Alterations of histone modifications by cobalt compounds

Qin Li, Qingdong Ke and Max Costa*

Department of Environmental Medicine, New York University School of Medicine, 57 Old Forge Road, Tuxedo, NY 10987, USA

*To whom correspondence should be addressed. Tel: +1 845 731 3515; Fax: +1 845 351 2118.
Email: max.costa@nyumc.org

In the present study, we examined the effects of CoCl₂ on multiple histone modifications at the global level. We found that in both human lung carcinoma A549 cells and human bronchial epithelial Beas-2B cells, exposure to CoCl₂ (≥200 μM) for 24 h increased H3K4me3, H3K9me2, H3K9me3, H3K27me3, uH2A and uH2B but decreased acetylation at histone H4 (AcH4). Further investigation demonstrated that in A549 cells, the increase in H3K4me3 and H3K27me3 by cobalt ions exposure was probably through enhancing histone methylation processes, as methionine-deficient medium blocked the induction of H3K4me3 and H3K27me3 by cobalt ions, whereas cobalt ions increased H3K9me3 and H3K36me3 by directly inhibiting JMJD2A demethylation activity in vitro, which was probably due to the competition of cobalt ions with iron for binding to the active site of JMJD2A. Furthermore, in vitro ubiquitination and deubiquitination assays revealed that the cobalt-induced histone H2A and H2B ubiquitination is the result of inhibition of deubiquitinating enzyme activity. Microarray data showed that exposed to 200 μM of CoCl₂ for 24 h, A549 cells not only increased but also decreased expression of hundreds of genes involved in different cellular functions, including tumorigenesis. This study is the first to demonstrate that cobalt ions altered epigenetic homeostasis in cells. It also sheds light on the possible mechanisms involved in cobalt-induced alteration of histone modifications, which may lead to altered programs of gene expression and carcinogenesis since cobalt at higher concentrations is a known carcinogen.

Introduction

Post-translational modifications of nucleosomal histones play critical roles in all aspects of eukaryotic chromosome dynamics, including replication, recombination, repair, segregation and gene expression. Such modifications include acetylation and methylation of lysines (K) and arginines (R), citrullination of arginines, phosphorylation of serines (S) and threonines (T), sumoylation and ubiquitination of lysines and adenosine diphosphate-ribosylation (1–3). Each modification can affect chromatin structure that may regulate gene transcription. Studies have shown that different histone modifications yield distinct functional consequences (2). For example, in general, trimethylation of histone H3 at K9 (H3K9me3), K27 (H3K27me3) and K36 (H3K36me3); dimethylation of histone H3 at K9 (H3K9me2); ubiquitination of histone H2A (uH2A) and the lack of histone H3 (AcH3) and H4 (AcH4) acetylation correlated with transcriptional repression in higher eukaryotes (4–8), whereas trimethylation of histone H3 at K4 (H3K4me3) and ubiquitination of histone H2B (uH2B) were associated with transcriptional activation (9). However, these modifications may interact with each other and their total sum may be the ultimate determinant of chromatin state that governs gene transcription.

The enzyme-catalyzed methylation of histone lysine is controlled by both methylation and demethylation processes. For example, the Set1-containing complex, complex proteins associated with Set1 (COMPASS), which is the yeast homolog of the human MLL complex, is required for mono-, di- and trimethylation of lysine 4 of histone H3. Both Cps60 and Cps40 components of COMPASS are required for proper histone H3 trimethylation (10). Suv39h family enzymes are responsible for trimethylation of H3K9 in vivo (11). The enhancer of zeste homolog 2 is a methyltransferase known to trimethylate H3K27 (12). Set2 is responsible for mediating H3 lysine 36 methylation in vivo (6). Two families of enzymes are capable of removing methyl groups from lysine residues in histone tails. One requires protonated nitrogen in the substrate for the demethylation reaction to occur, such as LSD1, which is a flavin-dependent amine oxidase and removes the methyl group from mono- or dimethylated H3K4 by catalyzing the oxidation of amine to an imine intermediate (13). The other does not require protonated nitrogen for demethylation, such as the JARID1 family, JMJD2 family and UTx, which all are JmJ-C-containing proteins and use Fe(II) and γ-ketoglutarate as cofactors to mediate an oxidation-based demethylation. JMJD1B specifically demethylates H3K4me3 (14). The family of JMJD2 includes JMJD2A/JHDM3A, JMJD2B, JMJD2C/GASC1 and JMJD2D, all of which can demethylate H3K9me3, whereas only JMJD2A and JMJD2C can also demethylate H3K36me3 (15). UTx has recently been discovered to exhibit H3K27 di- and trimethylation activity (16).

In spite of its discovery three decades ago (17), histone ubiquitination is still not well characterized, compared with histone acetylation and methylation. All the four core histones (H2A, H2B, H3 and H4) can be ubiquitinated, and the most prevalent forms of this modification are monoubiquitinated H2A (uH2A) and H2B (uH2B) (18). Ubiquitin is catalytically linked to the lysine 119 (K119) of H2A and lysine 120 (K120) of H2B (19,20).

Ubiquitination is a reversible process, and several studies have recently demonstrated that uH2A can be deubiquitinated by the enzyme Ubp-M in humans (21), and both uH2A and uH2B can be deubiquitinated by the enzyme complex of ubiquitin protease USP22, ATXNL7, ySgf11 homolog and ENY2 (ySus1 homolog) in humans (22). In yeast, uH2B can be deubiquitinated by the enzyme Ubp8 (23,24) and Ubp10/DOT4 (25). Ubp8 is a component of the SAGA histone acetyltransferase. It has been proposed that the Rad6-catalyzed monoubiquitination of histone H2B is followed by the recruitment of SAGA to the ubiquitinated nucleosome and subsequent deubiquitination of histone H2B, which is required to initiate transcription. Ubp10/DOT4 also targets monoubiquitinated histone H2B for deubiquitination. However, this enzyme exhibits deubiquitination activities involved in telomeric-associated gene silencing (26), indicating that such deubiquitinating enzymes have distinct functions in the regulation of gene expression via the targeting of histone H2B deubiquitination.

Cobalt is found in various ores and is universally used in the steel industry, being a major constituent of hard metal alloys. Its compounds are also used in the production of pigments, drying agents and catalysts (27). Cobalt is an essential element necessary for the formation of vitamin B12 (hydroxocobalamin); however, excessive administration of this trace element produces goiter and reduced thyroid activity (28). Radioactive isotopes of cobalt are used in industry, medicine and nuclear research. In nuclear power plants, ⁶⁰Co-containing alloys can be activated into radioactive ⁶⁰Co oxides and may become dispersed in the cooling water and contaminate workers. ⁶⁰Co is an important radioactive tracer and cancer-radiation oncology treatment agent (29). Occupational exposure to Co occurs mainly via inhalation leading to various lung diseases, such as pneumonitis, fibrosis and asthma (27). When administered by inhalation, cobalt significantly increased the incidences of combined malignant and benign tumors at multiple tissue sites in experimental animals. It caused lung tumors in male and female mice and female rats, as well as adrenal gland tumors in the female rats (30). However, the mechanism by which cobalt ions induce cancer remains unclear.

Recent research has indicated that disruption of epigenetic homeostasis affects gene expression that can be inherited through cell
division and is probably an important event in nickel-induced tumorigenesis. Although cobalt is an essential element for life in minute quantities, at higher levels of exposure it shows mutagenic and carcinogenic effects similar to nickel, including altering gene expression (31–33) and interfering with the cellular homeostasis of reactive oxygen species (34,35). Ca (33.36) and Fe (37.38). Thus, the possible effects of CoCl₂ on global histone modifications were examined in human lung carcinoma A549 cells and human bronchial epithelial Beas-2B cells, which are well-established models to study the epigenetic effects of metals (39–42).

In this study, cobalt ions increased both the levels of the chromatin gene-repressive marks (H3K9me3, H3K27me3, H3K36me3, and H2A2) as well as gene activation marks (H3K4me3 and H2B2) and decreased ACh4. The cobalt-induced increase in H3K4me3 and H3K27me3 may be operating through activation of histone methyltransferases, whereas the increase in H3K9me3 and H3K36me3 was attributed to the inhibition of a histone demethylase JMJD2A enzyme activity, which is probably due to the competition of cobalt ions with iron for binding to the active site of JMJD2A. Moreover, the induced H2A2 and H2B2 probably was caused by inhibition of the histone-deubiquitinating enzyme activity. Our study is the first to demonstrate the effects of cobalt ions on histone modifications and explore the possible mechanisms by which cobalt ions alter specific histone modifications. The results of this study will broaden our understanding on cobalt toxicity and carcinogenesis.

Materials and methods

Chemicals
CoCl₂, 6H₂O and all other reagents, unless specified, were purchased from Sigma–Aldrich (St Louis, MO).

Cell culture
Human lung carcinoma A549 cells were cultured in Ham’s F-12K medium (Invitrogen, Frederick, MD) and human bronchial epithelial Beas-2B cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen). Both media were supplemented with 10% fetal bovine serum (Omega Scientific, Tarzana, CA) and 1% penicillin/streptomycin (Invitrogen). Cells were maintained at 37°C as monolayers in a humidified atmosphere containing 5% CO₂. Cells were passaged at 70–80% confluence by trypsinization. When cell density reached ~70 to 80% confluence, they were treated with the cobalt ions.

Histone extraction
Cells cultured in the 150 or 100 mm dish were washed with ice-cold 1× phosphate-buffered saline (0.13 M NaCl, 7 mM Na₂HPO₄, 3 mM NaH₂PO₄ and 0.27 mM KCl, pH 7.4) twice and lysed with ice-cold radiolabeled preincubation assay buffer [50 mM Tris–HCl, pH 7.4, 1% Nonidet P-40, 0.25% Na-deoxycholate, 150 mM NaCl and 1 mM ethylenediaminetetraacetic acid (EDTA)] supplemented with a protease inhibitor mixture (Roche Applied Sciences, Indianapolis, IN) for 10 min on ice. The lysate was then collected and centrifuged at 10 000 g for 10 min. The supernatant was removed and the pelleted washed once in 10 mM Tris/13 mM EDTA buffer (pH 7.4) and spun down at maximum speed for 5 min. The pellet was then resuspended in 100 µl 0.4 N H₂SO₄. After at least 1.5 h of incubation on ice, the sample was centrifuged at ≥14 000g for 15 min. The resulting supernatant was recovered, then mixed with 1 ml of cold acetone and kept at ~20°C overnight. The histones were then collected by centrifugation at ≥14 000g for 15 min. After one wash with acetone, the histones were air-dried and redissolved in 4 M urea.

Whole-cell protein extraction
Cells cultured in six-well plates were washed with ice-cold 1× phosphate-buffered saline twice and lysed in 100 µl of boiling lysis buffer [10 mM Tris–HCl, pH 7.4, 1% sodium dodecyl sulfate (SDS) and 1 mM sodium orthovanadate] for 15 min. The cell lysates were transferred to an Eppendorf tube and sonicated to reduce viscosity by applying 10 second pulses using a Branson Sonifier 450. The samples were stored at ~20°C until use.

Western blot
The protein concentration was determined using Bio-Rad DC protein assay (Bio-Rad, Hercules, CA). Five micrograms of purified histones or 50 µg whole-cell proteins (WCL) were separated by SDS–polyacrylamide gel electrophoresis (PAGE) gel and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Antibodies against dimethyl H3K9, trimethyl H3K9, trimethyl H3K27, trimethyl H3K36, H3, acetyl-H4, H4, 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). Trimethyl H3K4 antibody was from Abcam (Cambridge, MA), and β-actin antibody was from Sigma–Aldrich. Anti-JMJD2A antibody was from Bethyl (Montgomery, TX). Horseradish peroxidase-conjugated antibodies or mouse secondary antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). The detection was accomplished by chemical fluorescence following an ECL Western blotting protocol (Amersham, Piscataway, NJ). After transfer to PVDF membranes, the gels were stained with Bio-safe Coomassie stain (Bio-Rad) to assess the loading of histones. The immunoblots were scanned and analyzed using ImageJ software, and values were normalized to those obtained in the control samples.

GeneChip assay
Total RNA was isolated from Co-exposed (200 µm, 24 h) and non-exposed cells using the TRIzol reagent (Gibco BRL, Rockville, MD). Double-stranded complementary DNA was synthesized with a Superscript cDNA Synthesis Kit (Gibco BRL) by using an oligo(dT)24 primer with a T7 RNA polymerase promoter site added to its 3’ end. The isolated complementary DNA was used for in vitro transcription using the Ambion T7 Megascript system (Austin, TX) in the presence of biotin-11-CTP and biotin-16-UTP (Enzo Diagnostics, Farmingdale, NY). A total of 25–50 µg of the complementary RNA product in buffer (40 mM Tris–acetate, pH 8.1, 100 mM potassium acetate and 30 mM magnesium acetate) was fragmented at 94°C for 35 min. This probe used for hybridization and mixed with histone DNA (0.1 mg/ml). Signals (400 ng) were applied to the GeneChip by using a chip loader and denatured at 94°C for 2 min. The chips were hybridized to the GeneChip array, which was then washed and scanned (Hewlett-Packard, GeneArray Scanner G2500A, Palo Alto, CA) according to the procedure developed by the manufacturer (Affymetrix, Santa Clara, CA). Aliquots of the complementary RNA hybridization mixtures (15 µg complementary RNA in 200 µl hybridization mix) were hybridized to a human GeneChip array, which was then washed and scanned (Hewlett-Packard, GeneArray Scanner G2500A, Palo Alto, CA) according to the procedure developed by the manufacturer (Affymetrix, Santa Clara, CA). The gene expression data were analyzed as previously (43). The extent of changes in gene expression was determined by dividing the mean intensity of each Co-exposed condition by the mean intensity of the control cells.

Reverse transcription–polymerase chain reaction
Either 0.5 or 1 µg of RNA was reversely transcribed to complementary DNA with the Superscript III First Strand Synthesis Super-Mix Kit for qRT–PCR (Invitrogen). Semiquantitative polymerase chain reaction (PCR) was then performed to amplify JMD1A, HMOX1, GADD45A, BNIP3L, and β-actin using the following primer pairs: JMD1A, 5′-AATCCAGATGCCCCATACCGG-3’ (forward) and 5′-CATACTCTCAAACCCACCGG-3’ (reverse); HMOX1, 5′-ACATCTGTGCCGCTTAGG-3’ (forward) and 5′-GTGGGGAAGGT-GAAGAAG-3’ (reverse); GADD45A, 5′-AAGGATGATTGAGGGGG-3’ (forward) and 5′-TGGCCGAAAACAAATAAG-3’ (reverse); BNIP3L, 5′-AATGTGGCTCCACCATCGTC-3’ (forward) and 5′-GAGAGTATGGTCTTGCAG-3’ (reverse). An aliquot of 2 µl was used in a final volume of 50 µl PCR mixture containing both the forward and reverse primers, deoxynucleoside triphosphates (10 mM each of dATP, dCTP, dGTP, and dTTP), 1.5 mM MgCl₂, 1× reaction buffer (5 µl), 1 U of Taq DNA polymerase (Roche, Indianapolis, IN). The PCR conditions were 95°C for 2 min, followed by 25 cycles (20 cycles for β-actin) of 30 s at 94°C, 45 s at 57°C and 1 min at 72°C. An aliquot of the PCR products was then analyzed by electrophoresis over 1.5% agarose gels containing ethidium bromide.

Generation and purification of the recombinant JMJD2A
The pFastBac HTb-Flag-JMJD2A bacmid DNA was kindly provided by Dr Yi Zhang (44). Sf9 insect cells were cultured in suspension in Grace complete medium (Invitrogen, Carlsbad, CA) supplied with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin (Grand Island, NY) at 27°C in dark. Purified recombinant JMJD2A bacmid DNA was transfected into Sf9 insect cells in SI-900 SFM (Invitrogen) medium by using Cellfectin Reagent (Invitrogen). Seventy-two hours post-transfection, the cell culture medium containing virus was collected and clarified by centrifuge at 500g for 5 min. The titer of the P1 viral stock (supernatant) was estimated and the P1 viral stock was used to infect Sf9 cells to generate a high-titer P2 baculoviral stock. The medium was collected and centrifuged at 500g for 5 min. The supernatant (P2 viral stock) was removed, and 100 µl SDS–PAGE loading buffer was added into the pellet (containing expressed recombinant protein), boiled at 95–100°C for 5 min, sonicated by applying 10 second pulses with a Branson Sonifier and centrifuged at 10 000g for 10 min. The supernatant was loaded into a 5% SDS–PAGE gel and subjected to western blot with anti-JMJD2A antibody. After the recombinant protein was verified, the titer of the P2 viral stock was determined by performing a viral plaque assay, and the viral stock was amplified by infecting cells until it reached a suitable titer (~1 × 10⁶ pfu/ml).
then the viral stock was used to infect insect cells to express flag-jmd2a protein on a large scale.

s9 cell suspension (1–2 × 10^6 cells/ml) was centrifuged at 500g for 5 min, and the pellet was resuspended in lysis buffer f (20 mM Tris–HCl, pH 8.0, 500 mM NaCl, 4 mM MgCl2, 0.4 mM EDTA and 20% glycerol) supplemented with protease inhibitor cocktail (roche) and 2 mM dithiothreitol (DTT). the lysate was then sonicated and centrifuged at 11 000 g for 10 min. the supernatant was collected and dialyzed against the lysis buffer f without DTT for 2 h at 4°C. the anti-FLAG M2 affinity gel (Sigma–Aldrich) was then used to purify the recombinant protein. the dialyzed protein solution was incubated with Flag-resin for 2 h in a rotator at 4°C and the Flag-resin was washed with buffer W (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 2 mM MgCl2, 15% glycerol and 0.01% Igepal CA-630) three times on a rotator (5 min each time). the recombinant protein was eluted with 3× Flag peptide in elution buffer (25 mM Tris–HCl, pH 8.0, 10% glycerol and 100 mM NaCl).

In vitro demethylation assays

Five micrograms (5 µg) of core histones (upstate) were incubated with purified recombinant JMJ2A in histone demethylation buffer [50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid–KOH (pH 8.0), 100 µM Fe(NH4)2(SO4)2, 1 mM α-ketoglutarate, 2 mM ascorbic acid and 1 mM phenylmethylsulfonyl fluoride] with or without CoCl2 at 37°C for 30 min. the demethylation reaction was terminated by the addition of SDS–PAGE loading buffer and subjected to western blot with anti-H3K9me3 and anti-H3K36me3 antibodies.

Protein extraction for in vitro ubiquitination and deubiquitination assays

cells cultured in 150 mm dishes were washed with ice-cold 1× phosphate-buffered saline 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 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 MgCl2, 10% glycerol, 1 mM DTT, 10 mM creatine phosphate, 0.1 µg/ml creatine kinase, 0.5 mM adenosine triphosphate, 1 µM ubiquitin aldehyde, 1 mg/ml methyl ubiquitin, 10 µM iodoacetemide, 1 µM okadic acid, 1 µg ubiquitin and 20 µg of protein extract. after incubation at 37°C for 1 h, the reaction was terminated by addition of SDS–PAGE loading buffer. the products were then boiled at 95–100°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 H2O were incubated with 10 µl deubiquitination mix containing 40 mM Tris (pH 7.4), 5 mM MgCl2, 10% glycerol, 1 mM DTT and 20 µg of protein extract. after incubation at 37°C for the indicated times or for 1 h if not indicated, the reaction was terminated by addition of SDS–PAGE loading buffer.

the products were then boiled at 95–100°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

CoCl2 alter multiple histone modifications at the global levels

to study whether cobalt ions alter epigenetic marks on histones, we treated human lung carcinoma A549 cells with 50, 200 and 300 µM of CoCl2 (for histone methylation and acetylation) or 250 and 500 µM of CoCl2 (for histone ubiquitination) for 24 h. Histones were then extracted from the cells, and the levels of selected commonly studied histone modifications, including histone methylation, acetylation and ubiquitination, were assessed by western blot analysis using very sensitive and specific antibodies. the results show that the higher doses (≥200 µM) of cobalt ions significantly increased global histone H3K4me3, H3K9me2, H3K9me3, H3K27me3 and H3K36me3 (fig. 1A), as well as uH2A and H2B (fig. 1B). Furthermore, when the cells were exposed to 0.5 mM CoCl2 for 24 h, di-uH2A appeared. in contrast, the global level of acetylation at histone H4 (AcH4) was

![Fig. 1. Alterations of histone modifications following cobalt ions exposure in A549 cells.](https://academic.oup.com/carcin/article-abstract/30/7/1243/2476820/Downloaded-from-https://academic.oup.com/carcin/article-abstract/30/7/1243/2476820)
decreased by cobalt ions exposure (Fig. 1C). Similar effects were also observed in the non-tumorigenic human bronchial epithelial Beas-2B cells. As shown in Fig. 2A–C, 24 h of exposure to 25, 50, 100 and 200 μM of CoCl₂ increased global histone H3K4me3, H3K9me2 and H3K27me3, but decreased AcH4 in Beas-2B cells, whereas only higher doses of CoCl₂ increased H3K9me3 (200 μM), H3K36me3 (50–200 μM), uH2A (150 μM) and di-uH2A (200 μM) in Beas-2B cells.

Cobalt ions increase H3K9me3 and H3K36me3 by inhibiting histone demethylation process

Histone lysines can be mono-, di- or trimethylated (2), and both the lysine’s position and its degree of methylation may play regulatory roles in different effector proteins that read chromatin modifications, which may result in distinct functional outcomes, including heterochromatin formation (45), X-inactivation (46), genomic imprinting (47) and gene silencing (39). Abnormal histone methylation has been associated with numerous human diseases (i.e. cancer) (48,49). Recent studies show that histone methylation is dynamically regulated by histone methyltransferases and demethylases. To study the possible mechanisms by which cobalt ions alter specific histone methylations, A549 cells were preincubated in methionine-deficient medium for 4 h prior to treatment with cobalt ions. Since methionine is a substrate for S-adenosylmethionine, which is a coenzyme involved in methyl group transfers and has a short half-life in cells (50), the methionine-deficient medium could decrease intracellular S-adenosylmethionine pool and consequently inhibit methyl transfer reactions. As shown in Fig. 3A and B, incubation of cells in methionine-deficient medium dramatically decreased global H3K4me3, H3K9me3, H3K27me3 and H3K36me3, indicating that the histone methylation reaction was inhibited by the withdrawal of methionine in the medium. The addition of 300 μM cobalt ions did not cause observable toxicity (e.g. cell detachment) in cells under methionine-depleted conditions, showing similar cellular toxicity as has been observed with 1 mM nickel ions (39). Under methionine-depleted conditions, the addition of cobalt ions failed to increase global H3K4me3 or H3K27me3 (Fig. 3A) but still elevated H3K9me3 and H3K36me3 compared with untreated cells (Fig. 3B). These results indicated that cobalt ions probably increased global H3K4me3 and H3K27me3 by enhancing the methylation process, in contrast, increased global H3K9me3 and H3K36me3 probably occurred by the inhibition of the demethylation process rather than activating the methylation process.

**Fig. 2.** Alterations of histone modifications following cobalt ions exposure in Beas-2B cells. (A–C) Beas-2B cells were exposed to the indicated concentrations of CoCl₂ for 24 h. Isolated histones (5 μg) were separated in 15% SDS–PAGE gels and subjected to western blotting with the indicated antibodies. The relative intensity of the bands was quantitated using ImageJ software and normalized to the loading control. The numbers quantitating the intensity of the bands were given below the corresponding bands. Histone H3 or H4 was used to assess the loading of the histones in western blot analysis. Similar data were obtained in at least two other independent experiments, and only one representative blot is shown here. me2 = dimethylation; me3 = trimethylation; AcH4 = acetylated histone H4; uH2A = ubiquitinated H2A; di-uH2A = diubiquitinated H2A.
of JMJD2A, supplemented with 100 Fe(II), 1 mM α-ketoglutarate and 2 mM ascorbic acid. The demethylation reaction was terminated by the addition of SDS–PAGE loading buffer and subjected to western blot with the antibody against H3K9me3 and H3K36me3. The results show that the recombinant Flag-JMJD2A can effectively remove methyl group from trimethylated histone H3K9 and H3K36 (Fig. 4B). To determine whether cobalt ions inhibited histone demethylation enzyme activity, 10 μM JMJD2A was preincubated with the indicated concentrations of CoCl2 for 10 min on ice prior to the addition of other demethylation reaction components. As shown in Fig. 4C, in the absence of CoCl2, the level of H3K9me3 and H3K36me3 was decreased ~90% after 30 min of demethylation reaction; however, in the presence of 1 μM CoCl2, the decreased histone methylation was attenuated, and the decreased histone methylation level recovered to 50% of original level when JMJD2A was preincubated with 6 μM CoCl2, with a molar ratio of Co2+ to JMJD2A of ~2. These results indicate that cobalt ions directly inhibited demethylase activity of JMJD2A in vitro.

The inhibition of JMJD2A demethylase activity by cobalt ions may be due to its interference with function of the iron moiety in this enzyme. To verify this hypothesis, 50, 100, 200 and 300 μM CoCl2 were first mixed with 100 μM Fe2+, α-ketoglutarate and other demethylation reaction components, and then incubated with 10 μl of JMJD2A at 37°C for 30 min. As shown in Fig. 4D, 100 μM or higher concentrations of CoCl2 significantly prevented the removal of the methyl group from H3K9 and H3K36. However, when 100 μM Fe2+ and α-ketoglutarate were preincubated with 10 μl of JMJD2A on ice for 10 min prior to the addition of other demethylation reaction component and CoCl2 (added last), addition of 100 μM CoCl2 did not increase the level of H3K9me3 or H3K36me3, compared with the absence of CoCl2 (Fig. 4E). These results suggest that Co2+ may compete with Fe2+ for binding to JMJD2A sequentially resulting in the inhibition of JMJD2A demethylase activity.

Cobalt ions do not increase the level of uH2A in the in vitro histone ubiquitinating 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 cobalt on any histone ubiquitinating enzyme activity. A schematic of the in vitro ubiquitination assay is illustrated in Fig. 5A. Excessive amounts of histone and ubiquitin (obtained commercially), supplemented with sufficient adenosine triphosphate, were incubated with cell lysate extracted from untreated A549 cells, in the absence or presence of cobalt ions (0.05 and 0.5 mM) or nickel ions (0.1 and 1 mM). After incubation at 37°C for 1 h, 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 Fig. 5B, incubation of the substrates with the cell lysate increased uH2A, concomitant with a decreased level of ubiquitin. This enhanced uH2A was not affected by the addition of various levels of CoCl2 or NiCl2 to the reaction mixture. These results indicated that cobalt or nickel ions-induced histone ubiquitination in the cell did not activate a histone ubiquitinating enzyme activity.

Cobalt ions inhibit histone-deubiquitinating enzyme activity in vitro

Inhibition of the histone-deubiquitinating enzyme could also give rise to increased cellular levels of histone ubiquitination. Thus, an in vitro deubiquitination assay was developed to assess this activity. Histones extracted from A549 cells that also contain ubiquitinated histones were incubated with untreated (control) cell lysate at 37°C, in the absence or presence of cobalt ions or nickel ions. As shown in Fig. 6A, incubation of histones with the cell lysate for 1 h led to a dramatic decrease in ubiquitinated H2A levels concomitant with a corresponding increase in free ubiquitin. Addition of CoCl2 or NiCl2 to the reaction prevented this loss. By comparison, when SDS–PAGE loading buffer was immediately added to the reaction at time zero, the levels of ubiquitinated H2A and ubiquitin were not affected. In addition, the level of uH2A remained unchanged, regardless of the presence of CoCl2 or NiCl2, when the lysate was excluded from the reaction (Fig. 6B). These data provide evidence that the altered levels of ubiquitinated H2A was directly mediated by a histone deubiquitinating enzyme in the cell lysate during the incubation.

In order to confirm that addition of CoCl2 or NiCl2 prevented the loss of uH2A in the deubiquitination reaction, a time-course study of deubiquitination reaction was conducted. The reactions were incubated at 37°C for 0.5, 1, 2, and 4 h, in the absence or presence of CoCl2 or NiCl2. As shown in Fig. 6C, consistent with the previous results, incubation of histones with control cell lysate led to a dramatic decrease of ubiquitinated H2A. About 50% of ubiquitinated H2A was lost at 0.5 h of incubation and it was undetectable at 1, 2 and 4 h. With the addition of CoCl2 or NiCl2, the levels of ubiquitinated H2A remained unchanged throughout.
We next assessed the level of uH2B in the in vitro deubiquitination assay in the presence and absence of CoCl2 or NiCl2. As shown in Fig. 6D, incubation of histones with the control A549 cell lysate resulted in the loss of uH2B. Addition of CoCl2 or NiCl2 significantly prevented this loss. The data demonstrated that cobalt ions, like nickel ions, inhibited deubiquitination of uH2A and uH2B in the in vitro deubiquitination assay. These results confirm that cobalt and nickel ions inhibited the histone-deubiquitinating enzyme activity, and thus may contribute to the earlier-observed increases in histone ubiquitination.

Modulation of gene expression by CoCl2 in A549 cells

As described in the Introduction, alteration of histone modifications is very important for the regulation of gene expression—gene transcription and gene repression. In this study, the results showed that exposure to Co2⁺ increased gene repression markers (H3K9me3, H3K36me3, H3K9me2, uH2A and lack of AcH4), as well as gene activation markers (H3K4me3 and uH2B) in both A549 and Beas-2B cells. We therefore used the GeneChip microarray technique to study the effects of Co compounds on global gene expression. A549 cells were exposed to 200 μM of CoCl2 for 24 h followed by GeneChip analysis. Microarray data showed that in response to Co compounds, cells not only increase but also decrease the expression of hundreds of genes that are involved in different cellular functions. Supplementary Table S1 (available at Carcinogenesis Online) lists the genes upregulated by Co2⁺, and supplementary Table S2 (available at Carcinogenesis Online) lists the genes downregulated by Co2⁺. For example, Co2⁺ upregulates the expression of genes functioning in tumorigenesis (i.e. CAIX and TDE2), signal transduction and trafficking (i.e. SLC2A3, SLC11A2 and SLC2A1), cell defense (i.e. BNP3L), protein expression and turn over (i.e. EEFL2, SU11, UBC and USP15), development, differentiation and proliferation (i.e. GDF15, VEGF, EGFR, ST13 and TNFRSF10B) and gene
transcription (i.e. MAFF and TCF3). Co\(^{2+}\) downregulates the expression of genes involved in tumor suppression (i.e. NBLL1 and MTUS1), as well as other genes such as RAB1B, a member of the Ras oncogene family, FARSLA Phenylalaine-tRNA synthetase like, NAB1, an EGR1-binding protein 1 and USP52.

We also selected some Co\(^{2+}\)-upregulated genes to be validated by semiquantitative PCR (reverse transcription–PCR). As shown in supplementary Figure 1S (available at Carcinogenesis Online), Co\(^{2+}\) increased the expression of genes in different functional classes, such as transcriptional activation (i.e. JMJD1A), cell defense (i.e. HMox1 and BNIP3L) and DNA repair and cell cycle checkpoint control (i.e. GADD45A).

**Discussion**

Cobalt compounds are possible human carcinogens, and chemical toxicity of cobalt has been proven in occupational exposure and oral intake for medical treatment; however, the underlying mechanisms are still not clear. Cobalt is a naturally occurring element that has properties similar to those of iron and nickel. Previous studies have shown that nickel exposure results in alteration of histone modifications in cells, and thereby disrupts epigenetic homeostasis (39,41). In this study, we demonstrated that cobalt exposure also changes histone modifications in A549 and Beas-2B cells. We found that exposure of A549 and Beas-2B cells to CoCl\(_2\) for 24 h increased H3K4me3, H3K9me2, H3K9me3, H3K27me3, H3K36me3, uH2A and uH2B, but decreased acetylation at histone H4 (AcH4).

Since inhalation is one of the main occupational exposure routes of cobalt and cobalt exposure causes lung inflammation and tumors in experimental animals, human lung carcinoma A549 cells and human bronchial epithelial Beas-2B cells were used in this study. The choice of the cobalt exposure concentrations used for the treatment of A549 cells was based on previous cell survival data and epidemiological studies. Davidson et al. (51) demonstrated that after exposed to 400 \(\mu\)M CoCl\(_2\) for 24 h, A549 cells still had a viability as high as 87.66 \(\pm\) 12.55%. Another study reported that the mean of cell viability following exposure of A549 cells to 250 and 500 \(\mu\)M of CoCl\(_2\) for 24 h was \(\sim\)86 and 78% (52). To compare the effects between A549 and Beas-2B cells, the same or lower concentrations of Co ions were used to treat Beas-2B cells. There was no apoptotic effect observed in either A549 or Beas-2B cells at the concentrations that were utilized. Urine concentrations have been used to monitor workers’ exposure to airborne cobalt. Epidemiological studies have shown that in the cobalt-grinding industry, the average urinary cobalt level among workers was 0.8–730 \(\mu\)g/l and the maximum was even >10 \(\mu\)M (53). Animal studies showed that 2 years of exposure to 1 mg/m\(^3\) airborne Co\(_{2}\)\(_{3}\)/H\(_{2}\)O significantly increase malignancy in both rats and mice (30). Therefore, the acute exposure concentration of cobalt used in this study were reasonable with regard to those levels that humans are exposed to in the occupational setting.

To study the mechanisms by which cobalt ions increase histone methylation, A549 cells were preincubated in the methionine-deficient medium prior to exposure to cobalt. Our data demonstrated that cobalt ions increased H3K4me3 and H3K27me3 by enhancing the methylation process, as was evident from the finding that cobalt ions did not increase H3K4me3 or H3K27me3 when the intracellular methylation process was transiently inhibited by the withdrawal of methionine from the medium. In contrast, cobalt ions elevated H3K9me3 and H3K36me3 by inhibiting the demethylation process since cobalt ions increased H3K9me3 and H3K36me3 even when the intracellular methylation process was suppressed by the absence of methionine.

As a major trimethylated H3K9/H3K36 demethylase, the role of JMJD2A in cobalt-induced H3K9me3 and H3K36me3 was investigated. Our data demonstrate that cobalt ions did not affect JMJD2A protein level but directly inhibited its demethylase activity. JmjC-containing JMJD2A binds to Fe\(^{2+}\) and \(\alpha\)-ketoglutarate at its catalytic-core region (residues 1–350) and is an iron- and \(\alpha\)-ketoglutarate-dependent oxygenase (54). Co and Fe are in the same group in the chemical periodic table, and they share a number of similar chemical properties. Studies have suggested that cobalt may replace essential divalent metal ions, such as magnesium, calcium, iron, copper and zinc, thus affecting important cellular functions (55). Therefore, it is reasonable to assume that the presence of cobalt ions may also interfere with the binding and/or function of Fe(II) in JMJD2A. When Co\(^{2+}\) was preincubated with JMJD2A or Fe\(^{2+}\) prior to the addition of other demethylation reaction components, Co\(^{2+}\) inhibited JMJD2A demethylase activity and prevented the removal of the methyl group from H3K9me3/H3K36me3 and increased levels of H3K9me3/H3K36me3, suggesting that cobalt ions compete with iron for binding to JMJD2A. However, when 100 \(\mu\)M Fe\(^{2+}\) and \(\alpha\)-ketoglutarate were preincubated with 10 \(\mu\)l of JMJD2A on ice for 10 min before the addition of other demethylation reaction components and CoCl\(_2\) (added last), the same amount of Co\(^{2+}\) (100 \(\mu\)M) failed to inhibit JMJD2A demethylase activity, suggesting that iron and \(\alpha\)-ketoglutarate had bound to JMJD2A and when this complex confronted the substrate (histone with trimethylated H3K9/H3K36), the demethylation reaction had already been initiated, such that the same amount of cobalt ions failed to replace iron and inhibit the demethylation reaction. These results indicated that cobalt ions increased the levels of H3K9me3/H3K36me3 by competing with iron for binding to JMJD2A thus directly inhibiting JMJD2A demethylase activity. Since there are several demethylases for H3K9me3 and H3K36me3 and JMJD2A is one of these, cobalt ions may also inhibit other histone demethylases, such as JMID2C, to increase the levels of H3K9me3 and H3K36me3.

In this study, exposure of cells to cobalt resulted in a remarkable increase of H2A and H2B histone ubiquitination demonstrating similar effects as have been found for nickel ions (41). The mechanism by
which cobalt increases global histone ubiquitination was investigated further. The results from the in vitro ubiquitination and deubiquitination assays showed that cobalt did not affect histone ubiquitination but prevented deubiquitination. Cobalt ions may inhibit one of aforementioned histone-deubiquitinating enzymes by unknown mechanisms. Further studies should focus on the use of recombinant deubiquitinating enzyme to assess the effect of cobalt ions on enzyme deubiquitinating activity in vitro.

The enzyme-catalyzed, post-translational modification of histones plays an important role in transcriptional processes. Disruption of the balance of histone modifications in Co-exposed cells could affect the normal expression of genes and contribute to Co-mediated carcinogenesis. In summary, this study provides the first evidence that cobalt disrupts epigenetic events by altering histone modifications, providing a novel view of cobalt’s potential mechanism of carcinogenesis. Further studies should be directed at how cobalt-induced alterations of histone modifications are involved in Co-mediated carcinogenesis and which critical genes are involved.

Supplementary material
Supplementary Figure 1S and Tables S1 and S2 can be found at http://carcin.oxfordjournals.org/

References

Funding
National Institutes of Environmental Health Sciences (ES014454, ES005512, ES000260); National Cancer Institute (CA16087).
Alterations of histone modifications


Received September 25, 2008; revised March 31, 2009; accepted April 7, 2008