) was obtained from INCO (Toronto, Canada); fetal bovine serum (FBS) and Eagle's MEM were purchased from

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BioWhittaker (Walkersville, MD). The luciferase assay substrate was purchased from Promega. Phospho-specific $p70^{S6k}$ (Thr^{389}) antibody, phospho-specific $p70^{S6k}$ (Thr^{421}/Ser^{424}) antibody, $p70^{S6k}$ antibody phospho-specific Akt (Thr^{308}) antibody, phospho-specific Akt (Ser^{473}) antibody, and Akt antibody were purchased from New England Biolabs (Beverley, MA). Wortmannin and Ly294002, PI-3K inhibitors, PD98059, a specific extracellular signal-regulated kinase pathway inhibitor, and rapamycin, a $p70^{S6k}$ pathway inhibitor, were purchased from Calbiochem (La Jolla, CA). LipofectAMINE was obtained from Life Technologies, Inc. (Rockville, MD).

Cell Culture. The JB6 P^+ mouse epidermal cell line, Cl41, was originally established by Dr. Nancy Colburn of the National Cancer Institute (33). Cl41 cells and their transfectants were cultured in monolayers at 37°C, 5% CO_2 using MEM containing 5% FCS (FBS), 2 mM L-glutamine, and 25 μg gentamicin/ml as described previously (28, 34).

Transient Transfection. Cl41 cells were cultured in a 6-well plate until they reached 85–90% confluence. Fifteen μl of LipofectAMINE reagent with 2 μg of VEGF-Luc or Mut-VEGF-Luc plasmid and 10 μg of dominant-negative form of HIF-1 or its vector plasmid DNA were used to transfect cells of each well in the absence of serum. After 10–12 h, the medium was replaced with 5% FBS MEM. Approximately 24–30 h after the beginning of the transfection, the cells were exposed to nickel and cultured for induction of luciferase activity.

Generation of Stable Cotransfectants. Cl41 cells were cultured in a 6-well plate until they reached 85–90% confluence. One μg of cytomegalovirus-neo vector and 15 μl of LipofectAMINE reagent with 2 μg of VEGF-Luc plasmid DNA mixed together with the dominant-negative mutant (SRα-Akt-T308A/S473A or Δp85) were used to transfect each well in the absence of serum. After 10–12 h, the medium was replaced with 5% FBS MEM. Approximately 30–36 h after the beginning of the transfection, the cells were digested with 0.033% trypsin, and cell suspensions were plated onto 75-ml culture flasks and cultured for 24–28 days with G418 selection (400 μg/ml). The stable transfectants were identified by measuring both the basal level of luciferase activity and the blocking activity of the relative kinase. Stable transfectants, Cl41 VEGF-Luc mass1, Cl41 VEGF-Luc Akt-T308A/S473A mass1 and Cl41 VEGF-Luc Δp85 mass1 were established and cultured in G418-free MEM for at least two passages before each experiment.

PI-3 Kinase Assay. PI-3 kinase activities were assayed as described in our previous reports (28, 29). Cells were cultured in monolayers in 100-mm dishes using normal culture medium. The media were replaced with 0.1% FBS MEM containing 2 mM L-glutamine and 25 μg of gentamicin/ml after the cell density reached 70–80%. Forty-five h later, the cells were incubated with fresh serum-free MEM for 3–4 h at 37°C. $NiCl_2$ or Ni_3S_2 was then added to cell cultures for PI-3K induction. Cells were washed once with ice-cold PBS and lysed in 400 μl of lysis buffer/plate [20 mM Tris (pH 8), 137 mM NaCl, 1 mM $MgCl_2$, 10% glycerol, 1% NP40, 1 nM DTT, 0.4 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride]. The lysates were centrifuged and the supernatants incubated at 4°C with 40 μl of agarose beads (conjugated previously with the monoclonal antiphosphotyrosine antibody Py20) overnight. Beads were washed twice with each of the following buffers: (*a*) PBS with 1% NP40, 1 mM DTT; (*b*) 0.1 M Tris (pH 7.6), 0.5 M LiCl, l mM DTT; and (*c*) 10 mM Tris (pH 7.6), 0.1 M NaCl, 1 mM DTT. Beads were incubated for 5 min on ice in 20 μl of buffer 3 and then 20 μl of 0.5 mg/ml phosphatidylinositol [sonicated previously in 50 mM HEPES (pH 7.6), 1 mM EGTA, 1 mM NaH_2PO_4] were added. After 5 min at room temperature, 10 μl of the reaction buffer were added [50 mM $MgCl_2$, 100 mM HEPES (pH 7.6), 250 μM ATP containing 5 μCi of [γ-^{32}P]ATP], and beads were incubated for an additional 15 min. Reactions were stopped by the addition of 15 μl of 4 N HCl and 130 μl of chloroform/methanol (1:1). After vortexing for 30 s, the solutions, 30 μl from the phospholipid-containing chloroform phase, were spotted onto TLC plates coated with silica gel H containing 1.3% potassium oxalate and 2 mM EDTA applied in H_2O/methanol (3:2). Plates were heated at 110°C for at least 3 h before use. Plates were then placed in tanks containing chloroform/methanol/NH_4OH/H_2O (600:470:20:113) for 40–50 min until the solvent reached the top of the plates. Plates were dried at room temperature and autoradiographed (28, 29).

Phosphorylation Assay for Akt and $p70^{S6k}$. Cl41 transfectants (3×10^4) were cultured in each well of 6-well plates to 70–80% confluence with normal culture medium. The cell culture medium was replaced with 0.1% FBS MEM with 2 mM L-glutamine and 25 μg of gentamicin and cultured for 33 h. Cells were incubated in serum-free MEM for 3–4 h at 37°C. Cells were exposed to $NiCl_2$ or Ni_3S_2 for various time points. Cells were 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 one of the antibodies, including rabbit phospho-specific Akt (Thr308) antibody, phospho-specific Akt (Ser473) antibody, nonphosphorylated Akt antibody, phospho-specific $p70^{S6k}$ (Thr^{389}), phospho-specific $p70^{S6k}$ (Ser^{421}/Ser^{424}), and nonphosphorylated $p70^{S6k}$ antibody. The Akt and $p70^{S6k}$ protein bands specifically bound to primary antibodies were detected using an antirabbit IgG-alkaline phosphatase-linked secondary antibody and an ECF Western blotting system (Amersham, Piscataway, NJ) (30).

Assay for HIF-1 Transactivation. Confluent monolayers of Cl41 VEGF-Luc mass1, Cl41 VEGF-Luc Akt-T308A/S473A mass1, or Cl41 VEGF-Luc Δp85 mass1 were trypsinized, and 8×10^3 viable cells suspended in 100 μl of medium were added into each well of a 96-well plate. Plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The cells were then exposed to $NiCl_2$ or Ni_3S_2 for the indicated times and dosages. The cells were extracted with lysis buffer, and luciferase activity was measured as described previously (29). The results were expressed as HIF-1 activity relative to untreated controls.

Assay for Cap43 Expression. Cl41 and its transfectants (3×10^4) 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 as indicated in the figure legends. 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 one of two antibodies, including rabbit-specific antibody against Cap43 or specific antibody against protein kinase Cα. The protein bands specifically bound to primary antibodies were detected using an antirabbit IgG-AP-linked secondary antibody and an ECF Western blotting system (35).

RESULTS

Induction of Phosphatidylinositol-3-kinase Activity in Mouse Epidermal C141 Cells by Nickel Compounds. It has been demonstrated that PI-3K activation plays an important role in tumor promotion by different chemical carcinogens in different systems (28, 29, 32, 36–38). Thus, it was important to determine whether PI-3K was involved in cell responses to nickel compounds. Shown in Fig. 1, *A* and *B*, are the results from PI-3K assays of C141 cells treated with Ni_3S_2 or $NiCl_2$. Both Ni_3S_2 and $NiCl_2$ induced marked activation of PI-3K in C141 cells in a time-dependent manner. This nickel-induced activation of PI-3K is abolished 100% when the cells are pretreated with wortmannin, a PI-3K inhibitor that covalently binds to Lys^{802} of the p110 catalytic subunit of PI-3K blocking phosphotransfer (39). It is also noted that PI-3K induction by insoluble Ni_3S_2 is stronger than that by soluble $NiCl_2$. These data demonstrated that nickel exposure is able to induce activation of PI-3K in Cl41 cells, suggesting that PI-3K may be involved in nickel-induced cell response.

PI-3K Activation is Required for HIF-Dependent Transcription by Nickel Compounds in Mouse Epidermal C141 Cells. It has been established previously that nickel-induced transformation of rodent cells leads to an increase in HIF-1 transcription in HOS human osteosarcoma cells and A549 human lung adenocarcinoma cells (17). The above results suggested that PI-3K is activated in Cl41 cells in response to nickel exposure. It was, therefore, of interest to determine the role of PI-3K in nickel-stimulated HIF-dependent transcription activity in mouse C141 cells. Because a previous study demonstrated that VEGF expression takes place in an HIF-1-dependent manner, we first established a Cl41 stable transfectant using VEGF-Luc. Fig. 2*A* indicates the inductive effect that Ni_3S_2 and $NiCl_2$ have on VEGF promoter-driven luciferase activity (Fig. 2*A*). Nickel exposure also results in accumulation of HIF-1α protein in C141 cells in a time-dependent manner as compared with medium control (Fig. 2*B*). Both $NiCl_2$ and Ni_3S_2 induced an increase in VEGF promoter-driven luciferase activity relative to medium controls (Fig. 2*A*). To demonstrate

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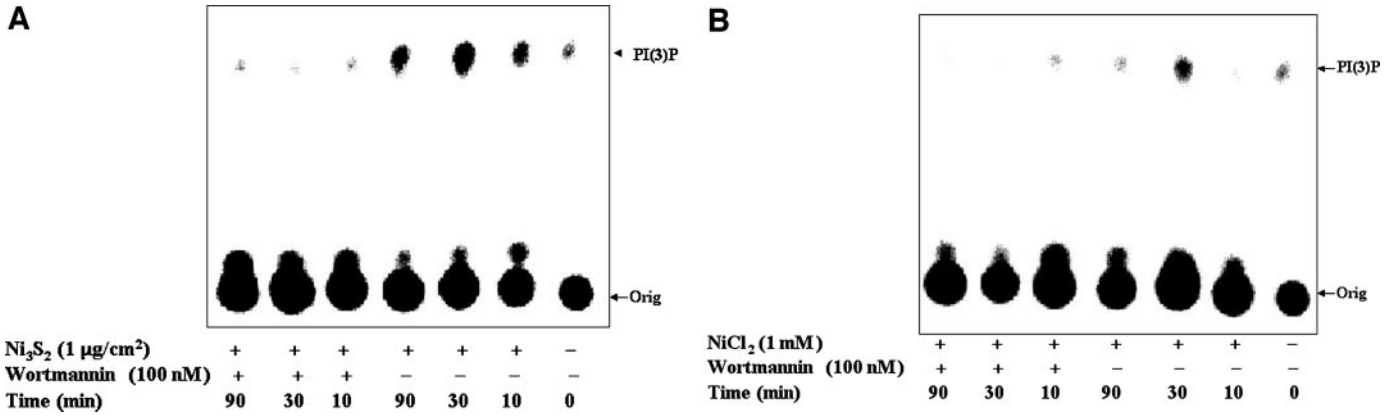

Fig. 1. Induction of phosphotidylinositol 3-kinase (PI-3K) activity by Ni_3S_2 (*A*) or $NiCl_2$ (*B*) in mouse epidermal Cl41 cells. Cl41 cells (8×10^5) were each seeded into 100-mm dishes. The media were replaced with 0.1% fetal bovine serum MEM after the cell density reached 70–80% confluency. Forty-five h later, the cells were incubated with fresh serum-free MEM for 3–4 h at 37°C. Cells were first pretreated with wortmannin (100 nM) for 30 min. Ni_3S_2 (1 μg/cm^2; *A*) or $NiCl_2$ (1 mM; *B*) was then added to cell cultures for PI-3K induction for the time periods indicated. Cells were washed once with ice-cold PBS and lysed in 400 μl of lysis buffer. PI-3K activity was measured as described in "Materials and Methods." The results presented are from one of three independent experiments. PI(3)P, phosphatidylinositol 3-phosphate; *Orig*, origin.

a specific role of HIF-1 in the VEGF promoter-driven luciferase induction, the transfection experiment with Mut-VEGF-Luc was carried out. The results showed that the induction of luciferase activity by nickel exposure in the Mut-VEGF-Luc construct transfected cells was inhibited by 87.5% as compared with the induction of luciferase activity in wild-type VEGF-Luc transfected cells (Fig. 2*C*). An essential and specific role of HIF-1α in nickel-induced VEGF promoter-driven luciferase activity is further confirmed by the finding that

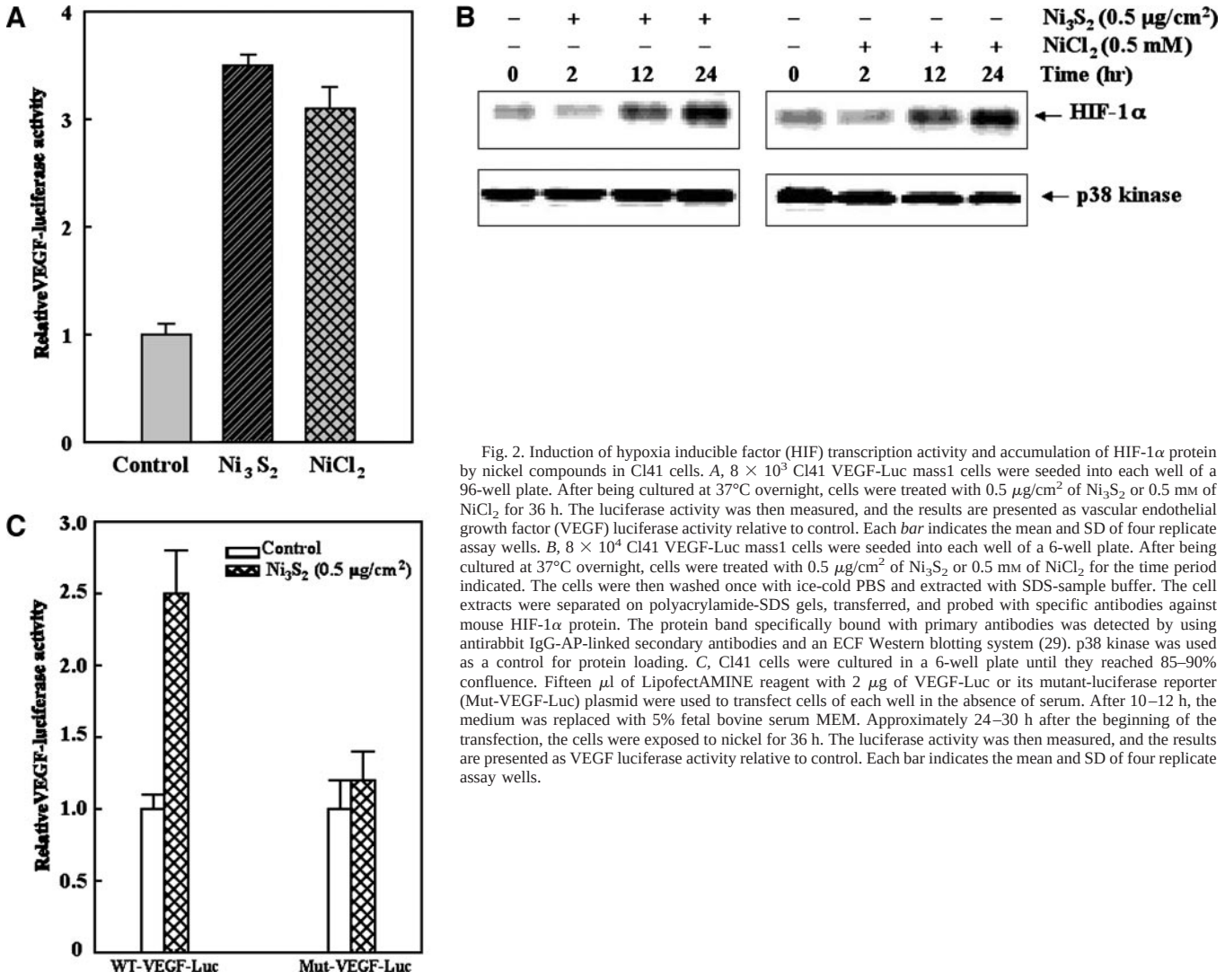

Fig. 2. Induction of hypoxia inducible factor (HIF) transcription activity and accumulation of HIF-1α protein by nickel compounds in Cl41 cells. *A*, 8×10^3 Cl41 VEGF-Luc mass1 cells were seeded into each well of a 96-well plate. After being cultured at 37°C overnight, cells were treated with 0.5 μg/cm^2 of Ni_3S_2 or 0.5 mM of $NiCl_2$ for 36 h. The luciferase activity was then measured, and the results are presented as vascular endothelial growth factor (VEGF) luciferase activity relative to control. Each *bar* indicates the mean and SD of four replicate assay wells. *B*, 8×10^4 Cl41 VEGF-Luc mass1 cells were seeded into each well of a 6-well plate. After being cultured at 37°C overnight, cells were treated with 0.5 μg/cm^2 of Ni_3S_2 or 0.5 mM of $NiCl_2$ for the time period 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 specific antibodies against mouse HIF-1α protein. The protein band specifically bound with primary antibodies was detected by using antirabbit IgG-AP-linked secondary antibodies and an ECF Western blotting system (29). p38 kinase was used as a control for protein loading. *C*, Cl41 cells were cultured in a 6-well plate until they reached 85–90% confluence. Fifteen μl of LipofectAMINE reagent with 2 μg of VEGF-Luc or its mutant-luciferase reporter (Mut-VEGF-Luc) plasmid were used to transfect cells of each well in the absence of serum. After 10–12 h, the medium was replaced with 5% fetal bovine serum MEM. Approximately 24–30 h after the beginning of the transfection, the cells were exposed to nickel for 36 h. The luciferase activity was then measured, and the results are presented as VEGF luciferase activity relative to control. Each bar indicates the mean and SD of four replicate assay wells.

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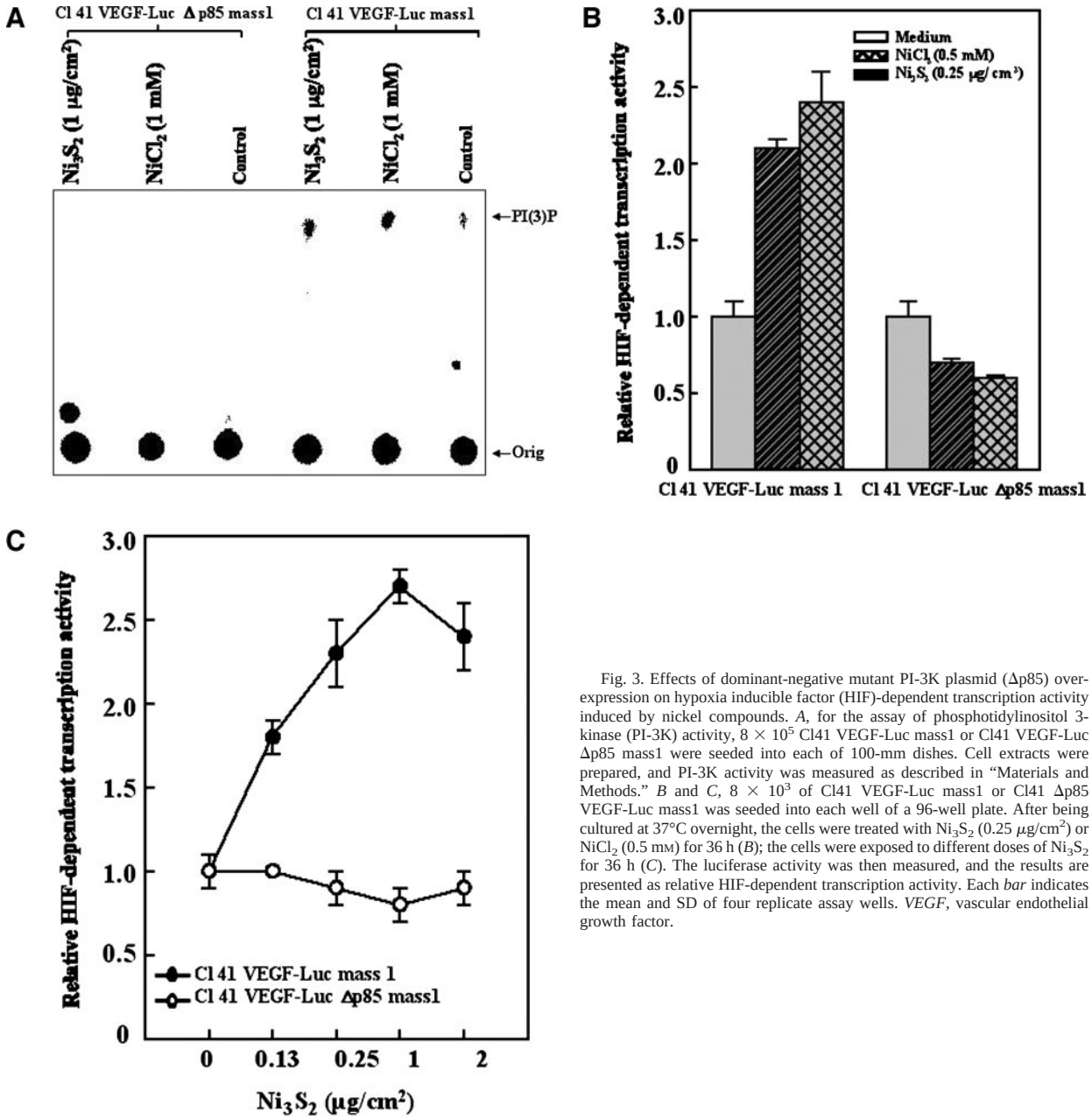

Fig. 3. Effects of dominant-negative mutant PI-3K plasmid (Δp85) overexpression on hypoxia inducible factor (HIF)-dependent transcription activity induced by nickel compounds. *A*, for the assay of phosphotidylinositol 3-kinase (PI-3K) activity, 8×10^5 Cl41 VEGF-Luc mass1 or Cl41 VEGF-Luc Δp85 mass1 were seeded into each of 100-mm dishes. Cell extracts were prepared, and PI-3K activity was measured as described in "Materials and Methods." *B* and *C*, 8×10^3 of Cl41 VEGF-Luc mass1 or Cl41 Δp85 VEGF-Luc mass1 was seeded into each well of a 96-well plate. After being cultured at 37°C overnight, the cells were treated with Ni_3S_2 (0.25 $\mu g/cm^2$) or $NiCl_2$ (0.5 mM) for 36 h (*B*); the cells were exposed to different doses of Ni_3S_2 for 36 h (*C*). The luciferase activity was then measured, and the results are presented as relative HIF-dependent transcription activity. Each *bar* indicates the mean and SD of four replicate assay wells. *VEGF*, vascular endothelial growth factor.

overexpression of a dominant-negative mutant HIF-1 inhibited nickel-induced VEGF promoter-driven luciferase activity by 73.3% (data not shown). These data strongly suggested that HIF-1 is essential for nickel-induced VEGF promoter-driven luciferase activity, suggesting that nickel-induced transactivation of VEGF expression is dependent on HIF-1 transactivation. To determine whether PI-3K plays a role in nickel-induced transactivation of HIF-1, we used dominant-negative mutant PI-3K, Δp85, to establish a stable transfectant, Cl41 VEGF-Luc Δp85 mass1. Transfection of Δp85 blocked nickel-induced PI-3K activation (Fig. 3*A*) and HIF-dependent transcription activity (Fig. 3, *B* and *C*), demonstrating that PI-3K activation plays a critical role in nickel-induced HIF-1 transactivation.

Overexpression of Dominant-Negative Mutant Akt Impaired Nickel-Induced HIF-1-Dependent Transcription Activity in Cl41 Cell. Because our results indicated that PI-3K is involved in signal transduction leading to HIF activation in response to nickel compounds, it was important to elucidate further PI-3K downstream signaling kinases. To determine whether Akt is a PI-3K downstream kinase that is involved in nickel-induced HIF transactivation, we established a stable transfectant, Cl41 VEGF-Luc Akt-T308A/S473A mass1. The results showed that nickel exposure results in increased phosphorylation of Akt at Thr^{308} and Ser^{473} (Fig. 4*A*). This increase in phosphorylation of Akt at Thr^{308} and Ser^{473} could be blocked by overexpression of the dominant-negative mutant Akt, SRα-Akt-T308A/S473A or overexpression of Δp85 (Fig. 4, *A* and *B*), revealing that Akt is a PI-3K downstream kinase in Cl41 cells. Blocking phosphorylation of Akt at Thr^{308} and at Ser^{473} by overexpressing SRα-Akt-T308A/S473A also dramatically inhibited nickel-induced HIF-dependent transactivation induced by nickel compounds in both a time- and dose-dependent manner (Fig. 5). These data indicated that Akt is not only a PI-3K downstream kinase in nickel-induced cellular responses but also plays a key role in nickel-induced HIF transactivation.

$p70^{S6k}$ Is Involved in Nickel-Induced Cellular Response, but Is Not Required for HIF Transactivation by Nickel Compounds. To test the role of another PI-3K downstream kinase, $p70^{S6k}$, in nickel-

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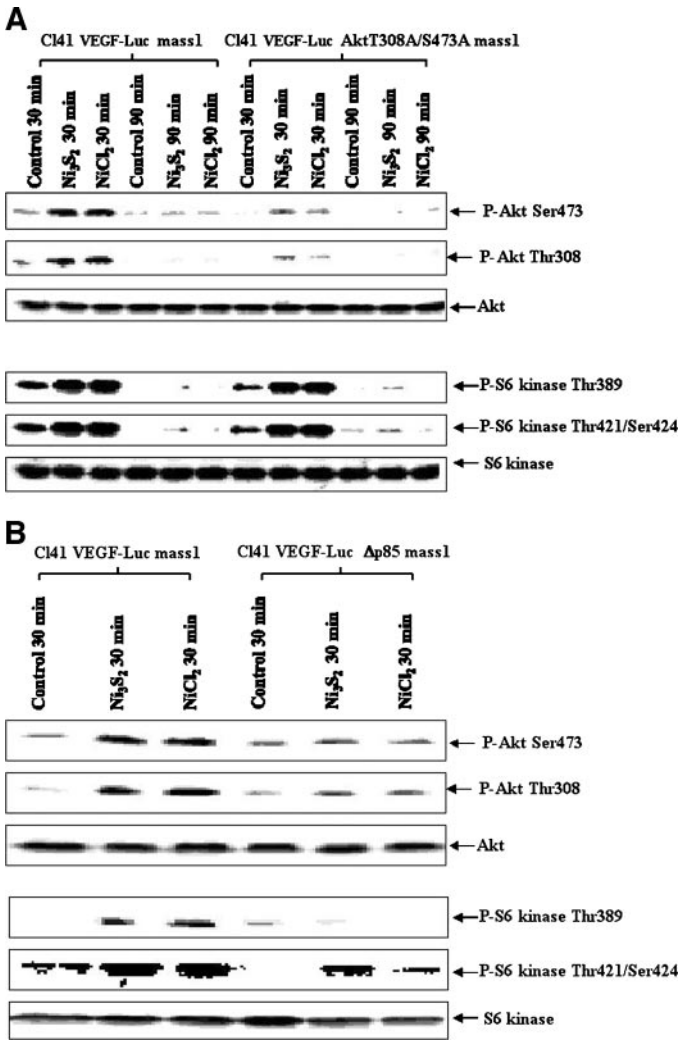

Fig. 4. Effects of overexpression of AktT308A/S473A (*A*) or dominant-negative mutant PI-3K (Δp85; *B*) on phosphorylation of Akt and $p70^{S6k}$ induced by nickel compounds. Subconfluent (90%) monolayers of Cl41 VEGF-Luc mass1, Cl41 VEGF-Luc AktT308A/S473A or Cl41 VEGF-Luc Δp85 mass1 in 6-well plates were subjected to either Ni_3S_2 (1 $\mu g/cm^2$) or $NiCl_2$ (1 mM) and cultured for the time points 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 one of the specific antibodies as indicated. The protein band specifically bound with primary antibodies was detected using antirabbit IgG-AP-linked secondary antibodies and an ECF Western blotting system (29). *VEGF*, vascular endothelial growth factor.

induced HIF activation, we investigated the possible activation of $p70^{S6k}$ in nickel-treated cells. As shown in Fig. 4, exposure of cells to either $NiCl_2$ or Ni_3S_2 resulted in an increase in phosphorylation of $p70^{S6k}$ at Thr^{389} and Thr^{421}/Ser^{424}. The increase in phosphorylation of $p70^{S6k}$ at Thr^{389} and Thr^{421}/Ser^{424} induced by nickel compounds was inhibited by overexpression of Δp85 (Fig. 4*B*), whereas overexpression of SRα-Akt-T308A/S473A did not show any inhibitory effect (Fig. 4*A*), indicating that $p70^{S6k}$ is a PI-3K, but not Akt, downstream kinase. Furthermore, inhibition of $p70^{S6k}$ by pretreatment of cells with rapamycin, which is able to inhibit $p70^{S6k}$ activation by impairing mammalian target of rapamycin, did not show any significant effects on HIF-1 transcription induced by $NiCl_2$ or Ni_3S_2, although it specifically impaired $p70^{S6k}$ phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} at the same doses (data not shown). The results indicated that $p70^{S6k}$ is activated by nickel compounds through a PI-3K-dependent and an Akt-independent pathway. However, this activation is not involved in HIF-1-dependent transcription stimulated by either $NiCl_2$ or Ni_3S_2 in mouse C141 cells.

Role of PI-3K, Akt, and $p70^{S6k}$ in Cap43 Induction by Nickel Compounds. Cap43 is a protein that is overexpressed in a variety of cancer cell types, including brain, breast, lung, and melanoma cancers (40, 41). Cap43 can also be induced by Ni_3S_2 and $NiCl_2$ in a HIF-dependent manner (40). With these facts in mind, we further probed the link between the signaling pathways leading to HIF transactivation and Cap43 protein induction. Fig. 6*A* shows that the induction of Cap43 in mouse epidermal C141 cells by either Ni_3S_2 or $NiCl_2$ occurs in a time-dependent manner. Considering the role of PI-3K, Akt, and $p70^{S6k}$ in nickel-induced HIF-dependent transcription, we evaluated the role of these kinases in nickel-induced Cap43 protein induction.

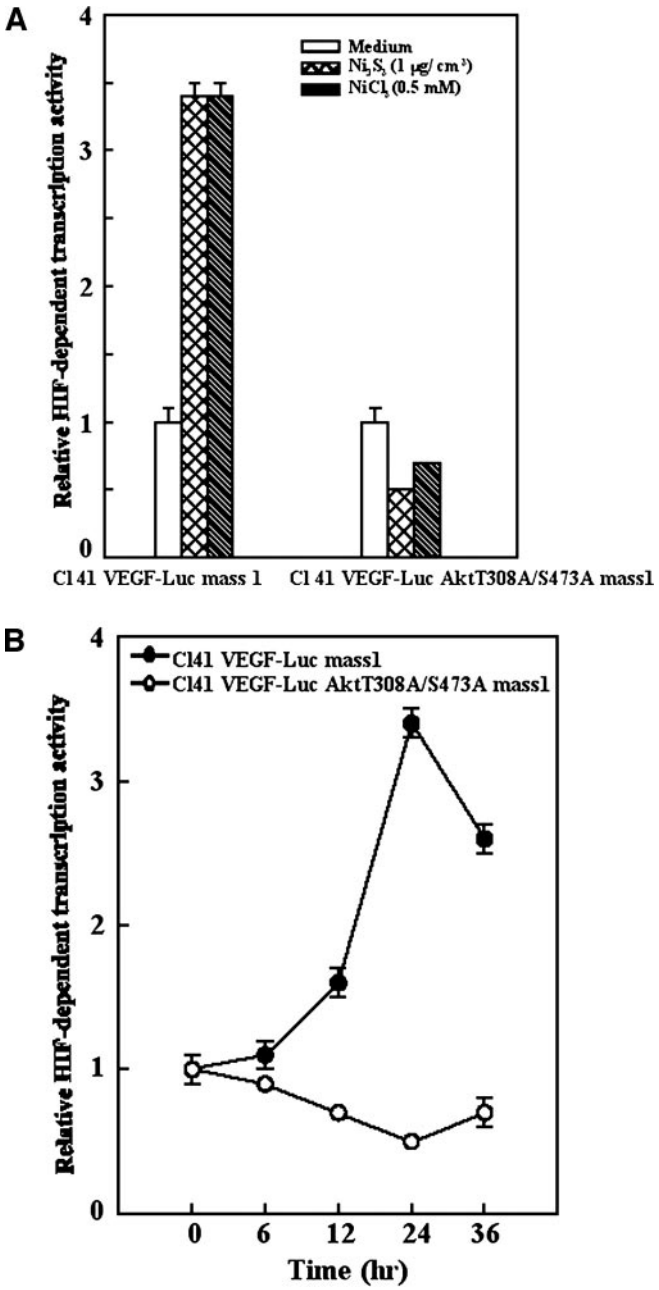

Fig. 5. Effects of overexpression of AktT308A/S473A on nickel-induced hypoxia inducible factor (HIF)-dependent transcription activity. Cl41 VEGF-Luc mass1 or Cl41 VEGF-Luc AktT308A/S473A mass1 (8×10^3) was seeded into each well of a 96-well plate. After being cultured at 37°C overnight, the cells were treated with (*A*) Ni_3S_2 (1 $\mu g/cm^2$) or $NiCl_2$ (0.5 mM) for 24 h or (*B*) Ni_3S_2 (1 $\mu g/cm^2$) for the time points indicated. The luciferase activity was then measured, and the results are presented as relative HIF-dependent transcription activity. Each *bar* indicates the mean and SD of four replicate assay wells. *VEGF*, vascular endothelial growth factor.

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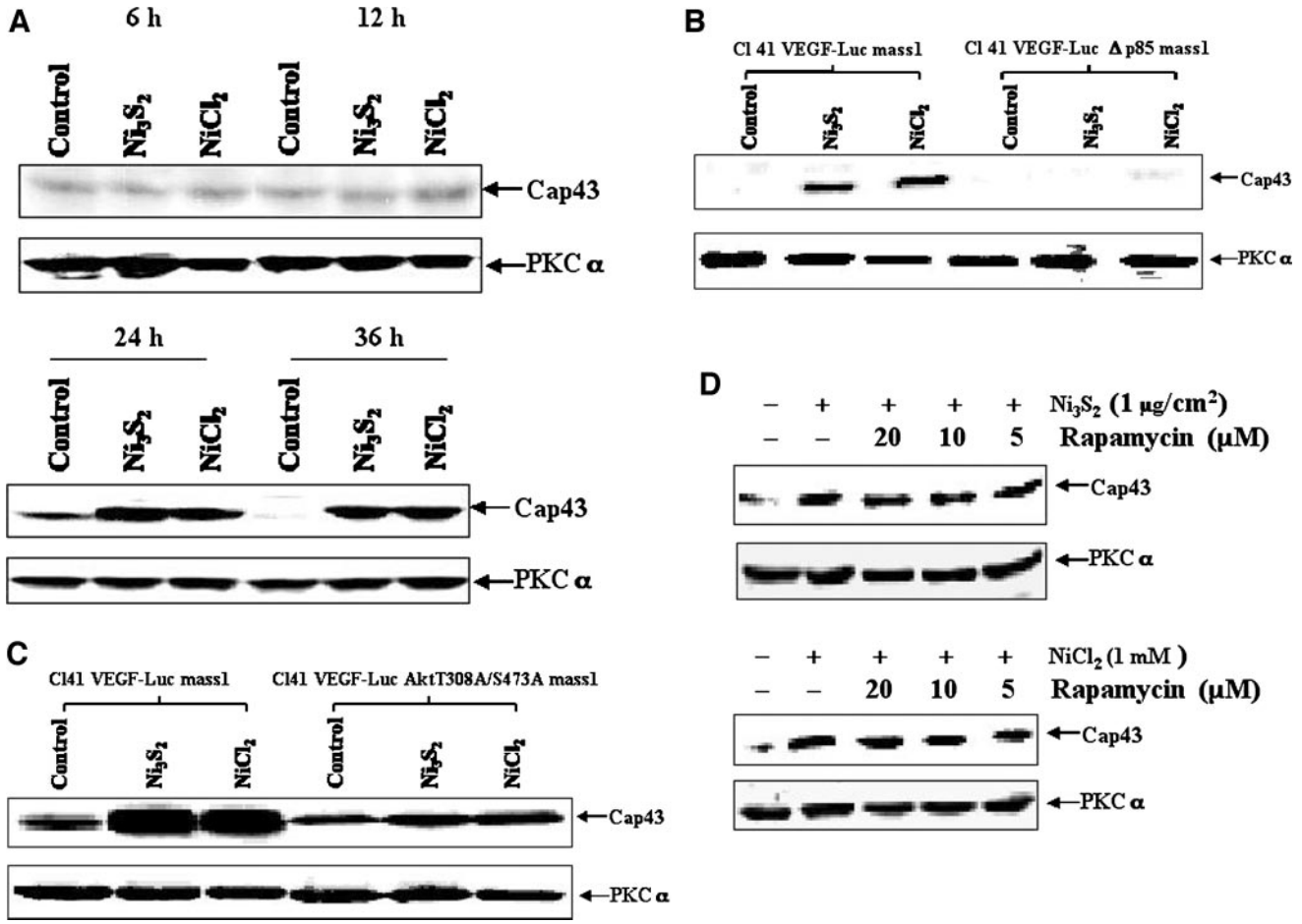

Fig. 6. Role of phosphotidylinositol 3-kinase, Akt, and $p70^{S6k}$ in Cap43 induction by nickel compounds. Cl41, Cl41 VEGF-Luc mass1, Cl41 VEGF-Luc AktT308A/S473A, or Cl41 VEGF-Luc Δp85 mass1 cells was cultured in monolayers in 6-well plates until 90% confluent. *A*, the cells were exposed to either Ni_3S_2 (1 $\mu g/cm^2$) or $NiCl_2$ (1 mM) for different time points as indicated. *B* and *C*, the cells were exposed to either Ni_3S_2 (1 $\mu g/cm^2$) or $NiCl_2$ (1 mM) for 24 h. *D*, The cells were pretreated with different concentrations of rapamycin as indicated for 30 min and then exposed to Ni_3S_2 (1 $\mu g/cm^2$) or $NiCl_2$ (1 mM). 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 polyclonal antibodies raised against Cap43. The Cap43 protein band specifically bound to the primary antibody was detected using an antirabbit IgG-AP-linked secondary antibody and an ECF Western blotting system (29). Protein kinase Cα was used as control for protein loading. *VEGF*, vascular endothelial growth factor. *Δp85*, dominant-negative mutant PI-3K plasmid. *PKC*, protein kinase C.

Consistent with nickel-induced HIF activation, overexpression of a dominant-negative mutant of either PI-3K or Akt inhibited nickel-induced Cap43 expression, although inhibition of $p70^{S6k}$ by rapamycin did not show inhibitory effects on Cap43 protein expression induced by nickel compounds (Figs. 6, *B–D*). The results indicate that the PI-3K-Akt pathway is required for Cap43 protein induction by nickel compounds and that $p70^{S6k}$ is not involved.

DISCUSSION

HIF-1α is induced in response to nickel compounds, although the details of the induction of this response are less well known. One area of particular interest is the signaling involved in the tumor promotion effects of nickel compounds, a signaling that is mediated by transactivation of transcription factors [*i.e.*, HIF-1 that activates the transcription of a variety of genes required for cell transformation, tumor promotion and progression, angiogenesis, altered metabolism, and apoptosis (42)]. Both soluble and insoluble nickel compounds have been shown to induce HIF-1α. To elucidate the signals leading from nickel exposure to induction of HIF-1, we investigated the effect of nickel compounds on HIF-dependent transcription. Our results indicated that exposure of mouse epidermal C141 cells to Ni_3S_2 or $NiCl_2$ led to an increase in HIF-dependent transcription activity and Cap43 protein expression. The increases in HIF-1 transactivation and Cap43 protein expression are mediated through PI-3K- and Akt-dependent pathways and a $p70^{S6k}$-independent pathway. This conclusion is based on the results provided by overexpression of the dominant-negative mutants, Δp85 and SRα-Akt-T308A/S473A, and pretreatment of cells with rapamycin.

It should be noted that the involvement of PI-3K/Akt signaling with respect to the regulation of HIF-1 is still somewhat controversial. It has been shown that a VEGF hypoxia-inducible pathway, regulated by HIF-1α, was distinct from a PI-3K-dependent pathway that itself only regulated basal levels of VEGF (43). This was supported by a study of HIF-1α levels in several different cell types indicating that HIF-1α levels were only moderately affected by the PI-3K/Akt pathway (44). Jiang *et al.* (32) demonstrated that insulin, epidermal growth factor, or an inactivation mutant in the tumor suppressor PTEN specifically increased the HIF-1α protein expression, but not HIF-1β in human cancer cell lines. This specific elevation of HIF-1α protein expression by insulin, epidermal growth factor, or inactivation mutant in PTEN was dramatically reduced by treatment of cells with PI-3K inhibitors, Ly294002 or wortmannin (32). This was consistent with the finding that PI-3K regulated HIF-1α protein levels (45).

Despite these facts, our results demonstrate a link between HIF-1 and the PI-3K/Akt pathway in mouse epidermal Cl41 cells treated

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with nickel compounds. When C141 cells were incubated with either $NiCl_2$ or Ni_3S_2, PI-3K activity was induced, and this activity was abolished by overexpression of Δp85. In a similar manner, HIF-dependent transcription activity was also attenuated by Δp85 overexpression. Moreover, overexpression of a dominant mutant of Akt also resulted in reduction of HIF transactivation by nickel compounds.

Taken together, these findings suggest that the PI-3K/Akt pathway plays a role in the downstream activation of HIF-dependent transcription in Cl41 cells treated with nickel compounds. Because rapamycin, an inhibitor of mammalian target of rapamycin, did not inhibit HIF-dependent transcriptional activation by nickel compounds, it would appear that the signaling for HIF-dependent transcription occurs through the PI-3K/Akt pathway and bypasses mammalian target of rapamycin/$p70^{S6k}$ pathway. Note that the activation of PI-3K/Akt pathway occurs at 30 min after nickel exposure, although a period of $>$12 h is required for HIF-dependent luciferase reporter transactivation. The time differences may be explained by the HIF-1 protein accumulation, transcription of HIF-dependent luciferase reporter gene, and translation, as well as modification of luciferase protein. This temporal delay also occurred in the MAPK (mitogen-activated protein kinases)-mediated activator protein-1-dependent luciferase reporter transactivation induced by 12-*O*-tetradecanoylphorbol-13-acetate, epidermal growth factor, and UV radiation (46–49). It has been reported that cobalt was able to prevent the binding of pVHL to HIF-1α protein by inhibiting prolyl-hydroxylase activity of pVHL that was required for proline hydroxylation of HIF-1α protein. Inhibition of the HIF-1α/pVHL association by cobalt leads to stabilized HIF-1α protein and HIF-1α transactivation (22, 23). We will further test whether prolyl hydroxylation may also be involved in nickel-induced HIF-dependent transcriptional activation in our future studies.

The second issue that we addressed involved the relationship between the PI-3K/Akt/$p70^{S6k}$ pathway and Cap43. Cap43, also called NDRG1, is a protein that was induced in response to hypoxia (hypoxia-mimic desferrioxamine and nickel) and increases in intracellular Ca^{2+} concentrations (40, 41). It was first cloned as a myc down-regulated gene and has been associated with both differentiation and growth arrest (50, 51). It has also been postulated as a tumor suppressor gene because it causes the reversal of malignant phenotype in colon cancer cells (52). More recently, it has been associated with Charcot-Marie-Tooth disease, a hereditary neuromuscular disorder (53). The mechanism of induction of Cap43 has yet to be clearly elucidated. The induction of Cap43/NDRG1 by Ca^{2+} was shown recently to take place via a hypoxia-independent pathway (54). In mouse epidermal Cl41 cells, nickel compounds are only able to induce transactivation of nuclear factor κB but not activator protein-1 (52), although nuclear factor κB activation by nickel compounds is not involved in Cap43 protein induction (52). Thus, our results confirm the observation that Cap43/NDRG1 is induced by nickel exposure. Additionally, we demonstrate the attenuation of nickel-induced Cap43/NDRG1 protein expression by inhibition of either PI-3K or Akt but not by inhibition of $p70^{S6k}$.

In summary, nickel induced HIF-dependent transcriptional activation in mouse epidermal C141 cells, an activity that was mediated through the PI-3K/Akt pathway. However, unlike other examples of the PI-3K/Akt pathway, HIF transactivation by nickel compounds in Cl41 cells was not mediated through $p70^{S6k}$. Finally, we demonstrate that nickel-induced Cap43 protein levels were also dependent on the PI-3K/Akt pathway.

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Nickel Compounds Act through Phosphatidylinositol-3-kinase/Akt-Dependent, p70 S6k -Independent Pathway to Induce Hypoxia Inducible Factor Transactivation and Cap43 Expression in Mouse Epidermal Cl41 Cells

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