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NICKEL COMPOUNDS INDUCE HISTONE UBIQUITINATION BY INHIBITING HISTONE DEUBIQUITINATING ENZYME ACTIVITY

Qingdong Ke, Thomas P. Ellen, and Max Costa

Department of Environmental Medicine and Pharmacology, NYU Cancer Institute, New York University School of Medicine, New York, New York 10016

Abstract

Nickel (Ni) compounds are known carcinogens but underlying mechanisms are not clear. Epigenetic changes are likely to play an important role in nickel ion carcinogenesis. Previous studies have shown epigenetic effects of nickel ions, including the loss of histone acetylation and a pronounced increase in dimethylated H3K9 in nickel-exposed cells. In this study, we demonstrated that both water-soluble and insoluble nickel compounds induce histone ubiquitination (uH2A and uH2B) in a variety of cell lines. Investigations of the mechanism by which nickel increases histone ubiquitination in cells reveal that nickel does not affect cellular levels of the substrates of this modification, i.e., ubiquitin, histones, and other non-histone ubiquitinated proteins. *In vitro* ubiquitination and deubiquitination assays have been developed to further investigate possible effects of nickel on enzymes responsible for histone ubiquitination. Results from the *in vitro* assays demonstrate that the presence of nickel did not affect the levels of ubiquitinated histones in the ubiquitinating assay. Instead, the addition of nickel significantly prevents loss of uH2A and uH2B in the deubiquitinating assay, suggesting that nickel-induced histone ubiquitination is the result of inhibition of (a) putative deubiquitinating enzyme(s). Additional supporting evidence comes from the similarity in the response of nickel ions with a known deubiquitinating enzyme inhibitor, iodoacetamide (IAA). This study is the first to demonstrate such effects of nickel ions on histone ubiquitination. It also sheds light on the possible mechanisms involved in altering the steady state of this modification. This study provides further evidence that supports the notion that nickel ions alter epigenetic homeostasis in cells, which may lead to altered programs of gene expression and carcinogenesis.

Keywords

nickel; histone ubiquitination; histone ubiquitination enzyme; histone deubiquitination enzyme

Introduction

The eukaryotic genome is packaged into chromatin, whose fundamental subunit is the nucleosome. Each nucleosome contains 146 base pairs of DNA wrapped around an octamer of histones (Luger *et al.*, 1997). Two copies of each of histone H2A, H2B, H3, and H4 form the histone core octamer. Post-translational modifications (i.e., acetylation, methylation, phosphorylation, ubiquitination, etc.) of the N- and C-terminal tails of these histones play an important role in regulating chromatin biology (Zhang and Reinberg, 2001). In spite of its

Address correspondence to: Max Costa, Ph.D., Department of Environmental Medicine, New York University School of Medicine, 650 First Avenue, New York, NY 10016, Fax: 845-351-2118, E-mail: costam01@nyu.edu.

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discovery three decades ago (Goldknopf *et al.*, 1977), histone ubiquitination is still not well-characterized, compared to histone acetylation and methylation.

All of the four core histones (H2A, H2B, H3, and H4) can be ubiquitinated, and the most prevalent forms of this modification are mono-ubiquitinated-H2A (uH2A) and H2B (uH2B) (Nickel and Davie, 1989). Approximately 10% of total H2A and 1–1.5% of H2B is ubiquitinated in a variety of higher eukaryotic cells. In fact, histone H2A is the most abundant ubiquitinated protein in mammals (West and Bonner, 1980). In contrast, uH2A has not been reported in the budding yeast *Saccharomyces cerevisiae*. Ubiquitin is linked to the lysine 119 (K119) of H2A and lysine 120 (K120) of H2B (K123 in yeast) (West and Bonner, 1980; Thorne *et al.*, 1987).

Recent studies on the functional significance of histone ubiquitination and the responsible enzymes have led to considerable advancement in our understanding (Minsky and Oren, 2004; Wang *et al.*, 2004; Zhu *et al.*, 2005). Unlike the most characterized function of protein ubiquitination, where four ubiquitin molecules are typically targeted to the protein, leading to degradation of the protein by the 26S proteasome, ubiquitination of histones often involves just one or two ubiquitin molecules covalently attached to the histones, resulting in mono- or diubiquitinated histone. This modification does not lead to the ubiquitin-targeted degradation of histones (Osley, 2004). Recent studies have found a variety of functional consequences of this modification, including gene activation, repression, and DNA repair (Minsky and Oren, 2004; Wang *et al.*, 2004; Cao *et al.*, 2005; Zhu *et al.*, 2005).

Nickel compounds are carcinogenic to both humans and experimental animals (IARC, 1990). Occupational exposure to nickel compounds has been linked to lung and nasal cancers (Doll, 1958; Doll *et al.*, 1970; Doll *et al.*, 1977). However, the mechanisms of tumor formation by nickel exposure remain unclear. Nickel compounds are found to be nonmutagenic in most conventional mutagenesis assays (Biggart and Costa, 1986; Biedermann and Landolph, 1987; Miura *et al.*, 1989; Kargacin *et al.*, 1993), but an epigenetic mechanism has been hypothesized to explain the potent carcinogenic effects of nickel ions (Lee *et al.*, 1995; Broday *et al.*, 1999; Sutherland *et al.*, 2001).

Exposure of cells to nickel compounds disrupts epigenetic homeostasis. Previous studies have shown that exposure of cells with nickel compounds [soluble nickel chloride (NiCl_2) and insoluble nickel subsulfide (Ni_3S_2)] results in intracellular accumulation of nickel ion, a loss of acetylation at all four core histones, and increased methylation at histone H3 lysine 9 (H3K9) at the global level (Chen *et al.*, 2006; Ke *et al.*, 2006; Ke, 2007). Research on the mechanisms by which nickel compounds alter histone modifications has revealed that nickel exposure decreases histone acetylation by inhibiting histone acetyltransferase (HAT) activity, but has no effects on histone deacetylases (HDAC) (Kang *et al.*, 2003). More recently, it has been shown that nickel increases methylation of H3K9 by inhibiting the activity of a histone demethylase, with no observable effect on methyltransferases (Chen *et al.*, 2006).

We have previously shown that exposure of human lung carcinoma A549 cells to soluble NiCl_2 increases intracellular levels of uH2A and uH2B (Ke *et al.*, 2006). Here, we extend this investigation to insoluble Ni_3S_2 and to a variety of cell lines including human hepatoma Hep3B cells, human lung epithelial Beas-2B, human cervix carcinoma HeLa cells, and mouse epidermal JB6 Cl41, to explore the mechanism by which nickel increases histone ubiquitination.

Materials and methods

Cell culture

Human lung carcinoma A549 cells were cultured in Ham's F-12 K medium (Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Omega Scientific, Inc., Tarzana, CA), and 1% penicillin/streptomycin (Grand Island, NY). Human hepatoma Hep3B and human cervix carcinoma HeLa cells were grown in MEM medium (Grand Island, NY) supplemented with 10% FBS and 1% penicillin/streptomycin. Mouse epidermal JB6 Cl41 and human lung epithelial Beas-2B cells were cultured in MEM medium supplemented with 5% FBS, 2 mM L-glutamine (Grand Island, NY), and 1% penicillin/streptomycin. All cells were maintained at 37°C as monolayers in a humidified atmosphere containing 5% CO₂. Cells were passaged at 70–80% confluence by trypsinization. All treatments were administered when cell density reached approximately 70–80% confluence.

Chemicals and antibodies

Ubiquitinated H2A antibody (anti-ubiquityl-Histone H2A, clone E6C5 mouse monoclonal IgM, catalog # 05–678) and H2B antibody (rabbit polyclonal IgG, catalog # 07–371) were purchased from Upstate (Lake Placid, NY). β -actin antibody was from Sigma (St. Louis, MO). Primary antibody-bound proteins were detected by using appropriate alkaline phosphatase (AP)- or horseradish peroxidase (HRP)-conjugated secondary antibody from Cell Signaling Technology and an enhanced chemifluorescent (ECF; for AP) or enhanced chemiluminescent (ECL; for HRP) Western blotting system from Amersham (Piscataway, NJ). All other reagents were obtained from Sigma unless otherwise specified.

Histone extraction

Cells cultured in the 150-mm dish were washed with ice-cold 1x PBS twice and lysed in 1 ml NIB buffer (10 mM Tris-HCl, pH 7.4, 2 mM MgCl₂, 3 mM CaCl₂, 1% Nonidet P-40) for 15 min. The pellet was collected by centrifugation at 10,000 $\times$ g for 10 min and lysed in 1 ml high salt NIB buffer (500 mM NaCl in NIB) for 15 min. The pellet was collected by centrifugation at 14,000 $\times$ g for 10 min and resuspended in 300 μ l 0.4 N H₂SO₄. After 90 min of incubation on ice, the supernatant was collected by centrifugation at 14,000 $\times$ g for 15 min, and mixed with 1.2 ml cold acetone overnight at –20°C. The histones were collected by centrifugation at 14,000 $\times$ g for 15 min and air-dried, and then were suspended in 4 M urea to make a final histone concentration of 1 mg/ml.

Whole cell protein extraction

Cells cultured in the 6-well plate were washed with ice-cold 1x PBS twice and lysed with 100 μ l lysis buffer (10 mM Tris-HCl, pH 7.4, 1% SDS, 1.0 mM sodium orthovanadate) for 15 min. The cells were transferred to an Eppendorf tube and sonicated to reduce viscosity by applying 10 one-sec pulses with a Branson Sonifier 450. The samples were stored at –20°C until use.

Western blot

The protein concentration was measured using the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA), and the proteins were separated by SDS-PAGE gel and transferred to a PVDF membrane. The gel was stained with Bio-safe Coomassie Stain (Bio-Rad) after transfer to assess protein loading. After blocking of the membrane for 1 hr with a 3% dry milk-TBS (0.1 M Tris, 150 mM NaCl) solution (TBS-MLK) freshly prepared, the membrane was incubated at 4°C with gentle agitation overnight in a solution of specific antibodies diluted in TBS-MLK. Membranes were washed by gentle agitation in TBS-T (0.1 M Tris, 150 mM NaCl, 0.1% Tween-20) three times (5 min per wash), and then incubated with appropriate AP- or HRP-conjugated secondary antibodies for 3 hr at 4°C with gentle agitation. After three washes with

TBS-T, the presence of secondary antibody was detected by chemical fluorescence following an ECF (for AP) or ECL (for HRP) Western Blotting protocol.

Protein extraction for *in vitro* ubiquitination and deubiquitination assays

Cells cultured in 150-mm dishes were washed with ice-cold 1x PBS twice and lysed with 1 ml ice-cold Pagano buffer, consisting of 20 mM Tris-HCl (pH 7.4), 2 mM DTT, 0.25 mM EDTA, 10 µg/ml leupeptin, and 10 µg/ml pepstatin. The suspension was kept on ice and sonicated as described above. After centrifugation at $14,000 \times g$ for 10 min, the supernatant was divided into aliquots and stored at -80°C .

In vitro ubiquitination assay

Five micrograms (5 µg) of histones extracted from cells as described above were incubated with 10 µl ubiquitination mix, containing 40 mM Tris (pH 7.4), 5 mM MgCl_2 , 10% glycerol, 1 mM DTT, 10 mM creatine phosphate, 0.1 µg/ml creatine kinase, 0.5 mM ATP, 1 µM ubiquitin aldehyde, 1 mg/ml methyl ubiquitin, 10 mM iodoacetamide (IAA), 1 µM okadaic acid, 1 µg ubiquitin, and 20 µg of protein extract. After incubation at 37°C for 1 hr, the reaction was terminated by addition of SDS-PAGE loading buffer. The products were then boiled at 100°C for 5 min and resolved in 15% SDS-PAGE gels, transferred to a PVDF membrane, and blotted with the anti-uH2A or anti-H2B antibodies.

In vitro deubiquitination assay

Five micrograms (5 µg) of extracted histones dissolved in H_2O were incubated with 10 µl deubiquitination mix containing 40 mM Tris (pH 7.4), 5 mM MgCl_2 , 10% glycerol, 1 mM DTT, and 20 µg of protein extract. After incubation at 37°C for the indicated times, or for 1 hr if not indicated, the reaction was terminated by addition of SDS-PAGE loading buffer. The products were then boiled at 100°C for 5 min and resolved on 15% SDS-PAGE gels, transferred to a PVDF membrane, and blotted with the anti-uH2A or anti-H2B antibodies.

Results

Nickel compounds induce ubiquitination of histone H2A and H2B in a variety of cell lines

Based on their water solubility, nickel compounds can be categorized as soluble [*i.e.*, nickel chloride (NiCl_2) and nickel sulfate (NiSO_4)] and insoluble [*i.e.*, nickel sulfide (NiS), nickel subsulfide (Ni_3S_2), and nickel monoxide (NiO)]. Occupational exposure to mixed soluble and insoluble Ni compounds has been associated with an increased risk of respiratory cancer (ICNCM, 1990). Since inhalation is the primary route for human exposure to nickel compounds, the human lung A549 cell line was utilized as the cell model for the present study.

The cytotoxicity of nickel compounds in A549 cells has been previously measured using MTT (Davidson *et al.*, 2003), cell growth (Ke *et al.*, 2006), and colony formation (Chen *et al.*, 2006) assays, all of which demonstrate that NiCl_2 at ≤ 1 mM does not significantly impact cell viability. For this report, the epigenetic effects of both soluble and insoluble nickel compounds have been studied. Thus, their impact on cell proliferation was first tested in A549 cells. A549 cells were treated with 0.1, 0.5, or 1.0 mM NiCl_2 and 0.5, 1.0, or 2.5 µg/cm² Ni_3S_2 for 24 hr or 48 hr. As shown in Figure 1A, treatment for 24 or 48 hr with either NiCl_2 at 0.5 and 1 mM or Ni_3S_2 at 0.5 and 1.0 µg/cm², show comparable toxicities to one another. We used these doses to expose cells throughout the remainder of this study.

A549 cells were treated with soluble NiCl_2 (0.25, 0.5, and 1 mM) or insoluble Ni_3S_2 (0.5 and 1 µg/cm²) for 24 hr. After exposure, the histones were isolated as described in the materials and methods, and Western blots were performed using antibody against uH2A. Consistent with the previous report, NiCl_2 exposure resulted in increased levels of uH2A in a dose-dependent

manner (Figure 1B). Similar increases of uH2A were found in cells treated with insoluble Ni₃S₂ (Figure 1B). This result indicates that soluble and insoluble nickel compounds share similar epigenetic effects in inducing histone ubiquitination in cells.

In order to study the effects of nickel compounds on histone H2A ubiquitination in terms of exposure time, a time-course study was conducted in which A549 cells were exposed to 1 mM NiCl₂ for 4, 8, 12, 24, 48, and 72 hr. As shown in Figure 1C, nickel exposure results in a time-dependent increase of uH2A. It was noticed that when the exposure time was extended up to 72 hr, an additional modification appeared, which migrated on the gel at about 8 kD (molecular weight of ubiquitin) above the uH2A in the blots. We presume this modification to be a second ubiquitin molecule ligated to the histone H2A molecule (di-uH2A).

To confirm this effect of nickel compounds on histone H2A ubiquitination in other cell types, several mouse and human cell lines, such as C141, Beas-2B, HeLa, and Hep3B, were exposed to NiCl₂ (0.5 and 1 mM). As shown in Figure 1D, although the extent of the increase of uH2A and the appearance of di-uH2A varies among different cell lines, nickel exposure leads to increased levels of uH2A in each.

We next investigated the effect of Ni compounds on histone H2B ubiquitination. Owing to the absence of an antibody specifically recognizing mono-ubiquitinated H2B, uH2B was monitored with anti-H2B in the blots. Anti-H2B recognizes both non- and uH2B, with the latter localizing about 8 kD above the former in the blots. A549 cells were exposed to soluble NiCl₂ (0.25, 0.5, and 1 mM) and insoluble Ni₃S₂ exposure (0.5 and 1 µg/cm²) for 24 h. As shown in Figure 1E, exposure of A549 cells to both forms of nickel compounds results in dose-dependent increases of uH2B. To confirm the effect of nickel on histone H2B ubiquitination, a different cell line, C141, was exposed to NiCl₂ (Figure 1F). Similar results were observed. Taken together, the above data show that nickel exposure, in both soluble and insoluble form, increases uH2A and uH2B in a variety of cell lines.

Next we examined the mechanism by which nickel induces histone ubiquitination. Although the function of histone ubiquitination differs from that of other well-characterized protein ubiquitinations, the conjugation and deconjugation of ubiquitin to and from the target protein occur by a similar enzymatically facilitated mechanism. Figure 2 shows a schematic of histone ubiquitination and deubiquitination by ubiquitinating enzymes (E1, E2, and E3) and deubiquitinating enzymes. The possible means by which nickel compounds affect the addition and removal processes were assessed. To investigate this, the components involved in the process of histone ubiquitination, *i.e.*, the cellular levels of the substrates (ubiquitin, H2A, and H2B), other non-histone ubiquitinated proteins, ubiquitinating enzyme activity, and deubiquitinating enzyme activity, were measured in nickel-exposed cells, or in *in vitro* assays.

Nickel does not affect the levels of ubiquitin, H2A, or H2B in cells

It is possible that nickel may increase histone ubiquitination by enhancing the cellular levels of ubiquitin, histone H2A and/or histone H2B. To evaluate the level of ubiquitin molecules in the nickel-exposed cells (A549), whole cell proteins were extracted as described in the materials and methods, and Western blot analysis was performed using an antibody against ubiquitin. As shown in Figure 3A, nickel exposure does not affect the cellular levels of ubiquitin (Ub). To assess the levels of total H2A and H2B, histones were isolated and subjected to SDS-PAGE separation, and the gel was stained with Coomassie Blue. As shown in Figure 3B, cellular levels of H2A and H2B are not affected by nickel exposure.

Since the data indicate that neither cellular levels of Ub nor those of histones H2A and H2B are affected by nickel exposure, nickel-induced histone ubiquitination is unlikely to be due to an increase in substrate.

Nickel does not affect the levels of non-histone ubiquitinated proteins in cells

Although nickel exposure does not result in changes in the cellular levels of ubiquitin, there was a possibility that nickel exposure causes a redistribution of cellular ubiquitin, and that ubiquitin molecules are transferred from other ubiquitinated proteins to H2A or H2B, resulting in increased levels of uH2A and uH2B, respectively. To evaluate this possibility, the levels of ubiquitinated proteins in nickel-exposed cells (A549) were analyzed by extracting whole cell proteins and subjecting them to Western blot, using an antibody against ubiquitin. As shown in Figure 4A, nickel exposure does not affect the cellular levels of non-histone ubiquitinated proteins; nor does it change the levels of ubiquitin.

Inhibition of the 26S proteasome by proteasome inhibitors such as MG132 has been shown to result in accumulation of cellular ubiquitinated proteins and depletion of free ubiquitin, concomitant with a decrease in levels of mono-ubiquitinated histones (Mimnaugh *et al.*, 1997). In order to confirm the effects of nickel on ubiquitin and ubiquitinated proteins, A549 cells were treated in parallel with the well-known proteasome inhibitor MG132 and NiCl₂. As shown in Figure 4B, A549 cells respond differentially to the two agents with regard to the levels of ubiquitin and ubiquitinated proteins. Consistent with previously reported results, when cells were exposed to MG132 for 2 or 4 hr, cellular non-histone ubiquitinated proteins increased dramatically; the levels of free ubiquitin decreased; and uH2A was depleted to undetectable levels (Mimnaugh *et al.*, 1997). In cells exposed to nickel for 2 or 4 hr, on the other hand, there was not much change. Importantly, in MG132-exposed cells, when time of exposure is extended to 8 hrs, levels of non-histone ubiquitinated proteins are enhanced, free ubiquitin increases, and uH2A becomes detectable, exceeding basal levels at 24 hrs, coincident with the appearance of di-uH2A. By comparison, in NiCl₂-exposed cells, non-histone ubiquitinated proteins and free ubiquitin remain unchanged, while levels of uH2A are enhanced in a time-dependent manner. Thus, it appears that nickel does not act as a typical proteasome inhibitor.

Collectively, the results demonstrate that nickel exposure does not affect the cellular levels of ubiquitinated non-histone proteins, implying that nickel-induced histone ubiquitination does not proceed via the transfer of ubiquitin molecules from non-histone ubiquitinated proteins to histones, in a cellular context.

Nickel does not enhance the level of uH2A in the *in vitro* ubiquitination assay

Increased histone ubiquitination may be due to an increased histone-ubiquitinating enzyme activity. Accordingly, an *in vitro* ubiquitination assay was developed to assess the effects of nickel on any putative histone-ubiquitinating enzyme activity. A schematic of the *in vitro* ubiquitination assay is illustrated in Figure 5A. Excessive amount of Histone and ubiquitin (obtained commercially), supplemented with sufficient ATP, were incubated with cell lysate extracted from untreated A549 cells, in the absence or presence of nickel ions (0.1 and 1mM). After incubation at 37 °C for 1 hr, the reaction was terminated by the addition of the SDS-PAGE loading buffer. The products were then subjected to Western blot using anti-uH2A antibodies. The same blots were also incubated with anti-Ub antibody to detect the level of ubiquitin.

As shown in Figure 5B, incubation of the substrates with the cell lysate leads to an increase of uH2A, concomitant with a decreased level of ubiquitin. This enhanced uH2A is not affected or further enhanced by the addition of various doses of NiCl₂ to the reaction mixture. These results indicate that nickel-induced histone ubiquitination in the cell does not operate by activation of a histone-ubiquitinating enzyme activity.

Nickel prevents the loss of uH2A in the *in vitro* deubiquitination assay

Inhibition of a histone-deubiquitinating enzyme could also give rise to an increase in cellular levels of histone ubiquitination. Therefore, an *in vitro* deubiquitination assay was developed to assess any nickel-inhibited histone- deubiquitinating enzyme activity. A schematic of the *in vitro* deubiquitination assay is shown in Figure 6A. Histones extracted from A549 cells (some of which are ubiquitinated) were incubated with cell lysate obtained from untreated A549 cells, in the absence or presence of NiCl₂. After incubation at 37°C for 1 hr, the reaction was terminated by addition of SDS-PAGE loading buffer. The products were then subjected to Western blot using anti-uH2A antibodies.

As shown in Figure 6B, incubation of histones with the cell lysate for 1 hr leads to a dramatic decrease in uH2A levels to undetectable levels, concomitant with a corresponding increase in free ubiquitin. However, when NiCl₂ (0.1 mM, 1 mM, and 10 mM) is included in the reaction, the observed decrease in ubiquitinated histones is almost entirely prevented. By comparison, when SDS-PAGE loading buffer is immediately added to the reaction at time zero, the levels of uH2A and ubiquitin are not affected. The level of uH2A remains unchanged regardless of the presence of nickel, when cell lysate is excluded from the reaction (data not shown), indicating that altered levels of uH2A is mediated directly by some deubiquitinating enzyme activity present in the cell lysate. Thus, the presence of nickel ions appears to block this enzymatic activity. In a dose-response study, NiCl₂ levels lower than 0.1 mM did not show such prevention (data not shown). It appears that 0.1 mM is the minimum concentration for NiCl₂ to inhibit the enzyme activity. Thus, NiCl₂ at 0.1 mM is used for the subsequent experiments.

In order to confirm that addition of nickel prevents the loss of uH2A in the deubiquitination reaction, a time-course study was conducted. The reactions were incubated at 37°C for 0.5, 1, 2, and 4 hr, in the absence or presence of NiCl₂. As shown in Figure 6C, incubation of histones with cell lysate in the absence of nickel leads to a dramatic decrease of ubiquitinated H2A: about 50% of uH2A is lost within 0.5 hr incubation, with total depletion by 1 hr. However, in the presence of nickel ions, the levels of uH2A remain unchanged throughout the time course. These results confirm that nickel ions inhibit the histone deubiquitinating enzyme activity, and in this way mediate the net increase of histone ubiquitination in cells.

Iodoacetamide (IAA) - a deubiquitinating enzyme inhibitor – induces ubiquitinated histones in cells and prevents the loss of ubiquitinated histone *in vitro*

In order to provide further evidence that nickel-induced histone ubiquitination occurs through inhibition of deubiquitinating enzyme activity, iodoacetamide (IAA) was tested in both cells and *in vitro* systems. IAA is a known deubiquitinating enzyme inhibitor, though the mechanism of its action is not clear. To test the effect of IAA on histone ubiquitination in cells, A549 cells were exposed to IAA (2.5, 5, or 10 μ M) for 24 hr. A dose curve shows IAA-induced uH2A dose-dependency (Figure 7A). Furthermore, di-uH2A appears when cells are treated with 10 μ M IAA for 24 hr (Figure 7A). Similarly, IAA exposure results in a dose-dependent increase in ubiquitination of histone H2B (Figure 7B). Similar results were obtained in C141 cells (data not shown). Thus, these results demonstrate that nickel exposure has the same effect as that of IAA, a known inhibitor of enzymatic deubiquitination. However, unlike nickel ions, IAA enhanced not only uH2A and uH2B in exposed cells, but also other ubiquitinated proteins in general, while the level of free ubiquitin molecule remained unchanged (Figure 7C).

Next, IAA was included in the *in vitro* deubiquitination assay reactions. As shown in Figure 7D, incubation of histones with the cell lysate for 1 hr leads to a loss of uH2A. Like nickel, when IAA is added to the reaction, this loss is greatly attenuated at all incubation times tested. Moreover, the combination of nickel with IAA does not further enhance the observed levels

of ubiquitinated H2A. We next assessed the level of uH2B in the *in vitro* deubiquitination assay in the presence and absence of NiCl₂ and IAA. As shown in Figure 7E, incubation of histones with the control A549 cell lysate results in the loss of uH2B. Adding NiCl₂ or IAA significantly prevents this loss. The data demonstrate that IAA, like nickel, inhibits deubiquitination of uH2A and uH2B in the *in vitro* deubiquitination assay.

Taken together, the results provide substantial evidence that the nickel-induced increase in the levels of both *in vivo* and *in vitro* histone ubiquitination occurs through the inhibition of (a) putative deubiquitinating enzyme(s). It strongly suggests that nickel is likely to act as a deubiquitinating enzyme inhibitor and cause increased uH2A and uH2B levels in cells that are exposed to it.

Discussion

Nickel compounds are potent carcinogens but they are only weakly mutagenic. Previous studies have shown that nickel exposure results in alteration of histone modifications in cells, and thereby disrupts epigenetic homeostasis. A loss of histone acetylation and an increased histone methylation on H3K9 were previously observed in nickel-exposed cells (Chen *et al.*, 2006; Ke *et al.*, 2006; Ke, 2007). In this study, it has been demonstrated that nickel compounds, both soluble NiCl₂ and insoluble Ni₃S₂, increase histone ubiquitination of H2A and H2B in A549 cells in a dose- and time-dependent manner. This effect has been observed in several other cell lines tested as well, such as C141, Hep3B, HeLa, and Beas-2B, indicating that nickel-induced histone ubiquitination is a universal phenomenon. Furthermore, we have observed that nickel exposure leads to an increased level of di-uH2A; however, at the present time it is unclear why various cell lines respond differently to nickel exposure in terms of the extent of change of their uH2A and di-uH2A levels.

The mechanism by which nickel ions elevate cellular histone ubiquitination was herein explored, by studying each of the components or steps involved in the process of histone ubiquitination or deubiquitination. The results show that nickel ions have no effect on the cellular levels of substrates for histone ubiquitination such as ubiquitin, histones, or other ubiquitinated proteins. This was confirmed through the comparison of nickel ions with the proteasome inhibitor, MG132, in which MG132 depleted free ubiquitin in the cell while raising the level of total protein ubiquitination, whereas nickel affected neither free ubiquitin levels nor non-histone ubiquitinated protein levels while increasing the levels of histone ubiquitination in the cell: their modes of action most assuredly diverge.

Subsequently, *in vitro* assays were developed to explore the effects of nickel ions on ubiquitinating and deubiquitinating enzyme activities. The results revealed that nickel ions do not affect ubiquitinating enzymes *in vitro*, whereas they most obviously prevent the deubiquitination of uH2A and uH2B, presumably by inhibiting deubiquitinating enzyme activities. Comparison of nickel ions with the known deubiquitinating enzyme inhibitor IAA in cells as well as *in vitro* further supports the notion that nickel ions act as an inhibitor of deubiquitination. However, unlike nickel ions, IAA enhanced not only uH2A and uH2B in exposed cells, but also other ubiquitinated proteins in general, while the level of free ubiquitin molecule remained unchanged. This indicates that IAA is a general deubiquitinating enzyme inhibitor while nickel ions are more specific, targeting (a) putative histone deubiquitinating enzyme(s) activity. The identification of (a) histone deubiquitinating enzyme(s), and understanding how nickel ion exposure leads to its (their) inhibition, will be the focus of future studies. Based on the evidence provided from this study, Figure 8 shows a model for the nickel-inhibition of deubiquitinating enzyme, and shift in the steady state of ubiquitinated histones toward higher multimers of ubiquitin.

The enzyme-catalyzed, post-translational modification of histones plays an important role in transcriptional processes. Disruption of the balance of histone modifications in nickel-exposed cells should be expected to affect the normal expression of genes and contribute to nickel-mediated carcinogenesis. Previous studies of the effect of nickel on transgene silencing in cell culture showed that nickel exposure of transgenic Chinese hamster G12 cells resulted in transgene silencing (Lee *et al.*, 1993). This nickel-induced transgene silencing did not involve mutation or deletion of any portion of the transgene (Lee *et al.*, 1995). Instead, increased DNA methylation and chromatin condensation (Lee *et al.*, 1995), as well as a broad loss of histone acetylation of all four core histones and an increase in histone H3K9 methylation were observed at the silenced transgene locus (Yan *et al.*, 2003; Ke *et al.*, 2006). This indicated that an epigenetic, rather than a mutagenic mechanism was involved in the nickel-induced transgene silencing. In another example, it has been shown that the endogenous gene SerpinA3g (a member of the mouse serpin family) was remarkably down-regulated by nickel exposure (34). Both histone deacetylation and increased dimethylated H3K9 were observed at the SerpinA3g gene locus (Zhao *et al.*, 2004).

The function of histone ubiquitination at the gene specific locus is still unclear. Nevertheless, the increased levels of uH2A and/or uH2B are likely to play an important role in nickel carcinogenesis. In agreement with this notion, it was previously reported that the levels of uH2A were highly upregulated in transformed human fibroblasts and keratinocytes compared to their normal counterparts (Vassilev *et al.*, 1995). Future studies should be directed at how nickel-induced histone ubiquitination changes are involved in nickel-mediated carcinogenesis, and which critical genes are involved. It is reasonable to postulate that nickel-induced histone modifications may interplay with the other nickel-induced epigenetic changes such as DNA methylation. Such changes may cause chromatin structure and gene transcription alterations, and ultimately contribute to nickel-mediated transformation and carcinogenesis.

Based upon the present and previous studies, we propose a preliminary model for the epigenetic effects of nickel compounds (Figure 9). Insoluble nickel compounds (i.e., NiS and Ni₃S₂) are taken up by cells through phagocytosis, and retained within tissues, resulting in nickel ion accumulation in the cell and the nucleus. On the other hand, soluble nickel compounds (i.e., NiCl₂ and NiSO₄) migrate freely through the cell and are easily cleared away from the exposed tissue by biological processes available in the cell's fluids. Nickel ions derived from nickel compounds taken up by the cell may bind tightly to proteins and amino acids of the cell. Nickel ions structurally and chemically resemble essential metal ions such as zinc, copper, iron, and manganese, and they can compete with these divalent metal ions for binding within molecular transport systems of the cell. Once in the cell, it is possible that an overwhelming amount of nickel ion replaces essential metal ions that are generally required for the structure and function of many enzymes, leading to their dysfunction. Effects on histone-modifying enzymes (which would contribute to nickel-induced epigenetic changes) in cells exposed to nickel have been documented voluminously over the past decade (22–26, 31). These include: inactivation of histone acetyltransferase (HAT) that may lead to histone deacetylation; inactivation of histone demethylase (HDM) that may increase histone methylation; inactivation of a histone deubiquitinase (HDUB) that could cause increases of histone ubiquitination; and, inactivation of DNA demethylase (DNDM) that may cause DNA hypermethylation. It is possible that the nickel-induced global alteration of histone deacetylation, histone methylation, histone ubiquitination, and DNA methylation may all work together to bring about an efficient repression of specific genes (i.e., tumor suppressor genes).

In conclusion, this study demonstrates that nickel-compound exposure increases histone ubiquitination, and that it does so through the inhibition of (a) putative deubiquitination enzyme (s) activity. This disruption of histone ubiquitination homeostasis by nickel ions is likely a key event in nickel carcinogenesis.

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Figure 1A.

Figure 1B.

Figure 1C.

Figure 1D.

Figure 1E.

Figure 1F.

Figure 1. Nickel compounds induce ubiquitination of histone H2A and H2B in cells

(A) The effects of NiCl₂ and Ni₃S₂ on cell proliferation in A549 cells. A549 cells were seeded one day before the treatment and treated with 0.1, 0.5, and 1 mM NiCl₂, or 0.5, 1.0, and 2.5 μg/cm² Ni₃S₂ for 24 hr or 48 hr. At the end of exposure, the cells were washed with 1x PBS, trypsinized and counted. The cell number from each treatment was compared with that from the untreated control. (B) A549 cells were treated with 0.25, 0.5, and 1.0 mM NiCl₂ or 0.5 and 1.0 μg/cm² Ni₃S₂ for 24 hr. Isolated histones (5 μg/treatment for detection of uH2A and 15 μg/treatment for detection of uH2B) were separated over 15% SDS-PAGE gels and subjected to Western blotting with the antibody against uH2A or H2B. The lower panels show the gels stained with Coomassie Blue after transfer to monitor for histone loading. (C) A549 cells were

treated with 1.0 mM NiCl₂ for 4, 8, 12, 24, 48, and 72 hr. (D) C141, Beas-2B, HeLa, and Hep3B cells were treated with 0.5 or 1.0 mM NiCl₂ for 24 hr. (E) A549 cells were treated with 0.25, 0.5, and 1.0 mM NiCl₂ or 0.5 and 1.0 µg/cm² Ni₃S₂ for 24 hr. (F) C141 cells were treated with 0.5 or 1.0 mM NiCl₂ for 24 hr.

Figure 2. Schematic of ubiquitination and deubiquitination of histone H2A and H2B by ubiquitinating enzymes (E1, E2, and E3) and deubiquitinating enzymes, respectively

Figure 3A.

Figure 3B.

Figure 3. Nickel does not affect the cellular levels of ubiquitin, H2A, or H2B

A549 cells were treated with 0.25, 0.5, and 1.0 mM NiCl₂ for 24 hr. (A) Whole protein extracts (40 μ g) were separated over 15% SDS-PAGE gels and subjected to Western blotting with antibody against ubiquitin (Ub). The same membrane was reblotted with the antibody against actin (loading control). (B) Isolated total histones (5 μ g) were separated over 15% SDS-PAGE gels and the gels were stained with Coomassie Blue.

Figure 4A.

Figure 4B.

Figure 4. Nickel does not affect the levels of total non-histone ubiquitinated proteins in cells

(A) A549 cells were treated with 1.0 mM NiCl_2 for 24 hr. Whole protein extracts (40 μg) were separated over 4–15% SDS-PAGE gels and subjected to Western blotting with antibody against Ub. The same membrane was reblotted with the antibody against actin (loading control). (B) A549 cells were treated with 5 μM MG132, or 1.0 mM NiCl_2 for 2, 4, 8, 12, and 24 hr. Whole protein extracts (40 μg) were separated over 4–15% SDS-PAGE gels and subjected to Western blotting with antibody against Ub. The same membrane was reblotted with the antibody against actin (loading control). Isolated histones (5 μg) were separated over 15% SDS-PAGE gels and subjected to Western blotting with antibody against uH2A. The lower panels show the gels stained with Coomassie Blue after transfer in order to monitor for histone loading.

Figure 5A.

Figure 5B.

Figure 5. Nickel does not enhance the level of uH2A in the *in vitro* ubiquitination assay
 (A) Schematic of the *in vitro* ubiquitination assay. (B) Histones (5 μ g) were incubated with ubiquitination mix containing ubiquitin (1 μ g), cell lysate (20 μ g) from A549 cells, and 0.5 mM ATP, in the absence or presence of NiCl₂ (0.1 and 1 mM). After incubation at 37°C for 1 hr, the reaction was terminated by addition of SDS-PAGE loading buffer and subjected to Western blot with the antibody against uH2A. The lower panels show the same membrane reblotted with the antibody against ubiquitin.

Figure 6A.

Figure 6B.

Figure 6C.

Figure 6. Nickel prevents the loss of uH2A in the *in vitro* deubiquitination assay

(A) Schematic of the *in vitro* ubiquitination assay. (B) Total histones (5 μ g) extracted from A549 cells were incubated with deubiquitination mix containing 20 μ g of cell lysate from A549 cells, in the absence or presence of NiCl₂ (0.1, 1, and 10 mM). Without incubation or after incubation at 37°C for 1 hr, the reaction was terminated by addition of SDS-PAGE loading buffer and subjected to Western blot with the antibody against uH2A. The same blots were reblotted with the antibody against ubiquitin (Ub). (C) Histones were incubated with 20 μ g of the cell lysate in the absence or presence of NiCl₂ (0.1 mM). After incubation at 37 °C for 0.5, 1, 2, or 4 hr, the reaction was terminated and samples processed as above.

Figure 7A.

Figure 7B.

Figure 7C.

Figure 7D.

Figure 7E.

Figure 7. Iodoacetamide (IAA) - a deubiquitinating enzyme inhibitor – induces ubiquitinated histones in cells and prevents the loss of ubiquitinated histone *in vitro*

(A) A549 cells were treated with IAA (2.5, 5, or 10 μ M) for 24 hr. Isolated histones (5 μ g) were separated over 15% SDS-PAGE gels and subjected to Western blotting with the antibody against uH2A. The lower panels show the gels stained with Coomassie Blue after transfer to monitor for histone loading. (B) A549 cells were treated with IAA (5 or 10 μ M) for 24 hr. Isolated histones (15 μ g) were separated over 15% SDS-PAGE gels and subjected to Western blot with the antibody against uH2B. The lower panels show the gels stained with Coomassie Blue after transfer to monitor for histone loading. (C) A549 cells were treated with 10 μ M IAA for 24 hr. Whole protein extracts (40 μ g) were separated over 4–15% SDS-PAGE gels and subjected to Western blotting with antibody against Ub. The same membrane was reblotted with the antibody against actin (loading control). (D) Total histones (5 μ g) were incubated with 20 μ g of protein extracts from A549 cells in the absence or presence of IAA (10 μ M) and/or

NiCl₂ (0.1 mM). After incubation at 37 °C for 0.5, 1, and 2 hr, the reaction was terminated by addition of SDS-PAGE loading buffer and subjected to Western blot with the antibody against uH2A. (E) Total histones (5 µg) were incubated with 20 µg of protein extracts from A549 cells in the absence or presence of IAA (10 µM) or NiCl₂ (0.1 mM). After incubation at 37 °C for 1 hr, the reaction was terminated by addition of SDS-PAGE loading buffer and subjected to Western blot with the antibody against H2B.

Figure 8. Model of the mechanism by which nickel induces histone ubiquitination

Figure 9. Model of the epigenetic effects of nickel compounds