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Hydrogen Peroxide Mediates Activation of Nuclear Factor of Activated T Cells (NFAT) by Nickel Subsulfide

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Abstract

Nickel compounds induce cell transformation in cell culture models and tumor formation in experimental animals. However, the molecular mechanisms by which nickel compounds induce tumors are not yet well understood. The present study found that exposure of cells to either Ni_3S_2 or $NiCl_2$ could result in specific transactivation of nuclear factor of activated T cells (NFAT), although it did not show any activation of p53 or AP-1. Furthermore, nickel compounds were also able to cause generation of reactive oxygen species (ROS). The scavenging of nickel-induced H_2O_2 with *N*-acety-L-cyteine (a general antioxidant) or catalase, or the chelation of nickel with deferoxamine, resulted in inhibition of NFAT activation. In contrast, pretreatment of cells with sodium formate (an ·OH radical scavenger) or superoxide dismutase (an $O_2^{\cdot-}$ radical scavenger) did not show any inhibitory effects. These results demonstrate that nickel compounds are able to induce NFAT activation, and that the mechanism of NFAT activation seems to be mediated by the generation of H_2O_2 by these metal compounds. This study should help us understand the signal transduction pathways involved in carcinogenic effects of these nickel compounds.

Introduction

Occupational exposure to nickel compounds frequently occurs in industrial processing (1, 2). Aerosols of nickel salts can be generated in electroplating and electrolysis areas of nickel refineries (1, 2). Nickel concentrations, as high as 0.2 mg/m^3 have been reported in these occupational environments (1, 2). Ambient exposure levels exceeding 1mg/m^3 have been reported in pyrometallurgical refining processes relating to exposure to nickel oxides, nickel sulfides, and nickel powder (1, 2). In addition, the release of nickel into the environment represents a potential for nonoccupational exposure (2). The average daily exposure to nickel by inhalation has been estimated to be as high as 0.2 and 0.4 g for rural and urban dwellers, respectively (3). The routes for nickel uptake include inhalation, ingestion, and dermal penetration (1, 2). Epidemiological studies have implicated occupational exposure to nickel compounds in the elevated incidence of some human cancers, such as lung and nasal (1, 2) cancer. The carcinogenic effects of nickel compounds have been supported by the findings that these compounds transform many human and rodent cells in cell culture models (4–6). Numerous studies have confirmed the carcinogenic potency of nickel compounds in experimental animal models (7). For example, a single injection of Ni_3S_2 can induce essentially a 100% incidence of tumor at the site of injection (8). Thus, nickel compounds are a somewhat unique class of carcinogens. However, the molecular mechanisms by which nickel cause cancers are not well understood, although several hypotheses have been described in previous studies (1–8).

A NFAT[2] was originally described as a transcription factor expressed in activated but not resting T cells (9, 10). The induction of NFAT in T cells required a calcium-activated signaling pathway and was blocked by CsA and FK506 (10). Growing evidence indicates that NFAT is not only a T cell-specific transcriptional factor, but also is expressed in a variety of lymphoid cells and in nonlymphoid tissue (10, 11). It has been reported that $NFAT_1$ and $NFAT_2$ mRNAs have been detected in brain, heart, skeletal muscle, testis, placenta, pancreas, small intestine, prostate, colon, and skin tumors as well as in lung (10). NFAT expression or NFAT-derived transactivation has also been described in several types of nonlymphoid cells, including mast, endothelial, neuronal, vascular smooth muscle, and liver-derived Chang cells (10).

Because previous studies have demonstrated that treatment of cells with nickel compounds could result in an increase of free intracellular calcium (12), which is an essential signal for NFAT activation, it is reasonable to associate the NFAT activation with carcinogenic effects of nickel compounds. Therefore, the present study investigated the NFAT activation and involvement of ROS-calcium-calcineurin pathway in NFAT transactivation by nickel compounds.

Materials and Methods

Plasmids and Reagents. CMV-neo vector plasmid and NFAT-luciferase reporter plasmid were constructed as described previously (9, 11, 13–15). $NiCl_2$ was purchased from Aldrich (Milwaukee, WI); Ni_3S_2 was obtained from INCO (Toronto, Canada); FBS, BAPTA-AM, and thapsigargin were purchased from Biomol (Plymouth Meeting, PA); nifedipine was from Calbiochem (La Jolla, CA); MEM and DMEM were from BioWhittaker (Walkersville, MD). Catalase, DMPO, NAC, NADPH, SOD, and sodium formate were purchased from Sigma Chemical Co. (St. Louis, MO); LipofectAMINE was from Life Technologies, Inc.; A23187, ionomycin, as well as CsA were purchased from Alexis Biochemicals (San Diego, CA).

Cell Culture. Mouse embryo fibroblast PW cells and its transfectants, PW NFAT mass1 and PW PG13 mass1, were cultured in DMEM with 10% FBS, 2 mM L-glutamine, and 25 μg of gentamicin/ml.

Generation of Stable Cotransfectants. PW cells were cultured in a six-well plate until they reached 85–90% confluence. One μg of CMV-neo vector and 15 μl of LipofectAMINE reagent, with 12 μg of NFAT-luciferase reporter plasmid DNA or PG-13-luciferase reporter plasmid DNA, 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

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[2] The abbreviations used are: NFAT, nuclear factor of activated T cells; CsA, cyclosporin A; ROS, reactive oxygen species; FBS, fetal bovine serum; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; NAC, ; SOD, superoxide dismutase; CMV, cytomegalovirus; ESR, electron spin resonance; AP-1, activated protein-1; NFκB, nuclear factor κB; TKF-α, tumor necrosis factor α; IL, interleukin.

G418 selection (800 μg/ml). The stable transfectants were identified by measuring basal level of luciferase activity. Stable transfectants, PW NFAT mass1 and PW PG13 mass1, were established and cultured in G418-free MEM for at least two passages before each experiment.

Assay for Transactivation of NFAT or p53. Confluent monolayers of PW NFAT mass1 or PW PG13 mass1 were trypsinized, and 5×10^3 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 $NiCl_2$, Ni_3S_2, or vanadate for the indicated times and dosages. The cells were extracted with lysis buffer, and luciferase activity was measured as described previously (16). The results were expressed as NFAT activity relative to untreated controls or p53 activity relative to untreated control (11, 17).

ESR Measurements. ESR spin trapping was used to detect short-lived free radical intermediates. This technique involves the addition-type reaction of a short-lived radical with a diamagnetic compound (spin trap) to form a relatively long-lived free radical product, the so-called spin adduct, which can be observed by conventional ESR. The intensity of the spin adduct signal corresponds to the amount of short-lived radicals trapped, and the hyperfine splittings of the spin adduct are generally characteristic of the trapped radical. ESR measurements were carried out using a Varian E9 ESR spectrometer and a flat-cell assembly. Hyperfine couplings were measured (0.1 Gauss) directly from magnetic field separation using potassium tetraperoxochromate (K_3CrO_8) and 1,1-diphenyl-2-picrylhydrazyl as reference standards. PW cells (1×10^6) were mixed with 100 mM DMPO, 100 μM NADPH, and $NiCl_2$ or Ni_3S_2. The reaction mixture was then transferred to a flat cell for ESR measurement as described previously (18).

Results

Induction of NFAT Transactivation in PW Cells by $NiCl_2$ or Ni_3S_2. To study the regulation of NFAT transcription activity by nickel compounds in cells, we generated a NFAT-luciferase reporter stable transfectant, PW NFAT mass1, by cotransfecting the NFAT-luciferase reporter plasmid and CMV-neo plasmid into mouse fibroblast cells. The results observed from this stable transfectant show that both compounds, $NiCl_2$ or Ni_3S_2 particulate, could induce marked activation of NFAT ($P < 0.05$; Fig. 1*A*). This activation seems to be time- and dose-dependent (Fig. 1, *B* and *C*). The maximum induction of NFAT activity by Ni_3S_2 occurred between 24 and 36 h after cells were exposed to Ni_3S_2 (Fig. 1*B*). To test whether Ni_3S_2-induced NFAT activation is transcription factor specific, we also exposed the p53-luciferase reporter stable transfectors, PW PG13 mass1, to Ni_3S_2. The results show that Ni_3S_2 induced only NFAT activity ($P < 0.05$) but not p53 activity ($P > 0.05$) in PW cells, whereas vanadate significantly induced activation of both NFAT and p53 in PW cells ($P < 0.05$; Fig. 2*A*). These findings were confirmed additionally by time-course and dose-response studies (Fig. 2, *B* and *C*). Results from using mouse 3T3 fibroblasts, mouse epidermal Cl 41 cells, and human B82 fibroblasts also showed that neither Ni_3S_2 nor $NiCl_2$ had any observed activation of transcription factors AP-1, p53, retinoic acid response element, or glucocorticoid response element (data not shown). These results demonstrate that nickel compounds are relative specific stimuli for NFAT transactivation.

Synergistic Effect of Thapsigargin on NFAT Activation Induced by $NiCl_2$ or Ni_3S_2 Thapsigargin is a cell-permeant chemical that induces the release of intracellular stored Ca^{2+} without hydrolysis of inositol-phospholipids via inhibition of endoplasmic reticulum Ca^{2+}-ATPase (19, 20). To study the role of intracellular Ca^{2+} in the activation of NFAT by nickel compounds, the effects of thapsigargin on NFAT activation induced by $NiCl_2$ or Ni_3S_2 were tested. The results showed that thapsigargin had significant synergistic effects on NFAT activation induced by $NiCl_2$ or Ni_3S_2 ($P < 0.05$; Fig. 3*A*), whereas this agent itself only had a slight effect on NFAT activation (data not shown). Previous studies have demonstrated that A23187 exhibited a synergistic effect with UV radiation or 12-*O*-tetradecanoylphorbol-13-acetate for NFAT induction (9, 11). To test the effect of the Ca^{2+} ionophore on NFAT activation induced by $NiCl_2$ or Ni_3S_2 in PW cells, we incubated PW NFAT mass1 with either A23187 or ionomycin. The results showed that both A23187 and ionomycin exhibited a synergistic augmentation of $NiCl_2$- or Ni_3S_2-

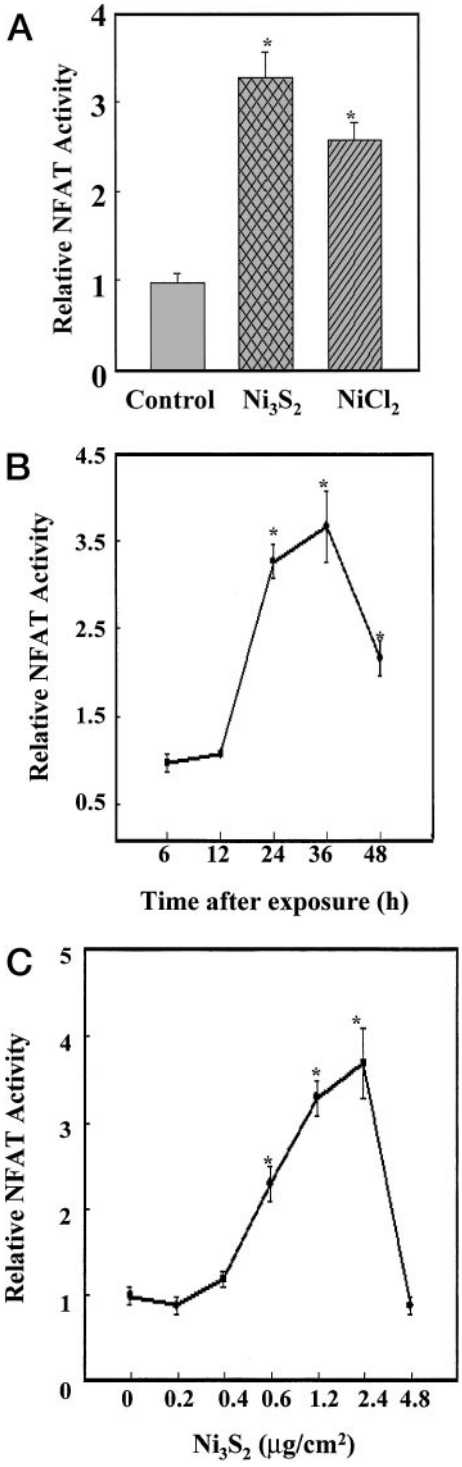

Fig. 1. Induction of NFAT-dependent transcription by Ni_3S_2 or $NiCl_2$ in PW cells. Cells (8×10^3) of PW NFAT mass1 were seeded into each well of 96-well plates. After being cultured at 37°C overnight, the cells were treated with 2 μg/cm^2 of Ni_3S_2 (8 μg/ml) or 1 mM of $NiCl_2$ for 24 h (*A*). *B*, for a time course study, the cells were exposed to 2 μg/cm^2 of Ni_3S_2 (8 μg/ml) 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 24 h; then the luciferase activity was measured. *Bars*, the mean and SD of four repeat assay wells. The results are presented as NFAT-dependent transcription activity relative to control. *, a significant increase from control ($P < 0.05$).

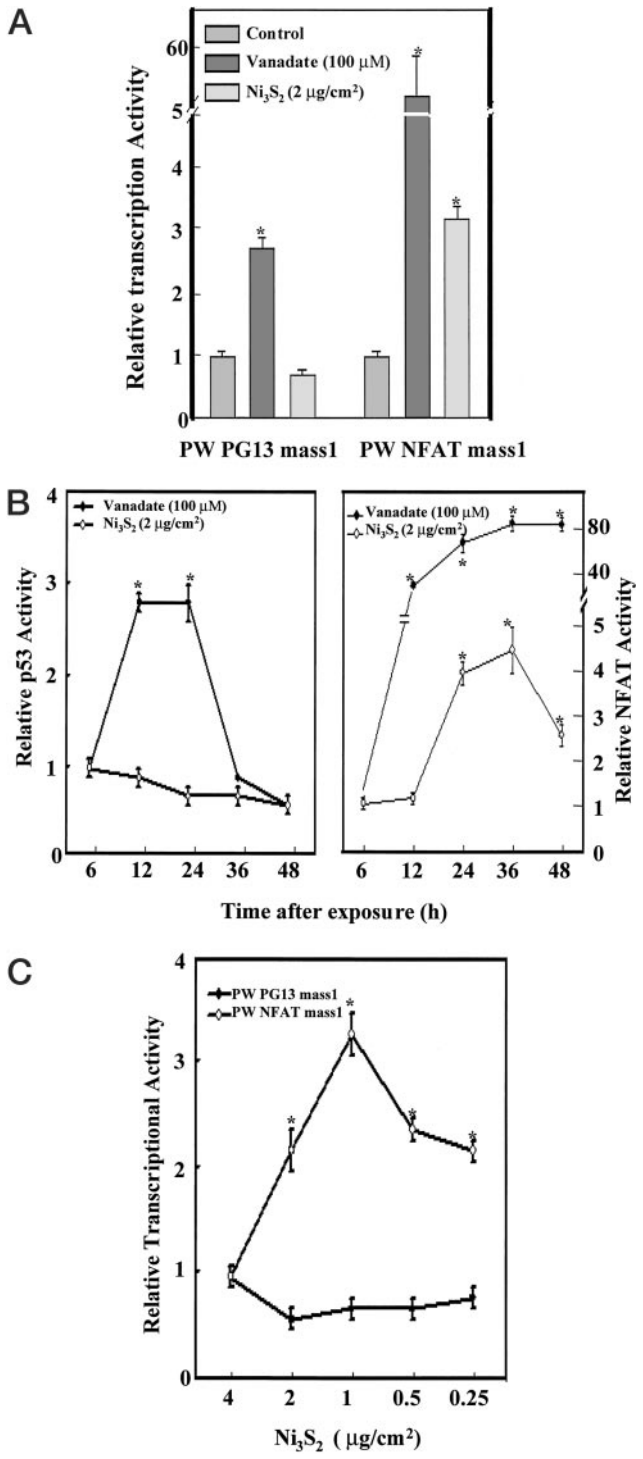

Fig. 2. Exposure of PW cells to Ni_3S_2 or $NiCl_2$ resulted in specific activation of NFAT but not p53. Cells (8×10^3) of PW NFAT mass1 or PW PG13 mass1 were seeded into each well of 96-well plates. After being cultured at 37°C overnight, the cells were treated with 2 μg/cm^2 of Ni_3S_2 (8 μg/ml) or vanadate (100 μM) for 24 h (*A*). *B*, for a time course study, the cells were exposed to 2 μg/cm^2 of Ni_3S_2 (8 μg/ml) or vanadate (100 μM) 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 24 h; then the luciferase activity was measured. *Bars*, the mean and SD of four repeat assay wells. The results are presented as NFAT-dependent or p53-dependent transcription activity relative to control. *, a significant increase from control ($P < 0.05$).

induced NFAT activation (data not shown). The role of intracellular Ca^{2+} and Ca^{2+} mobilization in NFAT activation induced by nickel compounds was confirmed additionally using nifedipine and 1,2-bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra-(acetoxymethyl)ester (BAPTA-AM), a specific Ca^{2+} channel blocker and a specific Ca^{2+} chelator, respectively (12, 21). Pretreatment of cells with either nifedipine or BAPTA-AM resulted in an inhibition of NFAT activation induced by Ni_3S_2 ($P < 0.05$; Fig. 3, *b* and *c*). Similar inhibition was observed using $NiCl_2$ (data not shown). These data demonstrate that elevation of intracellular calcium in PW cells plays an important role in the activation of NFAT in response to $NiCl_2$ or Ni_3S_2, suggesting that activation of NFAT by nickel compounds is through calcium-dependent pathways.

CsA Blocks NFAT Transactivation Induced by $NiCl_2$ or Ni_3S_2. Previous studies have demonstrated that the major NFAT activation pathway seems to involve a calcium/calmodulin-dependent phosphatase, calcineurin (10, 11, 22). To test the role of calcineurin in NFAT-dependent transcription activity induced by $NiCl_2$ or Ni_3S_2 in PW cells, CsA, a widely used pharmacological inhibitor of the phosphatase calcineurin, was used. Pretreatment of cells with CsA resulted in a dramatic inhibition of NFAT transactivation induced by Ni_3S_2 ($P < 0.05$; Fig. 3*d*). These data suggest that activation of calcineurin is required for NFAT activation induced by nickel compounds, and that nickel compounds activate the NFAT transcription activity in mouse embryo fibroblasts through a pathway similar to that in T cells.

Generation of ROS in PW Cells by $NiCl_2$ or Ni_3S_2. If ROS play some role in NFAT activation induced by $NiCl_2$ or Ni_3S_2, ROS should be generated after cells were exposed to $NiCl_2$ or Ni_3S_2. To test this possibility, $^{\cdot}OH$ generation was determined in PW cells exposed to $NiCl_2$ or Ni_3S_2 by ESR. PW cells (1×10^6) were mixed with 100 mM DMPO and 10 μg of Ni_3S_2 or 2 mM of $NiCl_2$ with or without antioxidants. The reaction mixtures were then transferred to a flat cell for ESR measurement as described previously (23). The data show that PW cells alone did not generate any detectable amount of free radicals (Fig. 4*A*), whereas PW cells exposed to $NiCl_2$ or Ni_3S_2 generated a strong ESR signal (Fig. 4*A*). The spectrum consists of a 1:2:2:1 quartet with hyperfine splitting of $a_H = a_N = 14.9$ Gauss, where a_N and a_H denote hyperfine splittings of the nitroxyl nitrogens and α-hydrogen, respectively. On the basis of these splittings and the 1:2:2:1 line shape, the spectrum was assigned to the DMPO-OH adduct, which is evidence of $^{\cdot}OH$ radical generation. This notion was supported further by the data from using ROS scavengers (Fig. 4*B*). Addition of SOD or catalase inhibited $^{\cdot}OH$ radical generation, indicating that $O_2^{\cdot-}$ and H_2O_2 were produced in the nickel-treated cells and involved in $^{\cdot}OH$ generation (Fig. 4*B*). Addition of sodium formate or ethanol, two $^{\cdot}OH$ radical scavengers, decreased the signal intensity (Fig. 4*B*). It may be noted that DMPO is a $^{\cdot}OH$ spin-trapping agent and is also a good $^{\cdot}OH$ scavenger. Sodium formate or ethanol reacted with $^{\cdot}OH$ in competition with DMPO. The intensity of the DMPO-OH signal depended on the reaction constants of sodium formate or ethanol and DMPO toward $^{\cdot}OH$ as well as their relative concentrations. Because the reaction rate of sodium formate with $^{\cdot}OH$ is comparable with that of DMPO, and the concentrations of sodium formate and DMPO are the same, it is expected that upon addition of sodium formate, the signal intensity of DMPO-OH would decrease by about 50%. It may also be noted that because $^{\cdot}OH$ is very reactive, there is no real specific $^{\cdot}OH$ scavenger available. The scavengers of $^{\cdot}OH$ only mean that their reaction rates with $^{\cdot}OH$ are relatively high. Other methods, such as footprinting, in combination with spin trapping may be used to verify further the $^{\cdot}OH$ generation. Nevertheless, the above results indicate that stimulation of PW cells with nickel compounds generated $^{\cdot}OH$ radicals, and the $O_2^{\cdot-}$ and H_2O_2 were involved in the mechanism of $^{\cdot}OH$ generation induced by nickel compounds.

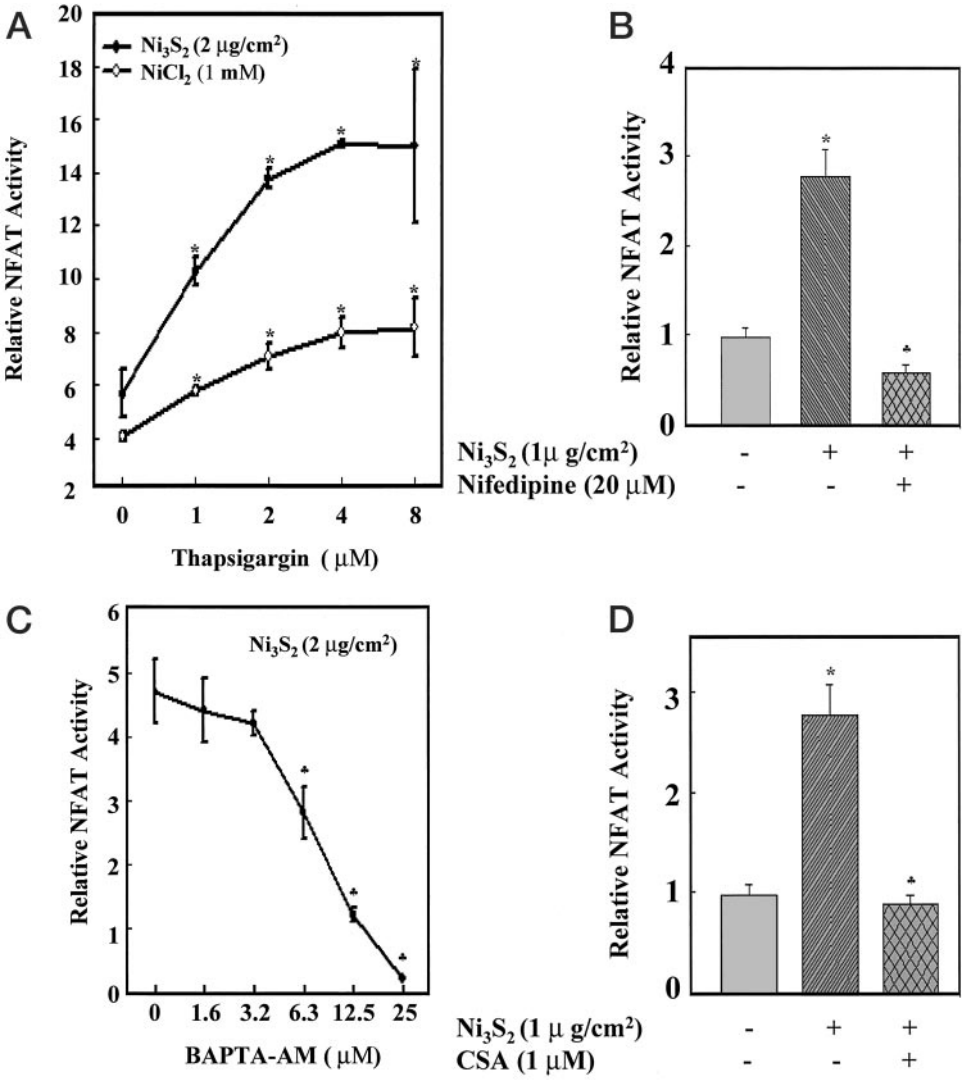

Fig. 3. Effects of thapsigargin, BAPTA-AM, nifedipine, or CsA on Ni_3S_2- or $NiCl_2$-induced NFAT activity. PW NFAT mass1 cells (8×10^3) suspended in 10% FBS DMEM medium were added to each well of 96-well plates. After being cultured at 37°C overnight, the cells were treated with 2 $\mu g/cm^2$ of Ni_3S_2 (8 $\mu g/ml$) or 1 mM of $NiCl_2$ plus different concentration of thapsigargin (*A*), nifedipine (20 μM; *B*); different concentrations of BAPTA-AM (*C*); or CsA (*D*) for 30 min, and sequentially exposed to Ni_3S_2 at the dose indicated. After being cultured for 24–30 h, the cells were harvested, and NFAT activity was measured by the luciferase activity assay. The results are presented as relative NFAT-dependent transcription activity. *Bars*, the mean and SD of assays from triplicate wells. *A*, *, a significant increase from Ni_3S_2 or $NiCl_2$ alone ($P < 0.05$); *B* and *C*, *, a significant increase from medium control alone ($P < 0.05$); ♣, a significant decrease from Ni_3S_2 ($P < 0.05$). *D*, *, a significant increase from medium control alone ($P < 0.05$); ♣, a significant decrease from Ni_3S_2 ($P < 0.05$).

Involvement of ROS in Activation of NFAT Induced by $NiCl_2$ or Ni_3S_2. To investigate the possible role of ROS in NFAT activation by $NiCl_2$ or Ni_3S_2, the effects of specific modifiers of ROS on nickel-induced NFAT activation were determined. The results showed that pretreatment of cells with deferoxamine, NAC, or catalase caused inhibition of $NiCl_2$- or Ni_3S_2-induced NFAT activation (Fig. 4, *C* and *D*), whereas addition of SOD, whose function is to scavenge $O_2^{\cdot-}$, did not show any significant inhibition of $NiCl_2$- or Ni_3S_2-induced NFAT activation, indicating that $O_2^{\cdot-}$ may not be required for nickel compound-induced NFAT activation (Fig. 4, *C* and *D*). In contrast, treatment of cells with sodium formate did not inhibit $NiCl_2$- or Ni_3S_2-induced NFAT activation, suggesting that $^{\cdot}OH$ may not play a significant role in $NiCl_2$- or Ni_3S_2-induced NFAT activation (Fig. 4, *C* and *D*). These data suggest that H_2O_2 generation by $NiCl_2$ or Ni_3S_2 plays a role in the induction of NFAT activity.

Discussion

The antigen-regulated, CsA-sensitive NFAT is not only expressed in lymphoid cells, as once thought, but also is expressed in other cells and organs, such as the skin and lung (10). The stimuli and regulation of NFAT in cells other than lymphoid cells, however, are not well understood. In this study, we investigated the possible activation of NFAT and the involvement of ROS in NFAT activation in cellular response to $NiCl_2$ or Ni_3S_2 in fibroblasts. Exposure of cells to $NiCl_2$ or Ni_3S_2 caused marked increases in NFAT-dependent transcription in a time- and dose-dependent manner. Cotreatment studies show that thapsigargin, A23187, or ionomycin augmented NFAT-mediated transcription synergistically in response to $NiCl_2$ or Ni_3S_2. Pretreatment of cells with nifedipine, BAPTA-AM, or CsA resulted in impairment of NFAT transactivation induced by $NiCl_2$ or Ni_3S_2. These results suggest that nickel compounds are able to induce NFAT activation. Furthermore, nickel compounds were also able to cause generation of ROS. The scavenging of nickel-induced H_2O_2 with NAC, catalase, and deferoxamine resulted in the inhibition of NFAT activation. In contrast, pretreatment of cells with sodium formate or SOD did not show any inhibitory effects. These results demonstrate that nickel compounds are able to induce NFAT activation and that nickel-induced NFAT activation seems to be mediated by H_2O_2 generated by these nickel compounds.

The activation of NFAT in T cells includes dephosphorylation, nuclear translocation, and an increase in affinity for DNA binding (10). Stimuli that elicit calcium mobilization result in rapid dephosphorylation of NFAT proteins and their translocation to the nucleus. These dephosphorylated proteins showed increased affinity for DNA binding (10). In T cells, transactivation of NFAT is regulated tightly in response to elevations of both the intracellular calcium ion (Ca^{2+}) and diacylglycerol after activation of phospholipase C (10). Increased intracellular calcium stimulates the activation of calmodulin (10). It is

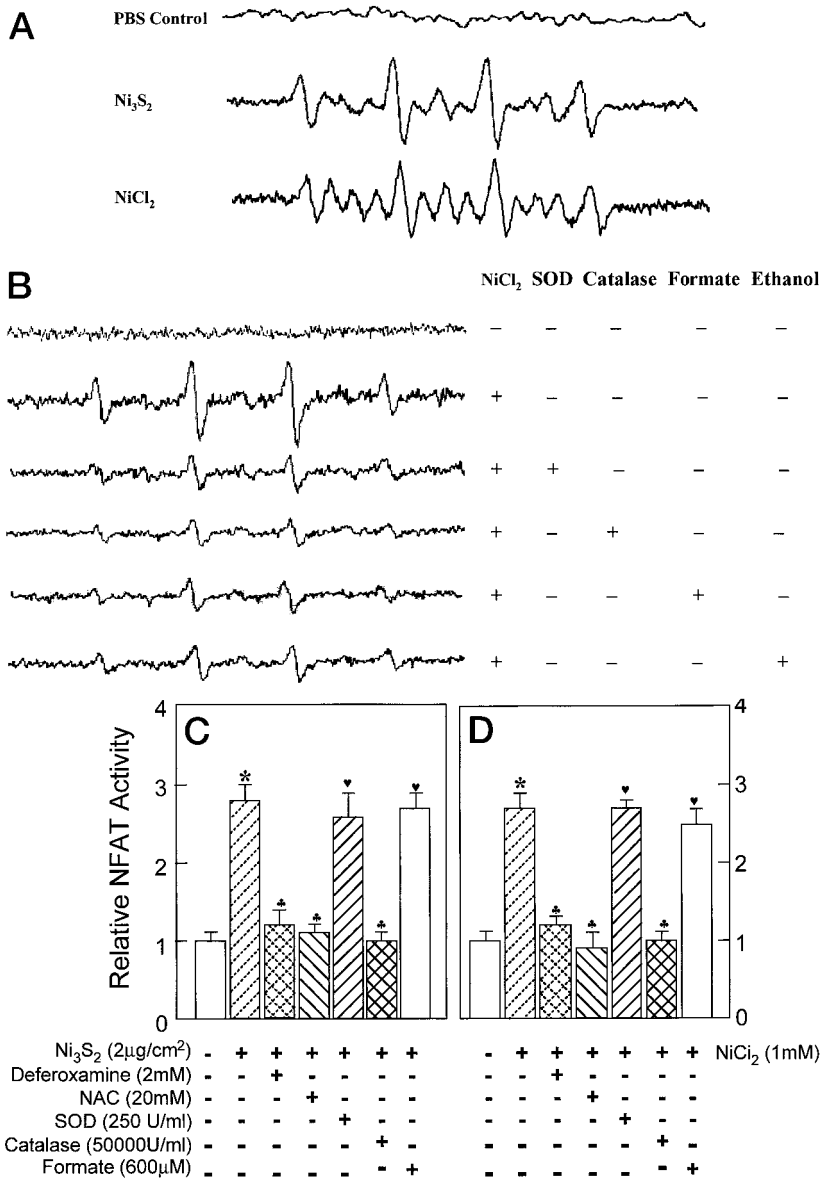

Fig. 4. Nickel-induced ˙OH generation in PW cells by ESR and the effects of free-radical modifiers on $NiCl_2$- or Ni_3S_2-induced NFAT activation. *A*, ESR spectra were recorded 1 h after mixing 1×10^6 PW cells, 100 mM DMPO, and 100 μM NADPH with or without Ni_3S_2 or $NiCl_2$. *B*, ESR spectra were recorded 1 h after mixing 1×10^6 PW cells, 100 mM DMPO, and 2 mM $NiCl_2$ with or without different ROS scavengers as indicated. The final concentrations of these scavengers were: SOD, 5 mg/ml; catalase, 2000 units/ml; or sodium formate, 200 mM; ethanol, 5%. *C* and *D*, PW NFAT mass1 cells suspended in 10% FBS DMEM were added to each well of 96-well plates and cultured overnight. The cells were pretreated with different free-radical modifiers at the concentrations indicated. The cells were then exposed to 2 $\mu g/cm^2$ of Ni_3S_2 (8 μg/ml; *C*) or 1 mM of $NiCl_2$ for 24 h (*D*). The NFAT activity was determined by the luciferase activity assay. The results are presented as relative NFAT activity. Columns and *bars*, the mean and SD from triplicate assays. *, a significant increase from medium control ($P < 0.05$); ♣, a significant decrease from nickel compounds ($P < 0.05$); ♥, no significant difference from nickel compounds ($P > 0.05$).

believed that the binding of calmodulin to a region near the COOH terminus of calcineurin displaces the auto-inhibiting domain and exposes the calcineurin active site (10). Activated calcineurin subsequently dephosphorylates the cytoplasmic NFAT proteins, leading to NFAT nuclear translocation (10, 22, 24). NFAT forms a heteromeric transcriptional coactivator complex with AP-1 that coinduces NFAT-dependent transactivation (10). It has also been reported that phosphorylation of NFAT is regulated by several protein kinases, including glycogen synthase kinase 3 and c-jun NH_2-teminal kinase 2 (10, 25, 26). Other than lymphocytes, NFAT activation has been demonstrated only in a few cell types, such as vascular smooth muscle cells, after induction with platelet-derived growth factor and liver-derived Chang cells after exposure to hepatitis B virus x protein (27, 28). Recently, we reported that UV irradiation resulted in activation of NFAT-mediated transcription through a calcium-dependent pathway in mouse epidermal JB6 cells as well as in mouse skin (11). The results obtained from the present investigation show that $NiCl_2$ or Ni_3S_2 alone could induce an increase in NFAT activity. Thapsigargin, A23187, or ionomycin augmented the NFAT-mediated transcription synergistically in combination with $NiCl_2$ or Ni_3S_2. Pretreatment of cells with BAPTA-AM, nifedipine, or CsA resulted in impairment of NFAT transactivation induced by $NiCl_2$ or Ni_3S_2. Therefore, the $NiCl_2$- or Ni_3S_2-induced NFAT transactivation seems to require an increase in free intracellular calcium and calcineurin activation.

Ca^{2+} is recognized as one of the most important intracellular second messages in all mammalian cells (29). Changes in intracellular cytoplasmic levels of Ca^{2+} have been shown to signal gene expression alternations associated with cell growth, differentiation, and apoptosis (29). It has been demonstrated that nickel exposure caused the release of stores of free intracellular Ca^{2+} in a variety of systems that is required for nickel-induced Cap-43 protein expression (12, 30). Although the detailed mechanism of Ca^{2+} release is unclear, we speculate that Ca^{2+} release from stores is mediated by H_2O_2 generation induced by nickel compounds. This speculation is supported by the findings that Ca^{2+} channels are activated and the intracellular Ca^{2+} is increased by exposure of cells to H_2O_2 (31, 32). It has been reported that H_2O_2 exposure caused mitochondrial membrane depolarization. This alternation may led to a change in the ability of mitochondria to take up and retain Ca^{2+} (33). Nevertheless, mitochondria do not store a significant amount of releasable Ca^{2+} (33) but rather modulate release from the inositol 1,4,5-triphosphate-sensitive pool during signaling (34, 35). Another potential source of Ca^{2+} in

cell response to H_2O_2 is attributable to annexia VI translocation and inactivation of plasma membrane Ca^{2+}-ATPase (31). Thus, we suggest that an increase in free intracellular Ca^{2+} may be mediated mainly by H_2O_2 generation, although we could not rule out the possible nickel effects on Ca^{2+} channels directly. The increase in free intracellular Ca^{2+} signal by nickel compounds (12, 30) also supports our findings that H_2O_2 plays a key role in nickel-induced NFAT activation.

ROS is one of the important determinants in the regulation of cell function pathways that can incorporate proliferation, apoptosis, transformation, and senescence (31, 32, 36–38). Intercellular levels of ROS are influenced by a number of endogenous and exogenous processes and regulated by several antioxidant enzymes (37). It is generally believed that the extracellular stimuli generate and/or require reactive free radicals or derived oxidant species to successfully transmit their signals to the nucleus (37–39). In addition to inducing cellular injury, such as DNA damage and lipid peroxidation, ROS also function as intracellular messengers (37–39). The nature of ROS-elicited cellular changes can be through two different pathways: (*a*) by the direct effect of ROS on the kinases or proteins, which can alter their conformation and activity; or (*b*) by the effect of cysteine-rich and redox-sensitive proteins, which play important roles in regulation of stress response (37). For example, oxidation of a specific cysteine residue of Ras may result in the activation of a downstream protein kinase. Accumulating data suggest a vital role of ROS in mediating cellular responses by various extracellular stimuli (37, 38). It has been reported that free radicals are involved in the production of cytokines, growth factors, and hormones, in the activation of nuclear transcription factors, in gene transcription, in neuromodulation, and in apoptosis (37, 38). For example, It has been reported that generation of H_2O_2 is required for platelet-derived growth factor signal transduction (40). The evidence suggesting the involvement of ROS in apoptosis includes: (*a*) the addition of ROS or the deletion of endogenous antioxidants can induce apoptosis; (*b*) apoptosis can be inhibited by endogenous or exogenous antioxidants in some cases; and (*c*) apoptosis is associated with increases in cellular ROS levels (41). Our recent data also demonstrated that increased intracellular H_2O_2 levels and activation of p53 activity were detected upon incubation of cells with vanadate. Pretreatment of cells with NAC or catalase prevented the increase in ROS, and resulted in the inhibition of p53 activation by vanadate (38). In contrast, increasing H_2O_2 levels with SOD or NADPH led to higher levels of p53 activation. These data suggest that H_2O_2 plays an essential role in vanadate-induced p53 activation (38). The important role of ROS in regulating signal transduction pathways is supported further by the data that the cells overexpressing catalase were unable to activate NFκB in response to TNF-α and okadaic acid (42). The catalase inhibitor aminotriazole restored the NFκB response (42). In contrast, overexpressing cytosolic SOD, which causes cytosolic H_2O_2 accumulation, potentiated NFκB response (42). All of those results strongly support the hypothesis that H_2O_2 is involved in activation of NFκB. The role of ROS in AP-1 induction is not only supported by the data that ROS mediated expression of *c-fos* and *c-jun*, the two major families of the AP-1 component, but also is supported by the inhibition of AP-1 activation by ROS scavengers (42). The results presented here demonstrate that increased intracellular H_2O_2 levels are associated with induction of NFAT activity by nickel compounds. The following experimental observations support this conclusion: (*a*) ESR measurements show that nickel compounds induce ROS generation in PW cells; (*b*), catalase, a scavenger of H_2O_2, inhibited nickel-induced NFAT activation; (*c*), sodium formate, an $^{\cdot}OH$ radical scavenger, did not affect nickel-induced NFAT activation; and (*d*) deferoxamine, a metal chelator, dramatically decreased the NFAT activation induced by nickel compounds, indicating a key role for nickel compounds in ROS generation and NFAT activation. Thus, H_2O_2 is involved in nickel-induced NFAT activation. Considering the results that a calcium-calcineurin pathway is also required for nickel-induced NFAT activation, we speculate that H_2O_2 may be an initiator for the calcium-calcineurin pathway for NFAT activation. This hypothesis was strongly supported by the previous findings that exposure of cells to H_2O_2 induces early release of Ca^{2+} from mitochondria into cytosol, which was mediated by annexin VI translocation and the inactivation of plasma membrane Ca^{2+}-ATPase (31, 32). It may be noted that both catalase and SOD are efficient H_2O_2 and $O_2^{\cdot-}$ scavengers. At relatively high concentrations, they can be used to study the generation of H_2O_2 or $O_2^{\cdot-}$ inside cells. Using a confocal microscope together with specific fluorescent dyes, we have shown that the addition of catalase or SOD reduced the H_2O_2 or $O_2^{\cdot-}$ generated inside the A549 cells under potassium dichromate stimulation to control level (43, 44). The generation of H_2O_2 and $O_2^{\cdot-}$ and the location of these ROS inside cells can be visualized.

Increasing evidence supports the hypothesis that ROS play a role in cancer development (37, 41, 45). In the JB6 cell model, ROS, especially $O_2^{\cdot-}$, have been demonstrated to be involved in signaling the cell transformation response (45). More and more data show that antioxidants may prevent or delay the development of some cancers (37, 42, 45). It is believed that ROS play a role in cancer development in both stages of initiation and promotion. ROS can cause structure alternations in DNA, such as bp mutations, rearrangement, deletions, insertions, and sequence amplification. Mutagenesis and DNA damage caused by ROS could contribute to the initiation of cancer. ROS can also affect cytoplasmic and nuclear signal transduction pathways. Through these pathways, ROS regulate gene expression (42). These effects are important in tumor promotion. Among the ROS, $O_2^{\cdot-}$ and H_2O_2 are nonreactive toward DNA bases (46), whereas $^{\cdot}OH$ generates a multiplicity of products from all four DNA bases, and this pattern seems to be a diagnostic "finger-print" of $^{\cdot}OH$ attack (46). If ROS cause damage to tumor-suppressor genes or enhanced expression of a proto-oncogene, the cells may become tumor cells. However, the development of human cancer depends on many other factors, including the extent of DNA damage, the DNA repair system, and gene regulating signals (37, 42, 45). NFAT is a transcription factor which has been reported to play an essential role in *IL-2* gene expression (10). NFAT activation is also found to be involved in the production of IL-1, IL-2, IL-3, IL-4, IL-5, Il-6, IL-8, IL-10, IL-13, IL-18, TNF-α, IFN-γ, and granulocyte macrophage colony-stimulating factor in a variety of cell types (10). The present study demonstrates that generation of ROS by $NiCl_2$ or Ni_3S_2 plays an important role in NFAT activation. Because previous studies have indicated that the generation of ROS and the expression of IL-8, TNF-α, and other cytokines are all related to cancer development (26, 28, 45), it is reasonable to associate NFAT activation with the carcinogenic effects of nickel compounds. This conjecture is supported by previous findings that CsA and FK506, the two most commonly used inhibitors for NFAT activation, exhibit a strong antitumor promotion activity by specifically targeting and blocking the activation of a Ca^{2+}-dependent phosphatase, calcineurin (10).

Crystalline nickel sulfide and nickel subsulfide compounds are extremely potent carcinogens in experimental animals (1–7). It has been demonstrated that a single injection at virtually any site in an animal will produce essentially a 100% carcinogenic response, whereas similar administration of water-soluble nickel compounds, such as $NiCl_2$ and amorphous nickel sulfide, does not yield a high incidence of cancer (2, 5, 8). This difference seems to be dependent on the surface charge of the particles, which affects phagocytosis by exposed cells (2, 5, 8). It has been found that the more negatively charged, the more actively particles were phagocytized by cells (2, 5,

8). It has also been demonstrated that amorphous nickel sulfide particles could become a carcinogen and could be actively phagocytized if their negative charges were increased by treatment with lithium aluminum hydride (2, 5, 7, 8). After phagocytosis, these particles are contained in vacuoles, which can fuse with lysosomes, resulting in the release of substantial amounts of nickel compounds into the cytoplasm and nuclei (2, 5, 7, 8). In contrast, a sufficiently high intracellular nickel concentration was not reached within certain long time with water soluble nickel compound (2, 5, 7, 8). This may be the reason that the high concentration of $NiCl_2$ (1 mM) is required to induce a similar level of NFAT activities by water-insoluble nickel compound in a cell culture model.

In conclusion, the present study demonstrates that $NiCl_2$ and Ni_3S_2 are inducers of NFAT activation and ROS generation. $NiCl_2$ or Ni_3S_2 induces NFAT activation through CsA-sensitive and H_2O_2- as well as calcium-dependent signal transduction pathways. The generated H_2O_2 by $NiCl_2$ or Ni_3S_2 may be the initiator of the calcium-calcineurin pathway for NFAT activation. Transactivation of NFAT in PW cells other than lymphocytes supports the hypothesis that NFAT activation may play an important role in $NiCl_2$- or Ni_3S_2-induced biological effects, such as tumor promotion.

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Hydrogen Peroxide Mediates Activation of Nuclear Factor of Activated T Cells (NFAT) by Nickel Subsulfide

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