

Involvement of L-Type Ca^{2+} Channel and Toll-Like Receptor-4 in Nickel-Induced Interleukin-8 Gene Expression

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ABSTRACT: The metal nickel (Ni^{2+}) is found everywhere in our daily lives, including coins, costume jewelry, and even nuts and chocolates. Nickel poisoning can cause inflammatory reactions, respiratory diseases, and allergic contact dermatitis. To clarify the mechanism by which nickel induces mediators of inflammation, we used the human acute monocytic leukemia THP-1 cell line as a model. Interleukin (IL)-8 promoter activity as well as gene expression were tested by luciferase assay and real-time polymerase chain reaction. The underlying mechanisms of nickel-induced IL-8 were investigated. We found that nickel induced IL-8 gene expression via the L-type Ca^{2+} channel, Toll-like receptor-4 (TLR-4) and nuclear factor $\text{NF-}\kappa\text{B}$ signal transduction pathways. Nickel activated $\text{NF-}\kappa\text{B}$ expression through extracellular signal-regulated kinase 1/2 phosphorylation and then increased IL-8 expression. Thus, the L-type Ca^{2+} channel and TLR-4 play important roles in nickel-induced inflammatory gene expressions. © 2014 Wiley Periodicals, Inc. *Environ Toxicol* 00: 000–000, 2014.

Keywords: nickel; TLR4; L-type Ca^{2+} channel; human acute monocytic leukemia

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INTRODUCTION

Nickel (Ni^{2+}) is widely used in coins, alloys, electroplating, the manufacture of alkaline batteries, and the chemical industry as a catalyst. Nickel metal trace elements are required for human growth. However, long-term excessive exposure to nickel can lead to nickel poisoning. Nickel compounds are taken up by the human body through ingestion, inhalation, skin contact, and other ways (Costa, 2002; Sivulka, 2005). Allergic contact dermatitis is caused by exposure to a substance such as nickel (Mattila et al., 2001; Nestle et al., 2002; Spiewak et al., 2007; Lidén et al., 2008). Nickel can induce delayed type T cell hypersensitivity and plays an important role in the development of autoimmune thyroiditis (Hybenova et al., 2010). Epidemiological studies demonstrated a significant correlation between nickel exposure and the risk of lung cancer (Andersen et al., 1996; De Palma et al., 2008; Grimsrud and Andersen, 2010). Occupational exposure to nickel compounds occurs in industrial processes, such as in electroplating factories and electrolytic nickel in refining factories, which produce nickel compounds contained in fog, thus increasing the incidence of cancer such as nasal cancer and lung cancer. Therefore, the lung cancer risk in long-term electrolysis workers was estimated to be twenty times higher than the risk in lung general population (Costa, 2002; Kasprzak et al., 2003; Grimsrud and Andersen, 2010), indicating that nickel plays an important role in the initiation and progression of cancer.

Interleukin (IL)-8 is a precursor inflammatory cytokine that has two main functions: neutrophil chemotaxis which causes inflammation and also granulocyte chemotaxis which causes their migration to sites of infection (Baggiolini and Clark-Lewis, 1992). IL-8 in immune-related cells, such as endothelial cells, macrophages, mast cells, and keratinocytes (Köhida and Csaba, 1998), can also induce a series of physiological reactions required for phagocytosis and migration, such as increasing the intracellular concentration of Ca^{2+} (Mahali et al., 2011), exocytosis (Yasuda et al., 2012), and respiratory bursts (Dias et al., 2011). Previous studies indicated that nickel can trigger activation of intracellular reactive oxygen species (ROS) (Oller, 2002; Stejskal et al., 2006; Freitas et al., 2010). IL-8 is a chemotactic agent for neutrophils, the accumulation of which may induce oxidative damage (Baggiolini and Clark-Lewis, 1992).

Occupational exposure to nickel induced DNA methylation that was associated with an increase in H3K4me3 and a decrease in H3K9me2, which then induced changes in global levels of posttranslational histone modifications (Arita et al., 2012). Nickel subsulfide (Ni_3S_2) also induced the methylation of retinoic acid receptor- β 2 (*RAR- β 2*), ras association (RalGDS/AF-6) domain family member 1 (*RASSF1A*), and cyclin-dependent kinase inhibitor 2A (*CDKN2A*) in Wistar

rats, resulting in loss of function or decreases in the *RAR- β 2*, *RASSF1A*, and *CDKN2A* genes (Zhang et al., 2011). Here, we report that nickel regulates IL-8 gene expression through TLR-4, the L-type Ca^{2+} channel, and their downstream extracellular signal-regulated kinase (ERK)1/2 as well as nuclear factor $\text{NF-}\kappa\text{B}$ signal pathways.

MATERIALS AND METHODS

THP-1 Cell Culture

The human acute monocytic leukemia THP-1 cell line was obtained from the Food Industry Research and Development Institute (FIRDI, Taiwan). Cells were cultured (at 37°C, in an atmosphere of 5% CO_2) in RPMI-1640 medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum and 1% penicillin-streptomycin. In this series of experiments, cells were treated with 200 μM NiCl_2 (Sigma, St. Louis, MO) in medium without serum.

Reverse-Transcription Polymerase Chain Reaction

RNA was isolated from THP-1 cells by Trizol (Invitrogen). A reverse-transcriptase reaction was performed on 1 μg of extracted total RNA using a reverse-transcriptase reaction kit (Invitrogen) according to the manufacturer's instructions. Complementary (c)DNA was used to determine the IL-8 gene expression level relative to a housekeeping β -actin gene as described previously (Chou et al., 2011). IL-8 primers (sense, CAGAGACAGCAGAGCACAC and antisense, AGTTCTTTAGCACTCCTTGGC) and β -actin primers (sense, ATCTCCTTCTGCATCCTGTCCGCAAT and antisense, CATGGAGTCCTGGCATCCACGAAAC) were used in the polymerase chain reaction (PCR).

Cell Transfection and Luciferase Assay

Lipofectamine 2000 (Invitrogen) and Opti-MEM medium (Invitrogen) were used to deliver the IL-8 promoter plasmid. IL-8 promoter plasmid was derived from previous study (Lin et al., 2013). Briefly, cells were treated with the drug after overnight transfection, and cells were then harvested using cell lysis buffer. A luciferase reporter assay kit (Promega, Madison, WI) was used to measure the luciferase activity of the reporter gene.

Western Blotting

Proteins (50 μg) from total cells that were lysed in lysis buffer (50 mM Tris-HCl at pH 7.6, 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecylsulfate [SDS], 0.1% deoxycholate, 2 mM EDTA, 2 mM NaF, 2 mM Na_3VO_4 , 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail) were analyzed by SDS-polyacrylamide gel

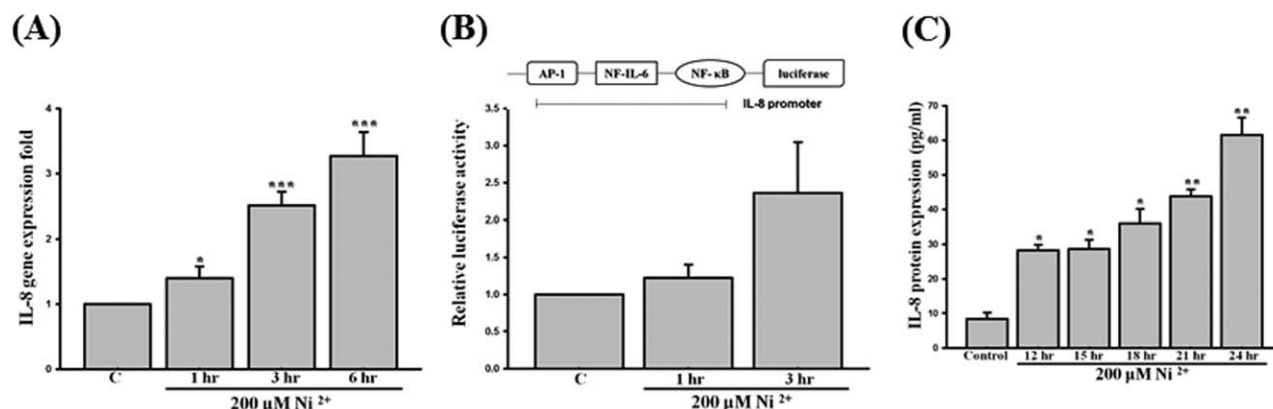


Fig. 1. Ni²⁺ induced IL-8 gene activation in a time-dependent manner. (A) THP-1 cells were stimulated with 200 μM NiCl₂ for 1, 3, and 6 h. Total RNA was extracted to determine IL-8 gene expression. (B) THP-1 cells were transiently transfected with 0.5 μg of the IL-8 promoter plasmid. After 24 h, cells were stimulated with 200 μM NiCl₂ for another 1 and 3 h. The luciferase activity and protein concentration were determined and normalized. (C) THP-1 cells were stimulated with 200 μM NiCl₂ for 12, 15, 18, 21, and 24 h, and the supernatant was collected for an ELISA analysis. Data are expressed as the mean ± SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the control.

electrophoresis on a 10% gel. Antibodies to IL-8 and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to ERK1/2 were diluted 1:1000. Antibodies to β-actin were diluted 1:10⁴. Then membranes were washed with phosphate-buffered saline with tween-20 (PBST) three times and incubated with a 1:6000 dilution of peroxidase-linked anti-mouse or anti-rabbit immunoglobulin G (Amersham Biosciences, UK) for 1 h at room temperature. Protein bands were detected using an enhanced chemiluminescence-plus Western Blot Detection System (Millipore, Bedford, MA).

Data Analysis

An analysis of variance (ANOVA) was applied to test the statistical significance. A p value of <0.05 , denoted by *, was considered statistically significant.

RESULTS

Ni²⁺ Induced IL-8 Gene Activation in a Time-Dependent Manner

A previous study indicated that IL-8 expression can be induced by nickel in human umbilical vein endothelial cells (Schmidt et al., 2010). To learn more about the mechanism by which nickel ions regulate IL-8 gene expression, we treated a human acute monocytic leukemia cell line (THP-1) with 200 μM NiCl₂. As shown in Figure 1, cells treated with 200 μM NiCl₂ showed increased activity of the IL-8 gene [Fig. 1(A)] and its promoter [Fig. 1(B)] in a time-dependent manner. IL-8 protein expression also increased with application of 200 μM NiCl₂ in a time-dependent manner [Fig. 1(C)].

Analysis of the Responsive Regions in the Promoter Area of the IL-8 Gene for Ni²⁺ Stimulation

To identify promoter sites that respond to Ni²⁺, a luciferase IL-8 promoter assay was used. THP-1 cells were transiently transfected with different mutants of IL-8 promoter plasmid. At a concentration of 200 μM, we found that Ni²⁺ evoked IL-8 promoter activation [Fig. 2(A)]. Importantly, IL-8 promoter with NF-κB mutation, leaving intact the NF-IL-6 and activator protein (AP)-1-binding sites, inhibited the response to Ni²⁺ [Fig. 2(B)]. The results suggested that NF-κB is an important transcription factor in Ni²⁺-mediated IL-8 expression.

Effects of NF-κB siRNA in Ni²⁺-Mediated IL-8 Expression

Our promoter activity data implied an important role of NF-κB in IL-8 gene expression. To further confirm to functional role of NF-κB, NF-κB siRNA (small interfering RNA) was used to knock down NF-κB expression. As shown in Figure 3, NF-κB siRNA blocked Ni²⁺-mediated IL-8 gene expression. These results indicated that NF-κB is an essential transcription factor in Ni²⁺-induced IL-8 gene activation.

Effects of ERK1/2 Phosphorylation in Ni²⁺-Induced IL-8 Expression

To identify whether ERK1/2 phosphorylation is involved in nickel-mediated IL-8 expression, THP-1 cells were stimulated with 200 μM NiCl₂, and Western blotting was used to analyze the phosphorylation of ERK1/2. As shown in Figure

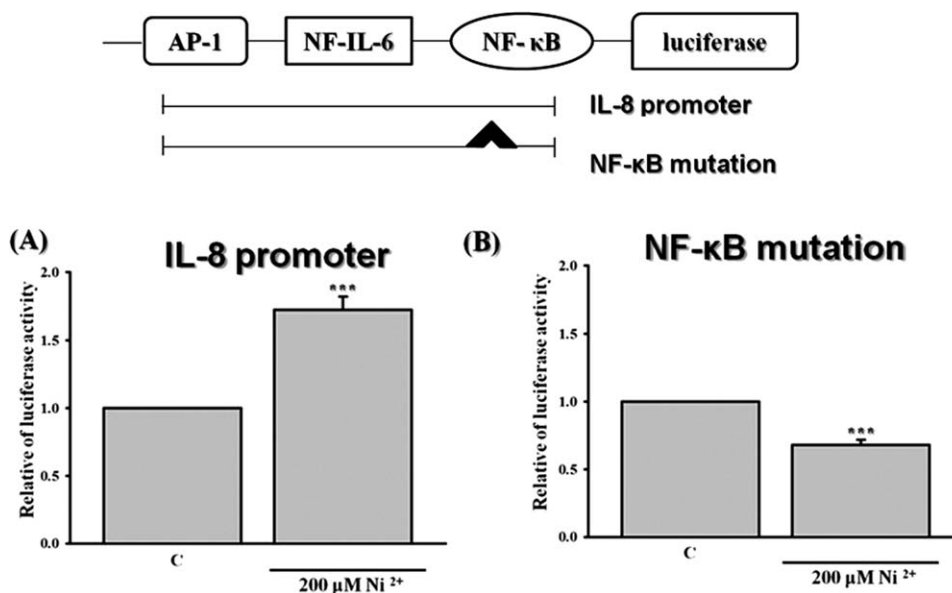


Fig. 2. Analysis of the responsive regions in the promoter area of the IL-8 gene for Ni²⁺ stimulation. (A, B) THP-1 cells were transiently transfected with promoter plasmids (IL-8 promoter and IL-8 promoter (NF-κB mutation)). After 24 h, cells were stimulated with 200 μM NiCl₂ for 3 h. The luciferase activity and protein concentration in cell lysates were determined and normalized. Data are expressed as the mean ± SEM of three independent experiments. *** $p < 0.001$ compared to the control.

4(A), ERK1/2 phosphorylation was induced by Ni²⁺ [Fig. 4(A)]. Importantly, the mitogen-activated protein kinase (MEK) inhibitor, PD98059, which is widely used to block ERK1/2 activation, suppressed IL-8 gene expression [Fig. 4(B)]. To further confirm whether ERK1/2 is essential for Ni²⁺-mediated NF-κB activation, we used a three-repeat NF-κB-binding site reporter as a reporter. As shown in Fig-

ure 4(C), pretreatment of cells with PD98059 blocked NF-κB activation induced by Ni²⁺. The results indicated that ERK1/2 is involved in Ni²⁺-induced IL-8 gene activation.

The Role of Membrane Targets in Ni²⁺-Induced IL-8 Gene Activation

To clarify the role of membrane targets in Ni²⁺-induced IL-8 gene activation, the store-operated calcium (SOC) channel inhibitor, 2-aminoethyl diphenyl borate (2-APB), the L-type Ca²⁺ channel inhibitor, nifedipine, and the TLR-4 inhibitor, CLI-095, were used in this study. We found that the SOC channel inhibitor, 2-APB (at 20 and 100 μM), did not inhibit Ni²⁺-induced IL-8 gene expression [Fig. 5(A)]. However, the L-type Ca²⁺ channel inhibitor, nifedipine (50 μM), and the TLR-4 inhibitor, CLI-095 (10 nM), significantly inhibited Ni²⁺-induced IL-8 gene expression [Fig. 5(B)]. In addition, both the TLR-4 and L-type Ca²⁺ channel inhibitors reduced Ni²⁺ mediated ERK1/2 phosphorylation [Fig. 5(C)]. These observations indicated that Ni²⁺ increased IL-8 expression mainly through the L-type Ca²⁺ channel and TLR-4 to stimulate ERK1/2 phosphorylation.

DISCUSSION

Several lines of evidence indicated that nickel induces inflammation and increases generation of intracellular ROS by cancer cells (Oller, 2002; Stejskal et al., 2006; Freitas et al., 2010). IL-8 is an important proinflammatory cytokine that induces a series of physiological responses required for

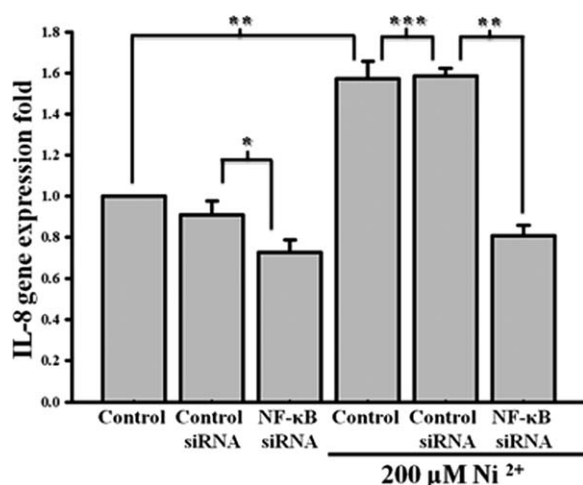


Fig. 3. Effects of NF-κB siRNA in Ni²⁺-mediated IL-8 expression. THP-1 cells were transfected by 10 nM NF-κB siRNA or control siRNA for 24 h. Then, cells were stimulated by 200 μM NiCl₂ for 3 h. Total RNA was extracted to determine IL-8 gene expression. Data are expressed as the mean ± SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

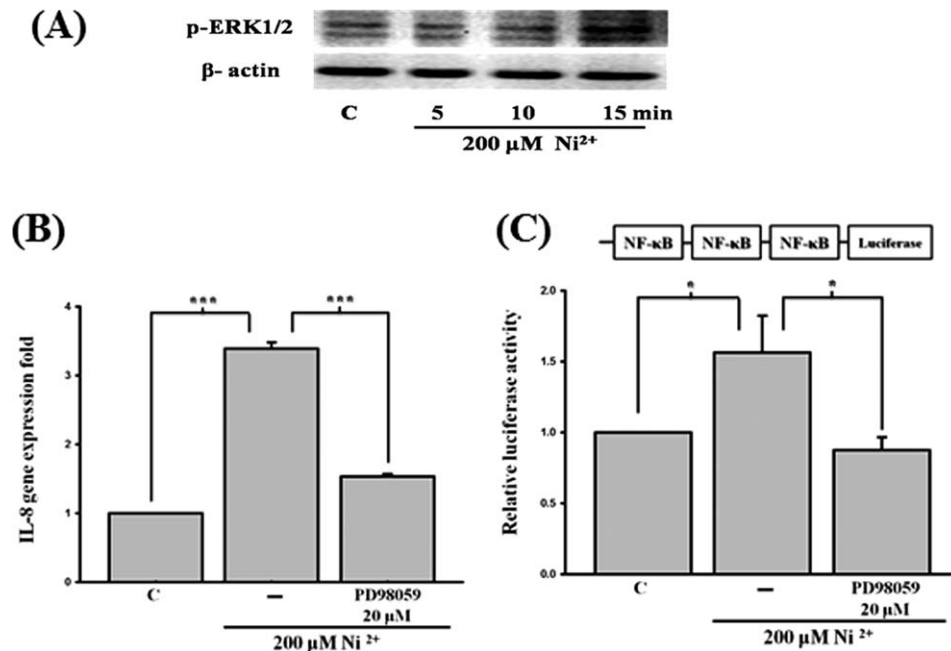


Fig. 4. Effects of ERK1/2 phosphorylation in Ni²⁺-induced IL-8 expression. (A) THP-1 cells were stimulated with 200 μM NiCl₂ for 5, 10, and 15 min. Cell lysates were collected for Western blot analysis. (B) THP-1 cells were pretreated with a MEK kinase inhibitor (PD98059) 20 μM for 30 min, then, stimulated with 200 μM NiCl₂ for 3 h. Total RNA was extracted to determine IL-8 gene expression by using RT-PCR. (C) THP-1 cells were transfected with three repeated NF-κB promoter. After 24 h, cells were pretreated with a MEK inhibitor (PD98059) 20 μM for 30 min, then stimulated with 200 μM NiCl₂ for 3 h. The luciferase activity and protein concentration in cell lysates were determined and normalized. Data are expressed as the mean ± S.E.M. of three independent experiments. * $p < 0.05$, *** $p < 0.001$ compared to the control.

phagocytosis and migration (van der Plas et al., 2009; Liapis et al., 2011; Paunel-Gorgulu et al., 2011). In our study, nickel can induce IL-8 gene expression by activating the ERK1/2 as well as NFκB signal transduction. L-type Ca²⁺ channel and TLR4 are important elements in the plasma membrane responsible for transmitting nickel signals to downstream inflammatory pathways (Fig. 6). Interestingly, divalent cation such as Pb²⁺ triggered downstream inflammatory gene activation through different mechanisms. IL-8 also can be induced by Pb²⁺ in human gastric carcinoma cell through the activation of transcription factor AP1 and ERK1/2 pathways (Lin et al., 2013). In addition, epidermal growth factor receptor (EGFR) is an important element in the plasma membrane responsible for transmitting Pb²⁺ signals to trigger downstream inflammatory cascades (Chang et al., 2011). Pb²⁺-mediated reduction of DNMT3a methylation was reported to involve in inflammatory gene activation (Tsai et al., 2014). The mechanisms by which divalent cations (Pb²⁺ and Ni²⁺) enter cells by different routes are unclear. Further studies are needed to address this question.

Daddaoua et al. (2013) reported that activation of IL-8 depended on c-Jun N-terminal kinase and NF-κB activation in intestinal epithelial HT29 cells. Indeed, NF-κB is an important transcription factor that regulates IL-8 gene expression. In uterine cell types, an NF-κB-binding site was reported to be necessary for upregulation of IL-8 gene

expression (Elliott et al., 2001). Combined with the results from NF-κB siRNA and a specific NF-κB inhibitor, we also noted that Ni²⁺ induced IL-8 gene expression via the transcription factor, NF-κB. However, mechanisms by which NF-κB activation is regulated are diverse. Several reports indicated that ERK1/2 is involved in activating NF-κB and IL-8 (Maulik et al., 2008; Zenali et al., 2009). Consistent with this, we observed that ERK1/2 phosphorylation was essential for nickel-mediated IL-8 expression.

The key cell-surface target through which nickels transmits its signal to trigger inflammatory reactions is still unclear. Both the calcium channel and TLR-4 were proposed (Blackburn and Highsmith, 1990; Funakoshi et al., 1997; Thompson et al., 2000; Schmidt et al., 2010). Considering the concept of metal ion mimicry, calcium channels are very likely to be transducers of nickel. Indeed, calcium channels have been considered as important mediators of inflammatory gene activation (Huang et al., 2011; Wang et al., 2012; Yang et al., 2013; Guo et al., 2013). In addition, pretreatment with the calcium channel blockers, verapamil and nifedipine, decreased nickel uptake by 20% (Funakoshi et al., 1997). Previous study also showed that nickel elicited contact hypersensitivity via human TLR4 (Schmidt et al., 2010). Our results reported that both the L-type Ca²⁺ channel inhibitor and the TLR-4 inhibitor blocked nickel-mediated IL-8 gene expression. Therefore, the L-type Ca²⁺

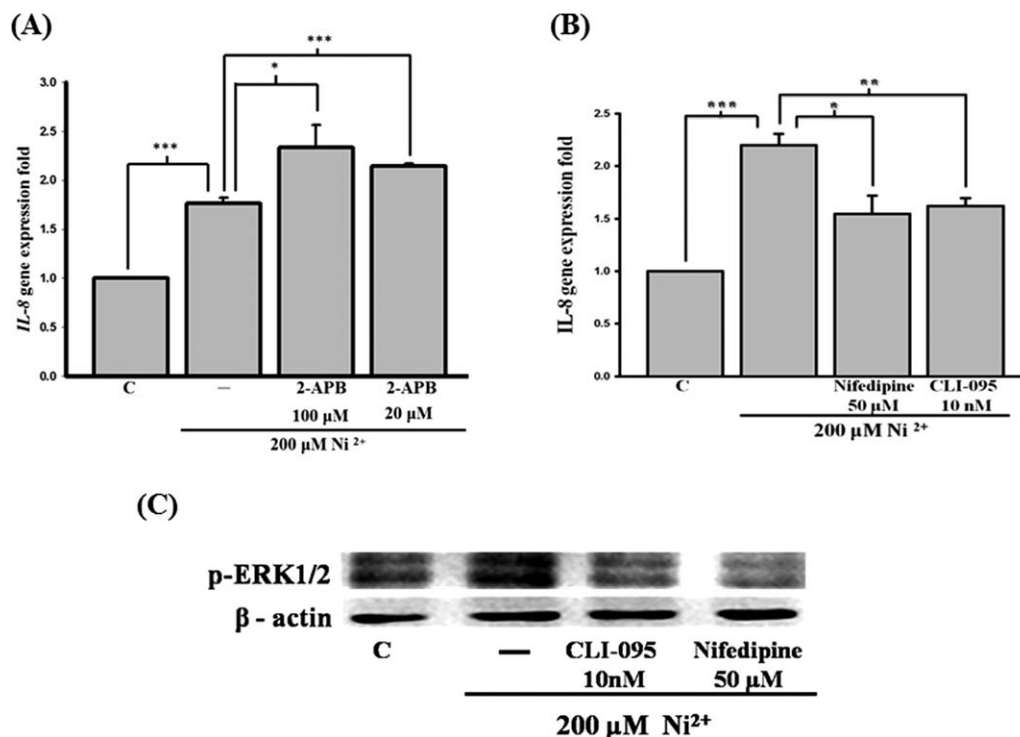


Fig. 5. The role of membrane receptors in Ni^{2+} -induced IL-8 gene activation. (A) THP-1 cells were pretreated a SOC channel inhibitor, 2-APB (20 μ M and 100 μ M) for 10 min, then stimulated with 200 μ M NiCl_2 for 3 h. Total RNA was extracted to determine IL-8 gene expression. (B) THP-1 cells were pretreated by L-type Ca^{2+} channel inhibitor, nifedipine (50 μ M) or a TLR-4 inhibitor, CLI-095 (10 nM) for 30 min, then stimulated with 200 μ M NiCl_2 for 3 h. Total RNA was extracted to determine IL-8 gene expression. Data are expressed as the mean $\pm$ SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (C) THP-1 cells were pretreated with a TLR-4 inhibitor, CLI-095 (10 nM) or L-type Ca^{2+} channel inhibitor, nifedipine (50 μ M) for 30 min, then stimulated with 200 μ M NiCl_2 . Cell lysates were collected for ERK1/2 and β -actin detection by Western blot analysis.

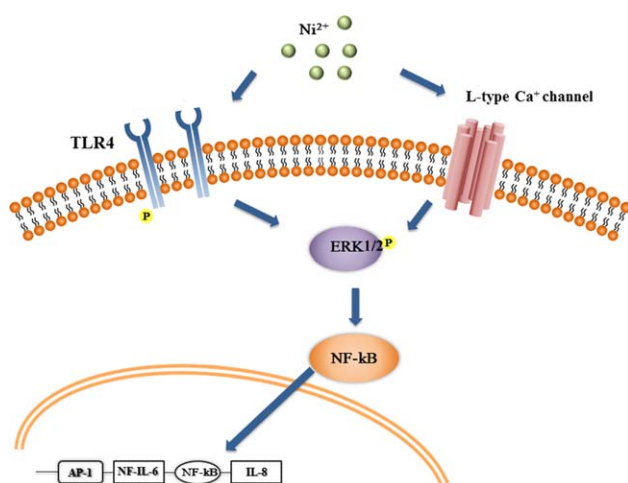


Fig. 6. Schematic representation of Ni^{2+} -mediated IL-8 gene action. Stimulation of THP-1 cells with Ni^{2+} via plasma membrane L-type Ca^{2+} channels and TLR-4 induced ERK1/2 phosphorylation and activates NF- κ B expression, which in turn triggered IL-8 gene activity. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://www.interscience.wiley.com).]

channel and TLR-4 are both needed for nickel-mediated IL-8 activation, at least in THP-1 cells. Whereas previous studies considered these signals to function independently, our studies reflect the importance of both the L-type Ca^{2+} channel and TLR-4 in responding to nickel stimulation. Further studies are necessary to understand whether the L-type Ca^{2+} channel and TLR-4 activation are mediated via other intrinsic pathways.

In summary, this study demonstrated that nickel mainly regulates IL-8 gene expression through TLR-4 and the L-type Ca^{2+} channel and the downstream ERK1/2-NF- κ B signaling pathways. Understanding the mechanisms between the cell-surface target and inflammatory gene activation may be helpful in identifying novel therapeutic targets for treating diseases caused by nickel toxicity.

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The authors declare that there is no conflict of interests regarding the publication of this paper.

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