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Essential role of PI-3K, ERKs and calcium signal pathways in nickel-induced VEGF expression

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Abstract

Exposure to a highly nickel-polluted environment has the potential to cause a variety of adverse health effects, such as the respiratory tract cancers. Since numerous studies have demonstrated that nickel generally has weak mutagenic activity, research focus had turned to cell signalling activation leading to gene modulation and epigenetic changes as a plausible mechanism of carcinogenesis. Previous studies have revealed that nickel compounds can induce the expression of vascular endothelial growth factor (VEGF), which is a key mediator of angiogenesis both in physiological and pathologic conditions. In the present study, we investigated the potential roles of PI-3K, ERKs, p38 kinase and calcium signalling in VEGF induction by nickel in Cl 41 cells. Exposure of Cl 41 cells to nickel compounds led to VEGF induction in both time- and dose-dependent manners. Pre-treatment of Cl 41 cells with PI-3K inhibitor, wortmannin or Ly294002, resulted in a striking inhibition of VEGF induction by nickel compounds, implicating the role of PI-3K in the induction. However, mTOR, one of downstream molecules of PI-3K, may not contribute to the induction because pre-treatment of Cl 41 cells with its inhibitor, rapamycin, did not show obvious decrease in nickel-induced VEGF expression. Furthermore, pre-treatment of Cl 41 cells with MEK1/2-ERKs pathway inhibitor, PD98059, significantly inhibited VEGF induction by both $NiCl_2$ and Ni_3S_2, whereas p38 kinase inhibitor, SB202190, did not impair the induction. Pre-treatment of Cl 41 cells with intracellular calcium chelator, but not calcium channel blocker, inhibited VEGF induction by nickel. Collectively these data demonstrate that PI-3K, ERKs and cytosolic calcium, but not p38 kinase, play essential roles in VEGF induction by nickel compounds. (Mol Cell Biochem **279:** 35–43, 2005)

Key words: calcium signalling, ERK, nickel, PI-3K, p38 kinase

Abbreviations: DMSO, dimethyl sulfoxide; ERK, extracellular signal–regulated kinases; FBS, fetal bovine serum; HIF-1, hypoxia-inducible Factor-1; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEM, Eagle's minimal essential medium; mTOR, mammalian target of rapamycin; PI-3K, phosphotidylinositol 3-kinas; TGF-β1, transforming growth factor beta 1; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; VEGF, vascular endothelial growth factor

Introduction

Nickel compounds are known to be widely distributed in the occupational and non-occupational environment. Epidemiological data have shown that exposure to nickel compounds is associated with immunological sensitization, epithelial dysplasia, asthma, fibrosis and lung cancer [1]. At the cellular level, nickel stimulates signalling cascades that increase the expression of profibrotic proteins, inflammatory cytokines, and genes involved in hypoxic responses, such as

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plasminogen activator inhibitor-1, IL-8, VEGF, and Cap43 (NDRG1) [2–5]. In addition, nickel suppresses the expression of the tumor suppressor thrombospondin-1 [6]. Changes in the expression levels of these genes may contribute to pathologic effects of nickel, including cancers.

Vascular endothelial growth factor (VEGF), initially discovered in 1983 [7] and cloned in 1989 [8], is a key player of angiogenesis in health and disease [9]. It has been well accepted that VEGF-mediated angiogenesis plays a crucial role in tumor progression [10]. The expression of VEGF can be induced by low oxygen tension, growth factors, and some metals, such as nickel compounds [4, 11–12]. VEGF induction under hypoxia conditions has been described as a part of the general response of a cell to low oxygen tension. A series of studies have shown that nickel can induce a hypoxia-like response [13–16]. Our previous results indicated nickel-activated hypoxia-inducible factor-1α (HIF-1α), a key mediator of the cellular response to low oxygen tension [17], plays an essential role in VEGF induction in Cl 41 cells [14]. Nickel has also been reported to affect other signal pathways by activating upstream signalling cascades [14, 18–20]. These include stimulation of calcium-dependent signalling [18], as well as activation of PI-3K [14] and MAPKs [19, 20]. However, attribution of these signal pathways to VEGF induction has not been clearly demonstrated. Thus, in this study, we addressed the potential role of PI-3K, MAPKs and calcium signalling in VEGF induction by nickel, and found that chemical inhibitors such as wortmannin, Ly294002, PD98059, but not SB202190, and a calcium chelator, but not the calcium channel blocker, significantly inhibited nickel-induced VEGF expression. These results demonstrated that PI-3K, ERKs and cytosolic calcium, but not p38 kinase, are essential for VEGF induction by nickel compounds.

Materials and methods

Cell culture and reagents

Mouse epidermal JB6 Cl 41 cell line and its VEGF-luciferase reporter stable transfectant (Cl 41 VEGF mass1) were cultured in monolayers at 37 °C, 5% CO_2 using Eagle's minimal essential medium (MEM) containing 5% fetal bovine serum (FBS), 2 mM L-glutamine, and 25 μg of gentamicin/ml [14]. Human lung bronchoepithelial A549 cells were grown in Ham's F-12K medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. The cultures were dissociated with trypsin and transferred to new 75-cm^2 culture flasks (Fisher, Pittsburgh, PA) from one to three times per week. FBS was purchased from Life Technologies, Inc.; MEM was from Calbiochem (San Diego, CA); luciferase assay substrate was from Promega. Nickel chloride ($NiCl_2$) was purchased from Aldrich (Milwaukee, WI); nickel subsulfide (Ni_3S_2) was obtained from INCO (Toronto, Canada); nickel sulfide (NiS) was purchased from Alfa (Ward Hill, MA); the luciferase assay substrate was purchased from Promega. Wortmannin and Ly294002, PI-3K inhibitors, PD98059, a specific inhibitor for MEK1/2-ERKs pathway; rapamycin, a $p70^{S6k}$ pathway inhibitor, SB202190, a p38 inhibitor, Nifedipine and BAPTA-AM, calcium inhibitors, were purchased from Calbiochem (La Jolla, CA).

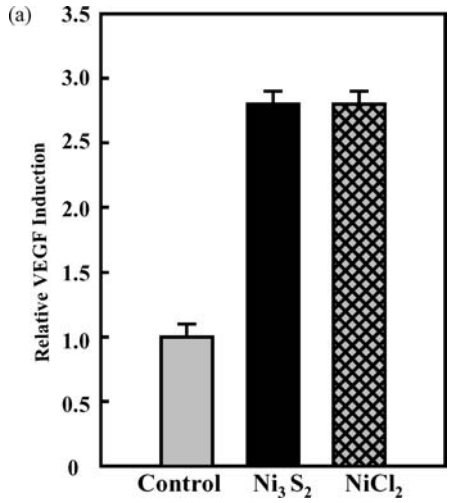
VEGF gene reporter assay

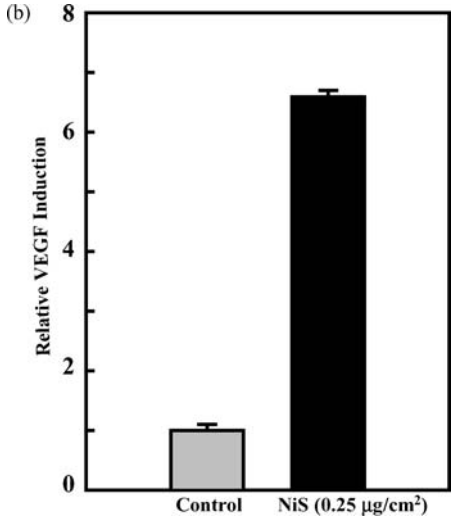
Confluent monolayers of Cl 41 VEGF-Luc mass1, a human VEGF-luciferase reporter Cl41 cell table transfectant, were trypsinized, and 8×10^3 viable cells suspended in 100 μl MEM supplemented with 5% FBS were added to each well of 96-well plates. The plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2. After the cell density reached 80–90%, the cell culture medium was replaced with an equal volume of MEM supplemented with 0.1% FBS and 2 mM L-glutamine [14]. 12 h later, cells were pre-treated with signal pathway inhibitors of interests, and then exposed to nickel compounds and cells were assessed for VEGF induction. Following selected time intervals, the cultures were extracted with 50 μl lysis buffer, and luciferase activity was measured using Promega Luciferase assay reagent with a luminometer (Wallac 1420 Victor2 multipliable counter system). The results are expressed as VEGF activity relative to control medium (relative VEGF activity) [17].

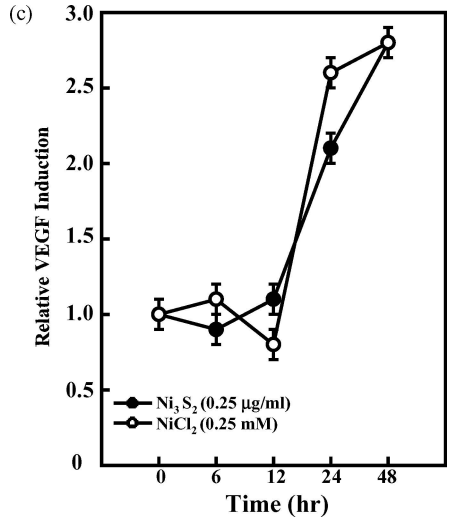
PI-3 kinase assay

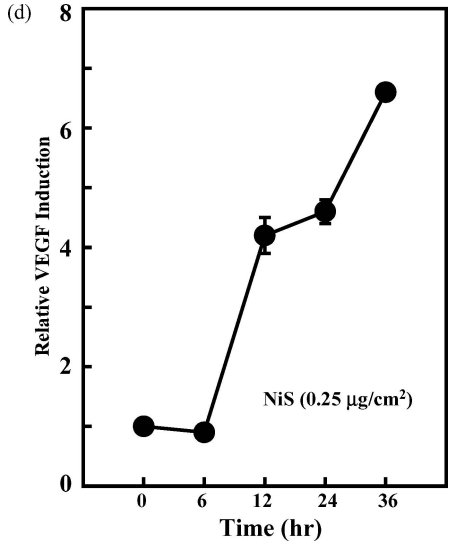
PI-3 kinase activity was assayed as described in our previous reports [21, 22]. Cells were cultured in monolayers in

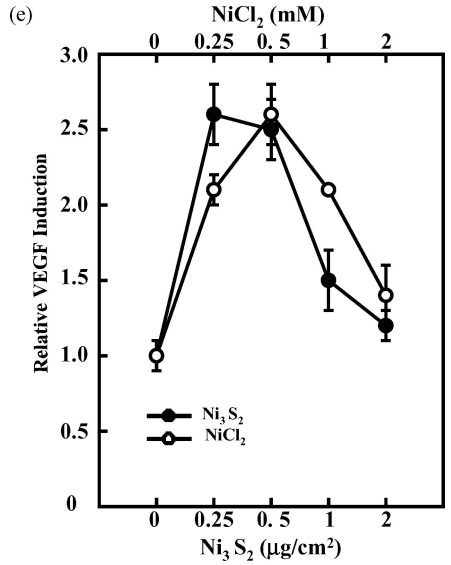
Fig. 1. VEGF induction by nickel compounds in mouse epidermal Cl 41 cells. 8×10^3 of Cl 41 VEGF mass1 cells were seeded into each well of 96-well plates, and cultured in 5% FBS MEM at 37 °C. After the cell density reached 80–90%, (a) and (b) the cells were exposed to 0.25 mM $NiCl_2$, 0.25 μg/cm^2 Ni_3S_2 or 0.25 μg/cm^2 NiS for 24 h; (c) and (d) the cells were exposed to 0.25 mM $NiCl_2$, 0.25 μg/cm^2 Ni_3S_2 or 0.25 μg/cm^2 NiS for 0∼48 h. (e) and (f) the cells were exposed to various concentrations of $NiCl_2$, Ni_3S_2 or NiS for 24 h; The cells were extracted with lysis buffer, and luciferase activity was measured using Promega Luciferase assay reagent with a luminometer (Wallac 1420 Victor2 multipliable counter system) after addition of 50 μl of lysis buffer for 30 min at 4 °C. The results are expressed as VEGF induction relative to medium control. Each bar indicates the mean and standard deviation of triplicate assay wells.

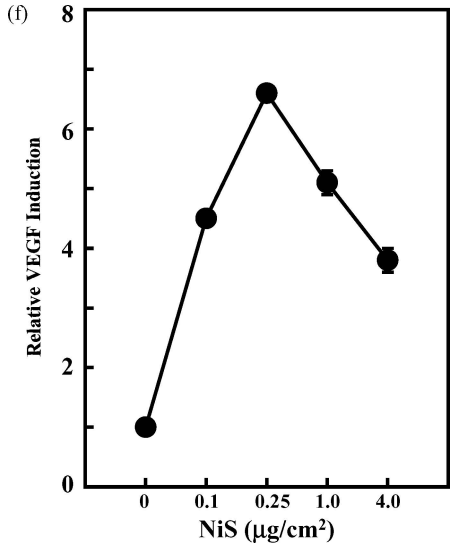

(a)
Relative VEGF Induction
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
Control
Ni3 S2
NiCl2


(b)
Relative VEGF Induction
8
6
4
2
0
Control
NiS (0.25 μg/cm2)


(c)
Relative VEGF Induction
3.0
2.5
2.0
1.5
1.0
0.5
0
Ni3 S2 (0.25 μg/ml)
NiCl2 (0.25 mM)
0
6
12
24
48
Time (hr)


(d)
Relative VEGF Induction
8
6
4
2
0
NiS (0.25 μg/cm2)
0
6
12
24
36
Time (hr)


(e)
NiCl2 (mM)
0
0.25
0. 5
1
2
Relative VEGF Induction
3.0
2.5
2.0
1.5
1.0
0.5
0
Ni3 S2
NiCl2
0
0.25
0. 5
1
2
Ni3 S2 (μg/cm2)


(f)
Relative VEGF Induction
8
6
4
2
0
0
0.1
0.25
1.0
4.0
NiS (μg/cm2)

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%. 45 h later, the cells were incubated with fresh serum-free MEM for 3–4 h at 37 °C. $NiCl_2$ or Insulin was then added to cell cultures and cells were assessed 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 mM DTT, 0.4 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride]. The lysates were centrifuged and the supernatants incubated overnight 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, 1mM 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 was 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, 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).The 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. The plates were dried at room temperature and autoradiographed.

Statistical analysis

The significance of the difference between treated and untreated groups were determined with the Student's *t*-test. The results are expressed as mean ± S.E.M.

Results

Induction of VEGF in mouse epidermal Cl 41 cells by nickel components

VEGF is a key mitogen for endothelial cells, which plays a crucial role in tumor angiogenesis during tumor promotion and progression [10]. It has been reported that VEGF can be induced by nickel compounds in cancer cells [23]. Because of its key role in angiogenesis, we investigated the signal pathways involved in VEGF-induction by nickel in Cl 41 cells, a valuable model for tumor promotion studies, and such studies may shed some light on elucidation of the molecular mechanisms of nickel-induced pathological effects, such as carcinogenesis. As shown in Fig. 1, treatment of Cl 41 cells with either $NiCl_2$, Ni_3S_2 or NiS resulted in VEGF induction in a dose- and time-dependent manner (Fig. 1). These results are consistent with previous reports [14], suggesting that nickel compounds are potent for VEGF expression.

Requirement of ERKs, but not p38K, for VEGF induction by nickel components in Cl 41 cells

To elucidate the signalling pathways involved in nickel-induced VEGF expression, we first focused on the MAPKs pathways, including ERKs and p38K based on the fact that nickel was able to activate ERKs and p38 kinase, and that MAPKs play a key role in the activation of transcription factors and other regulatory proteins involved in the regulation of expression of numerous genes [24–25]. Cl 41 cells were pre-treated with PD98059, an inhibitor of MEK1/2-ERKs pathway, and SB202190, an inhibitor of p38K, and then exposed to nickel compounds and subsequently the expression of VEGF was assessed. The results indicated that pre-treatment of Cl 41 cells with PD98059 resulted in a striking inhibition of nickel-induced VEGF expression (Figs. 2a and b). The expression induced by $NiCl_2$ and Ni_3S_2 was completely blocked by PD98059 at 50 μM and 100 μM respectively. However, the p38 kinase inhibitor, SB202190 did not show any inhibitory effect on VEGF induction (Fig. 2a and b). In contrast, low concentrations of SB202190 enhanced Ni_3S_2-induced VEGF expression (Fig. 2b). This data indicated that ERKs, but not p38K, were required for nickel-induced VEGF expression.

Involvement of calcium signalling in VEGF induction by nickel components in Cl 41 cells

It has been reported that ion channels and intracellular calcium contribute to hypoxia-mediated VEGF activation in the human fetal kidney cell line 293 cells [26]. Considering the fact that nickel can also stimulate calcium-dependent signals, it was likely that calcium signalling may also contribute to VEGF induction by nickel compounds. Here we demonstrated that BAPTA-AM pre-treatment totally suppressed VEGF expression induced by both $NiCl_2$ and Ni_3S_2 in Cl 41 cells (Figs. 3a and b). However, Nifedipine did not show any inhibitory effect on VEGF induction by nickel, instead

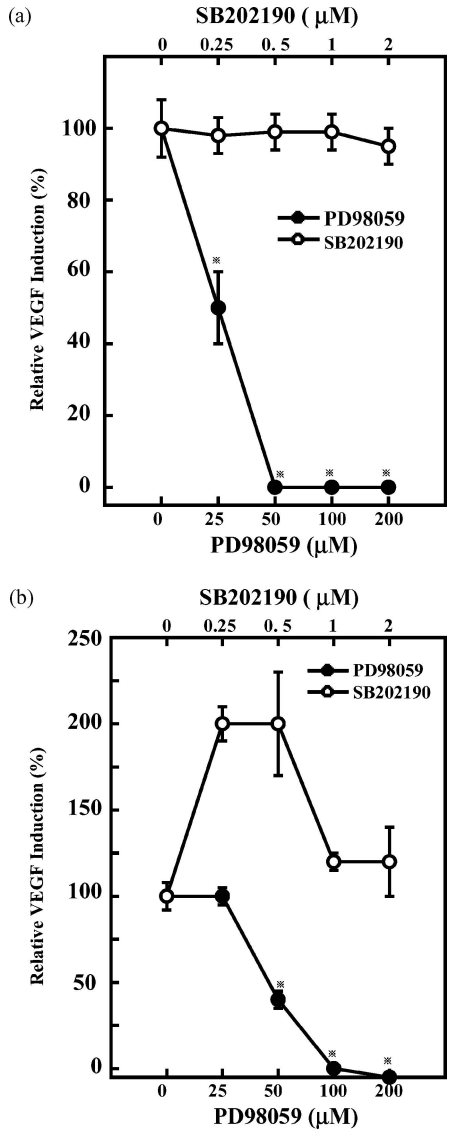


Fig. 2. Requirement of ERK, but not p38K for nickel-induced VEGF expression. 8×10^3 of Cl 41 VEGF mass1 cells were seeded into each well of 96-well plates, and cultured in 5% FBS MEM at 37 °C. After the cell density reached 80–90%, the cells were first pre-treated with various concentrations of PD98059 or SB202190, and then exposed to 1 mM $NiCl_2$ (a) or 1 μg/cm^2 Ni_3S_2 (b) for 24 h. The cells were extracted with lysis buffer, and luciferase activity was measured using Promega Luciferase assay reagent with a luminometer (Wallac 1420 Victor2 multipliable counter system) after addition of 50 μl of lysis buffer for 30 min at 4 °C. The results are expressed as VEGF induction relative to medium control. Each bar indicates the mean and standard deviation of triplicate assay wells. The asterisk (*) indicates a significant decrease from vanadium control ($p < 0.05$).

it slightly augmented the VEGF induction by both $NiCl_2$ and Ni_3S_2 (Figs. 3a and b). These data suggest that cytosolic calcium signalling, but not extracellular calcium influx, is required for nickel-induced VEGF expression.

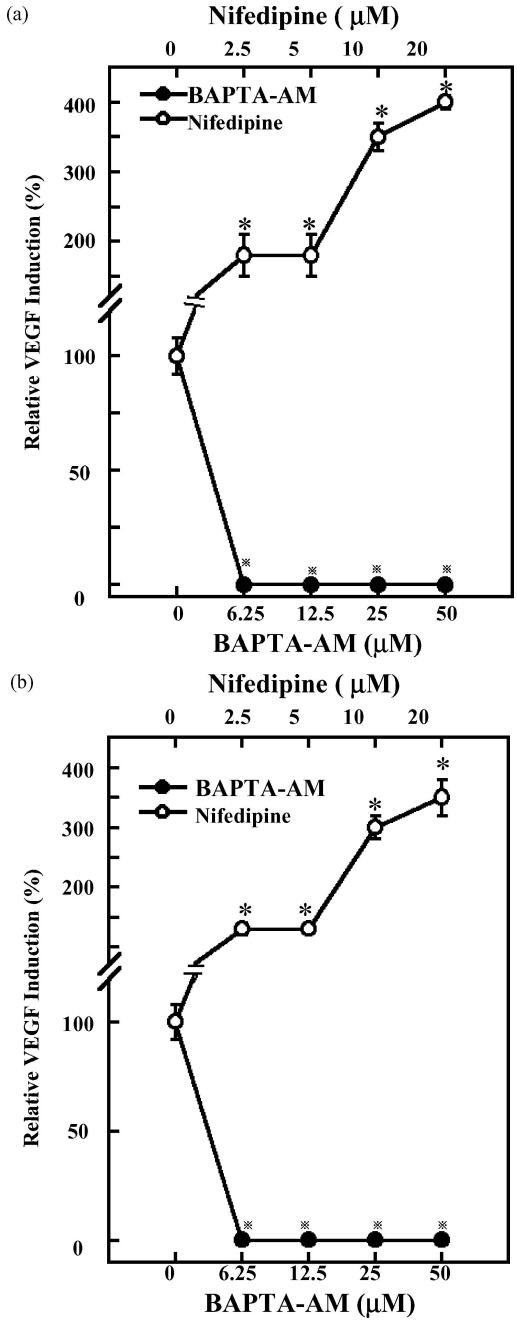
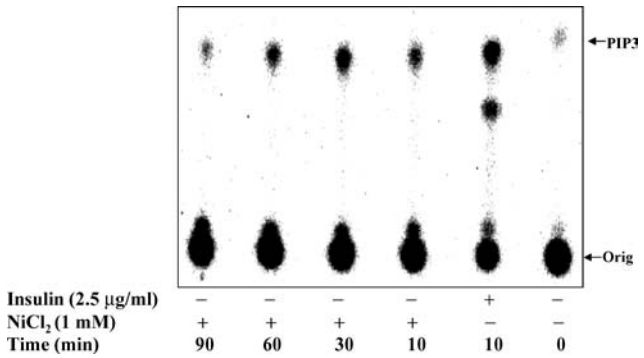


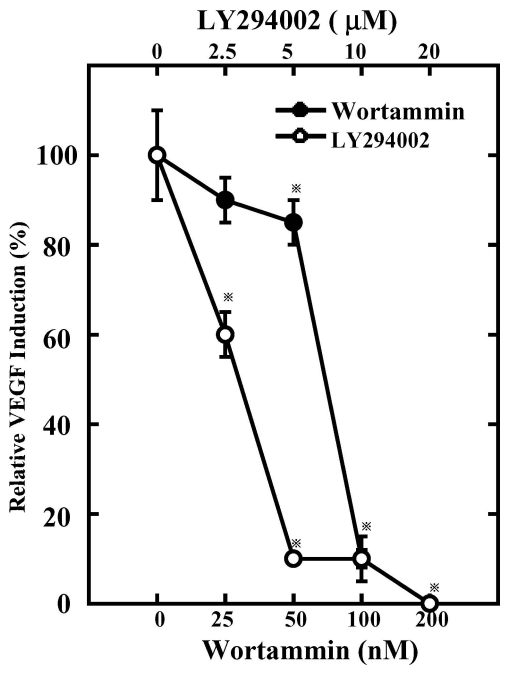
Fig. 3. Involvement of calcium signalling in nickel-induced VEGF expression. 8×10^3 of Cl 41 VEGF mass1 cells were seeded into each well of 96-well plates, and cultured in 5% FBS MEM at 37 °C. After the cell density reached 80–90%, the cells were exposed to 1 mM $NiCl_2$ (a) or 1 μg/cm^2 Ni_3S_2, (b) for VEGF induction after pre-treatment with calcium channel blocker nifedipine or intracellular calcium chelator BAPTA-AM for 12 h, and then were extracted with lysis buffer. The luciferase activity was measured using Promega Luciferase assay reagent with a luminometer (Wallac 1420 Victor2 multipliable counter system) after addition of 50 μl of lysis buffer for 30 min at 4 °C. The results are expressed as VEGF induction relative to medium control. Each bar indicates the mean and standard deviation of triplicate assay wells. The asterisk (*) indicates a significant decrease from nickel control ($p < 0.05$).

PI-3K activation and its role in VEGF induction by nickel components

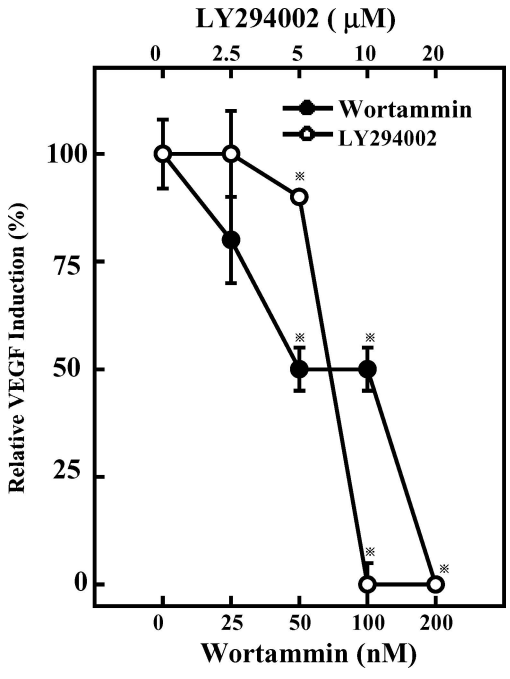
Numerous studies have demonstrated that PI-3K plays a role in cell proliferation, survival, and malignant transformation [27]. Our previous study has demonstrated that nickel compounds could activate PI-3K in Cl 41 cells [14]. Here we also found that nickel activated PI-3K in human lung A549 cells (Fig. 4a), showing that activation of PI-3K by nickel is not only in Cl41 cells, but also in various cell lines, such as A549 cells. Pre-treatment of Cl 41 cells with PI-3K inhibitors (Ly294002 and wortmannin), resulted in a significant reductions in VEGF expression induced by both $NiCl_2$ and Ni_3S_2 (Figs. 4b and c), which demonstrated that PI-3K activation also plays an important role in nickel-induced VEGF expression in Cl41 cells. It has been reported mTOR, one of the



(a)



(b)



(c)

Fig. 4. PI-3K activation and its role in VEGF induction by nickel compounds. A549 cells or Cl 41 VEGF mass1 cells were seeded into 100-mm dishes or each well of 96-well plates, respectively. After the cell density reached 80–90%, (a) A549 cells were exposed to 1 mM $NiCl_2$ for 20 min. PI-3K activity was measured as described in the section on "Materials and methods," (b) and (c) Cl 41 VEGF mass1 cells were first pre-treated with PI-3K inhibitors, and then exposed to 1 mM $NiCl_2$ or 1 $\mu g/cm^2$ Ni_3S_2 for 24 h, (d) and (e) Cl 41 VEGF mass1 cells were first pre-treated with rapamycin, then exposed to 1 mM $NiCl_2$ (d) or 1 $\mu g/cm^2$ Ni_3S_2 (e) for 24 h. The luciferase activity was measured using Promega Luciferase assay reagent with a luminometer (Wallac 1420 Victor2 multipliable counter system. The results are expressed as VEGF induction relative to medium control. Each bar indicates the mean and standard deviation of triplicate assay wells. The asterisk (*) indicates a significant decrease from nickel control ($p < 0.05$).

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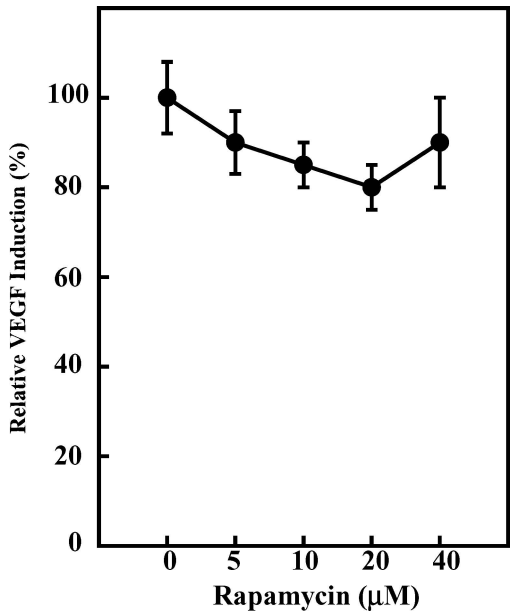
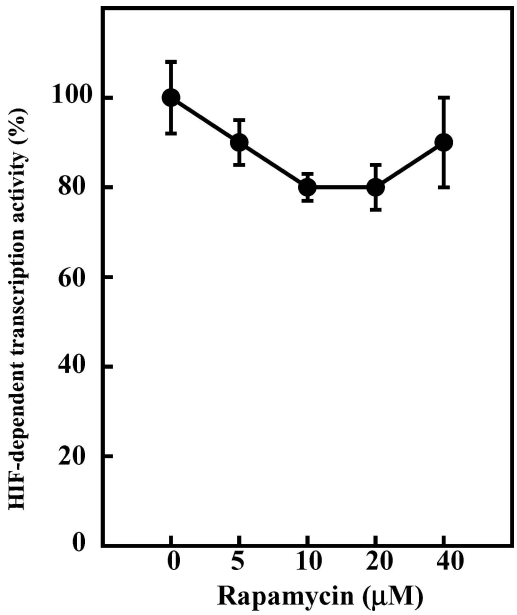


(d)



(e)

Fig. 4. (*Continued*)

downstream signal molecules of PI-3K, could induce VEGF expression in mouse embryonic fibroblasts [28], but nonetheless, mTOR did not appear to be involved in VEGF induction by nickel because pre-treatment of Cl 41 cells with its chemical inhibitor, rapamycin, did not show significant reduction in the induction of VEGF by both $NiCl_2$ and Ni_3S_2 (Figs. 4d and e).

Discussion

Available epidemiological data demonstrated that exposure to nickel compounds caused a variety of pathologic effects, including immunological sensitization, epithelial dysplasia, asthma, fibrosis and lung cancer [1]. The exact mechanisms of these pathologic effects induced by nickel are not fully understood. These mechanisms are likely to involve alteration of signal pathways, and modulation of genes expression, and chromatin remodeling.

Vascular endothelial growth factor (VEGF), a key player of angiogenesis, has been reported to be induced at the early stages of tumor formation in several types of experimental models, such as the RIP1-Tag2 pancreatic β-cell islet carcinoma in transgenic mice [29, 30]. Inhibition of VEGF expression and its receptor function dramatically decreases the tumor growth, invasion, and metastasis in animal models [31]. VEGF expression can be induced by low oxygen tension, growth factors, and some metals including nickel [4, 11–12], but the precise mechanisms underlying VEGF regulation by different stimulation are not fully explored. It seems that signal pathways involved in VEGF induction are dependent on the type of cells and stimuli. HIF-1α is key mediator of VEGF induction under hypoxia condition. It has been reported that inhibition MEK1/2 by the specific chemical inhibitor, PD98059, blocks ERKs-induced VEGF expression in CCL39 fibroblasts and Ras-transformed fibroblasts [32, 33]. PI-3K inhibitor LY294002 was able to strongly suppressed VEGF expression in Ras-transformed epithelial cells, but not Ras-transformed fibroblasts [33]. Our present study indicated that VEGF expression can be induced by $NiCl_2$, Ni_3S_2 (Fig. 1a, and b) and NiS in a dose- and time-dependent manner in Cl 41 cells. The induction was significantly impaired by pre-treatment of Cl 41 cells with MEK/ERKs inhibitor, PD98059 (Figs. 2a and b), cytosolic calcium chelator, BAPTA-AM (Figs. 3a and b) and PI-3K inhibitors, Wortammin and LY294002 (Figs. 4a and b). In contrast, the p38K inhibitor (Figs. 2a and b), SB202190, and extracellular calcium influx blocker (Figs. 3a and b), nifedipine, didn't show any inhibitory effect. These results demonstrated that PI-3K, MEK1/ERKs, and calcium signal are involved in VEGF induction by nickel compounds in Cl 41 cells.

mTOR is the downstream molecule of PI-3K signal cascade. mTOR seems not be involved in regulating VEGF induction by nickel compounds in Cl 41 cells because its inhibitor, rapamycin, did not show obvious inhibitory effect (Figs. 4d and e). MAP kinase families (JNKs, p38K, and ERKs) have been reported to be activated by nickel

compounds [19, 20]. ERKs can modulate HIF-1 or HIF-2 activity and VEGF expression in several cell types [34], and JNK is required for hypoxia-induced AP-1 activation [35]. p38 kinase has been previously characterized as a proapoptotic kinase. Recent studies demonstrated that p38 kinase may play a role in regulation of tumorigenesis through key cellular growth-control mechanisms [36]. Some new data suggested that p38 kinase may be a tumor suppressor with critical contributions in the negative regulation of cell cycle progression. In this study, we found that pre-treatment of Cl 41 cells with p38 kinase inhibitor, SB202190, enhanced VEGF expression induced by Ni_3S_2. It will be of interest to investigate how p38 negatively regulate VEGF expression and if the down-regulation of VEGF attributes to the tumor suppressor function of p38 kinase.

Calcium signal is one of the most important second message. Calcium signals have been implicated in a vast array of cellular functions ranging from short-term responses, such as contraction and secretion, to longer-term control of transcription, cell division and cell death. In most cells, the generation of cytosolic calcium signals is complex and involves two interdependent and closely coupled components: the release of calcium from stores in the endoplasmic reticulum and influx of extracellular calcium [37]. Previous studies have shown that hypoxia was able to elevate intracellular calcium independent on extracellular calcium influx in human endothelial cells, suggesting that intracellular calcium store release is important for hypoxia signalling [38]. Actually, Metzen *et al.* reported that intracellular calcium store release, but not extracellular calcium influx, is involved in hypoxia-induced VEGF expression [39]. Consistent with these reporters, we also found that intracellular calcium, but not extracellular calcium influx, is essential for VEGF induction by nickel compounds.

In summary, we demonstrated that PI-3K, ERKs and cytosolic calcium, but not p38 kinase, play essential roles in VEGF induction by nickel compounds. These data may provide some hints to find some targets for prevention of nickel-induced pathological effects.

Acknowledgments

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