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Inhibition of core histones acetylation by carcinogenic nickel(II)

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Abstract

Nickel, a well-established human carcinogen, was shown to decrease acetylation of histones H4 and H3 in cultured cells. Such a decrease is expected to suppress gene expression. However, nickel is known to not only suppress but also enhance the expression of many genes. So, perhaps, nickel can alter histone acetylation in a more complex way? In a first step of testing this presumption, we examined acetylation status of histones H2A, H2B, H3 and H4, in human (HAE) and rat (NRK) cells exposed to nickel(II) under various conditions. In both cell lines, acetylation of all four histones was down-regulated by nickel(II) in a concentration- and time-dependent manner. Acetylation of histone H2B was suppressed to greater extent than that of the others, with histone H3 being relatively least affected. The analysis of acetylation status of each of the four lysine sites at the N-terminal tail of histone H2B revealed decreases consistent with those observed in the total acetylation patterns, with the K12 and K20 residues being markedly more affected than K5 and K15 residues. Thus, the decrease in acetylation was to some degree site specific. In NRK cells, the observed uniform down-regulation of histone acetylation was consistent with a marked suppression of global gene transcription measured as [^{3}H]-uridine incorporation into mRNA. However, in HAE cells, global RNA expression was transiently increased (in 24 h) before dropping below control after longer exposure (3 days). In conclusion, the effects of Ni(II) on histone acetylation are inhibitory, with their extent depending on the dose and exposure time. This uniform inhibition, however, is not consistently reflected in global RNA expression that in HAE cells may include both increase and decrease of the expression, clearly indicating the involvement of factors other than histone acetylation. The observed effects may contribute to neoplastic transformation of Ni(II)-exposed cells. (Mol Cell Biochem **279:** 133–139, 2005)

Key words:

Abbreviation: HAE cells, human airway epithelial 1HAEo-cells; HAT, histone acetyl-transferase; NRK cells, rat kidney tubular epithelial NRK-52E cells; PBS, 150 mM saline buffered with 50 mM phosphate, pH 7.4

Introduction

Nickel is a well-established human occupational carcinogen. The mechanisms underlying its carcinogenicity have been widely studied in experimental animals and cultured cells [1–3]. They include promutagenic DNA damage [4, 5] and inhibition of its repair [6, 7], interference with Ca(II) signal transduction [8], and structural/functional effects on chromatin [9–11]. The basic structural unit of the latter is nucleosome, which consists of four core histones (H2A, H2B, H3 and H4) and DNA that is wrapped around them. The structural and functional organization of chromatin has been linked to the post-translational modifications of histones, including acetylation, methylation, phosphorylation, ubiquitination, and the recently discovered sumoylation [12, 13]. The acetylation status of specific lysine residues in histones is one of the major determinants of chromatin-based regulation of gene expression. In most reports, it has been argued that

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increased levels of histone acetylation (hyperacetylation) are associated with increased transcriptional activity, whereas decreased levels of acetylation (hypoacetylation) are associated with repression of gene expression. However, most recent studies show a more complex picture. For example, analysis of histone acetylation in *S. cerevisiae* revealed unique patterns of both hyperacetylated and hypoacetylated states of specific individual N-terminal lysines in histones H2A, H2B, H3 and H4 within the promoter and coding regions of genes [14]. So, it is a distinct combination of hypo- and hyperacetylation of specific lysine residues of different histones, rather than global up- or down-regulation of histone acetylation that serves as regulatory code for gene expression.

Nickel is known to affect chromatin structure and to up-regulate or down-regulate expression of certain genes. General inhibition of gene expression has been functionally associated with nickel-mediated deacetylation of histones H3 and H4 [11, 15–17]. The deacetylation was, in turn, likely to result from inhibition by nickel of histone acetyltransferase (HAT) [15]. However, nickel is known to not only suppress but also enhance the expression of certain genes [1, 18] that does not seem to be consistent with the inhibition of histone acetylation, mentioned above. So, perhaps, nickel could alter histone acetylation in a more complex way? In a first step of testing this presumption, we investigated acetylation status of histones H2A and H2B, which had not been studied before, and of histones H3 and H4 – for comparison under the same treatment conditions, in cells grown for extended periods of time with various concentrations of nickel. To relate histone acetylation status with gene transcription, cells treated in the same way were also tested for incorporation of [^{3}H]-uridine into global mRNA. To account for possible cell origin-related differences, the experiments were performed on human airway epithelial 1HAEo- cells, and rat kidney tubular epithelial NRK-52E cells. After finding histone H2B to be relatively most sensitive to nickel-induced deacetylation, we extended our observations on the acetylation status of its four individual N-terminal lysines: K5, K12, K15, and K20.

Materials and methods

Cell culture and nickel treatment

The human airway epithelial 1HAEo- (HAE)* cell line was obtained from Dr Gruenert (University of California, San Francisco, CA) [19]; the normal rat kidney tubular epithelium NRK-52E (NRK) cells were American Type Culture Cells, ATCC-CRL 1571. HAE cells were grown on 15-cm plastic dishes pre-coated with LHC basal medium (Biosource, Camarillo, CA) supplemented with 100 μg/ml of bovine serum albumin (Invitrogen, Carlsbad, CA) and 30 μg/ml of collagen (Cohesion, Palo Alto, CA) in Earle's modified essential medium (EMEM), containing 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA), 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 μ/ml penicillin. NRK cells were grown on 15-cm plastic dishes in Dulbecco's modified Eagle's medium, containing 5% fetal bovine serum (Gemini Bio-Products), 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 μ/ml penicillin. Both cultures were maintained at 37 °C in humidified air with 5% CO_2. To determine the total N-terminal acetylation of individual histones, the cells were cultured in at least triplicate with 0.4, 0.6, 0.8 and 1.0 mM Ni(II), added as nickel(II) acetate (J. T. Baker, Phillipsburg, NJ), for 12 and 24 h, or with 0.1, 0.2, 0.3 and 0.4 mM Ni(II) for 3 and 5 days. The same time and concentration regimens were applied for evaluation of acetylation of individual lysines in histone H2B. Cells cultured without Ni(II) were used as controls. All cells were harvested by scraping at maximum 90% confluence after being washed twice with ice-cold PBS, pH 7.4. Details of culture and treatments for testing RNA expression are described in the "Newly transcribed RNA" section, below.

Extraction of histones and gel electrophoresis

Histones were isolated using the following procedure: At the end of experiment, cells were washed twice with ice-cold PBS and harvested by scraping. Cell nuclei were released at 4 °C by suspending cells from one dish in 500 μl of lysis buffer containing 10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 0.5% NP40, 20 ng/ml trichostatin A (Sigma, St. Louis, MO), 1 mg/ml protease inhibitor cocktail (Complete, Roche, Indianapolis, IN), followed by incubation for 15 min. on ice. Then, the samples were vortexed, washed once with lysis buffer and with 10 mM Tris, pH 7.4, supplemented with protease inhibitor coctail and centrifuged at 700 × g for 5 min. After the centrifugation, supernatant was discarded and histones were extracted from the pellet in suspension with three volumes of extraction buffer (20% glycerol, 0.5 M HCl, 0.1 M 2-mercaptoethylamine-HCl) incubated for 2–3 h on ice. Finally, the samples were centrifuged at 12,000 × g for 10 min. The histone-containing supernatant was collected and concentration of protein measured using the Bradford method with albumin calibration (Pierce, Rockford, IL). Equal amounts of histone extracts (10 g protein/lane) were separated by polyacrylamide gel electrophoresis on 14% Novex Tris-Glycine gels (Invitrogen, Carlsbad, CA) under reducing conditions provided by 10% v/vβ-mercaptoethanol. Gels were run in Novex Tris-Glycine SDS running buffer (Invitrogen) at 80–130 V for 2 h.

Western blotting

Proteins separated by electrophoresis were transferred at 4 °C onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA) using 25 mM 3-[(1,1-dimethyl-hydroxyethyl) amino]-2-hydroxypropanesulfonic acid (AMPSO) buffer, pH 9.5, at 40 V for 2 h. Blots were first blocked with 5% fat-free milk in TTBS buffer (Tris-buffered saline containing 0.1% Tween 20, pH 7.4) for 2 h, and then incubated with appropriate primary antibody overnight at 4 °C. The bands of acetylated histones H2A, H2B, H3, and H4 were visualized with respective primary rabbit antibodies against all N-terminal acetylation sites of each histone, purchased from Upstate Biotechnology (Lake Placid, NY). In addition, the bands of histone H2B were visualized with the use of a series of primary rabbit antibodies against the individual lysines known to be acetylated in its N-terminal tail: K5, K12, K15, and K20 (Upstate). Anti-rabbit antibodies, labeled with horseradish peroxidase (Cell Signaling Technology, Beverly, MA) served as the secondary antibodies together with Super Signal (Pierce, Rockford, IL) reagents to detect the chemiluminescent signal. Coomassie Brilliant Blue and Western blot immunostaining for total histone H2B (anti-H2B antibody from Upstate) served as loading controls. To quantify some of the effects, the intensities of bands on selected Western blots were evaluated by densitometry using the LabWorks software (UVP Bioimaging Systems, Upland, CA).

Newly transcribed RNA assay

To relate the effect of nickel on histone acetylation with that on RNA synthesis, HAE and NRK cells were cultured in 60 mm plastic dishes, at least three dishes per treatment/time point, for 24 h with 0.4 and 0.8 mM Ni(II) and for 3 days with 0.2 and 0.4 mM Ni(II). Before the end of nickel treatment, [^{3}H]-labeled uridine (specific activity16.2 Ci/mmol; Moravek Biochemicals, Brea, CA), was added to the culture medium to obtain 1 μCi/ml and incubated for the last three hours of each experiment. After the cells were washed twice with ice-cold PBS, harvested by scraping, and centrifuged for 5 min. at 1000 $\times$ g, RNA was isolated with a silica gel-based columns RNeasy Mini Kit and Qiashredder Kit (Qiagen, Valencia, CA) according to manufacturer's protocol. The isolated RNA was resuspended in 80 μl of RNase-free water. RNA concentration was measured spectrophotometrically, and 30-μl aliquots were used for counting the radioactivity (Ecoscint scintillation fluid, National Diagnostics, Atlanta, GA; Beckman LS 6000TA instrument). The level of [^{3}H]-labeled uridine incorporation per microgram of RNA was calculated for each treatment, and the effect of nickel and time was expressed in percentages versus the incorporation level in untreated cells.

Results

Effect of nickel on histone acetylation

Western blot analysis of acetylation of the N-terminal lysines of core histones H2A, H2B, H3 and H4 revealed a general trend of the acetylation levels to decrease with Ni(II) concentration and exposure time in both HAE and NRK cells (Fig. 1). Interestingly, the decline in acetylation of histone H2B was more pronounced than that in the other histones, especially after shorter exposures. The longer, 3- and 5-day, exposures revealed, in turn, a relatively lowest sensitivity of histone H3 to Ni(II)-mediated inhibition of acetylation. For example, densitometric measurements of the bands allowed for estimation of the decline in histone H2B acetylation to approximately 80, 65, 25, and 5% of the control in HAE cells cultured with 0. 4 mM Ni(II) for 12 h, 24 h, 3 days, and 5 days, respectively; whereas the respective numbers for acetylated histone H3 were approximately 100, 80, 40, and 30%.

Effect of nickel on acetylation of individual lysines in histone H2B

Because of its relatively highest sensitivity to Ni(II)-mediated deacetylation, histone H2B was used as a model for testing acetylation status of four individual lysines at the H2B N-terminus in cells grown with Ni(II). The results are exemplified in Fig. 2. As shown in this figure, lysines 5 and 15 were barely affected, except for an apparent increase of K5 acetylation in HAE cells, (but not NRK cells) exposed for 24 h to 0.8 and 1.0 mM Ni(II). In contrast, acetylation of lysines 12 and 20 was reduced in a Ni(II) concentration- and time-dependent manner, more so in HAE than in NRK cells.

Effect of nickel on RNA transcription

To relate the observed inhibition of histone acetylation with nickel effect on gene transcription, we cultured HAE and NRK cells with various concentrations of nickel for different periods of time and measured incorporation of [^{3}H]-labeled uridine into newly transcribed RNA. The results are shown in Fig. 3. As expected, in NRK cells, under conditions in which Ni(II) induced histone deacetylation, RNA synthesis was likewise suppressed. However, in HAE cells, Ni(II) effect was clearly biphasic and included a transient stimulation of RNA synthesis followed by inhibition at longer exposure time. This pattern was not consistent with the uniform

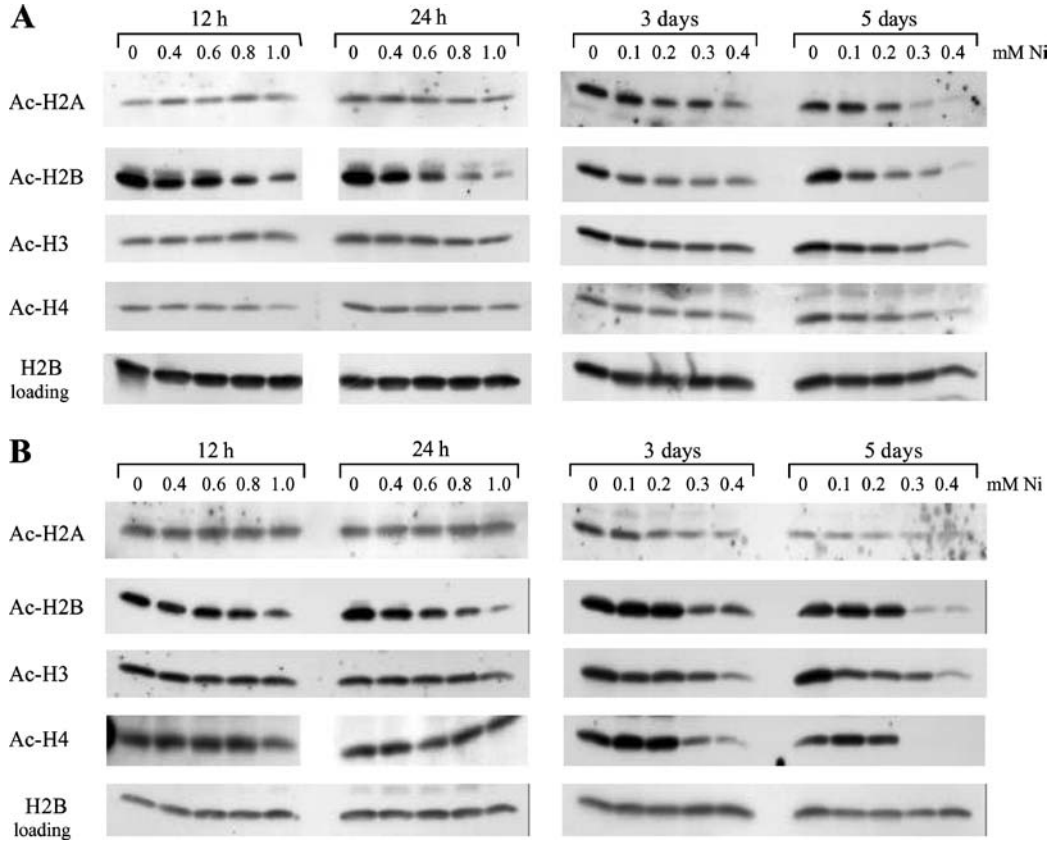

Fig. 1. Western blot analysis of global acetylation levels of N-terminal loci of core histones in HAE (A) and NRK (B) cells cultured with increasing Ni(II) concentrations for various periods of time. The gels were loaded with 10 μg protein per lane of nuclear histone extracts. Histone H2B band visualized with antibodies against unmodified variant of this histone served as an additional loading control.

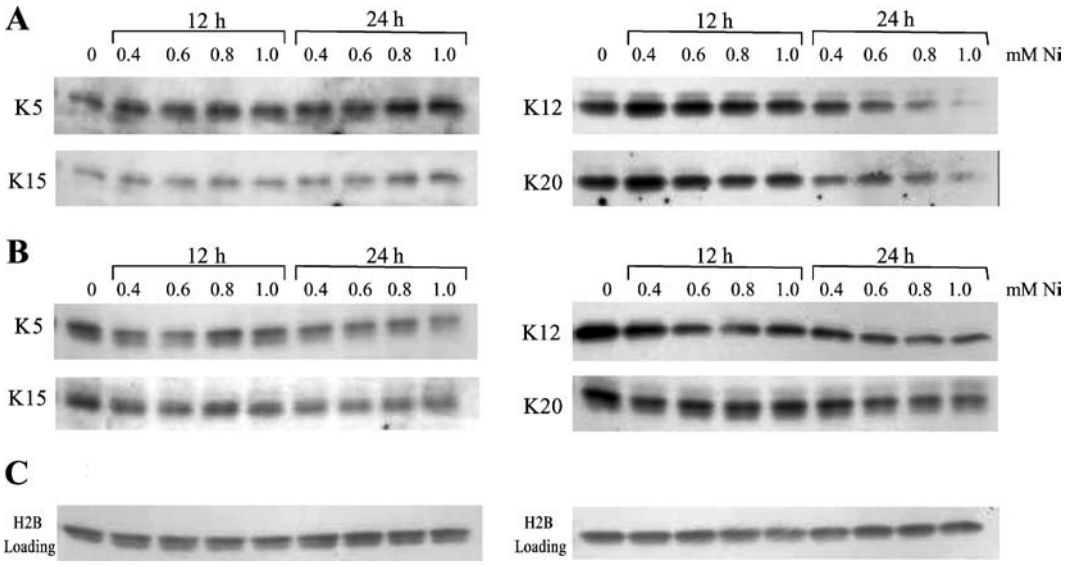

Fig. 2. Western blot analysis of individual acetylation sites, lysines 5, 12, 15, and 20, in the N-terminal tail of histone H2B isolated from HAE (A) and NRK (B) cells cultured with increasing Ni(II) concentrations for 12 and 24 h. The gels were loaded with 10 μg protein per lane of nuclear histone extracts. Histone H2B band visualized with antibodies against unmodified variant of this histone served as additional loading control (C).

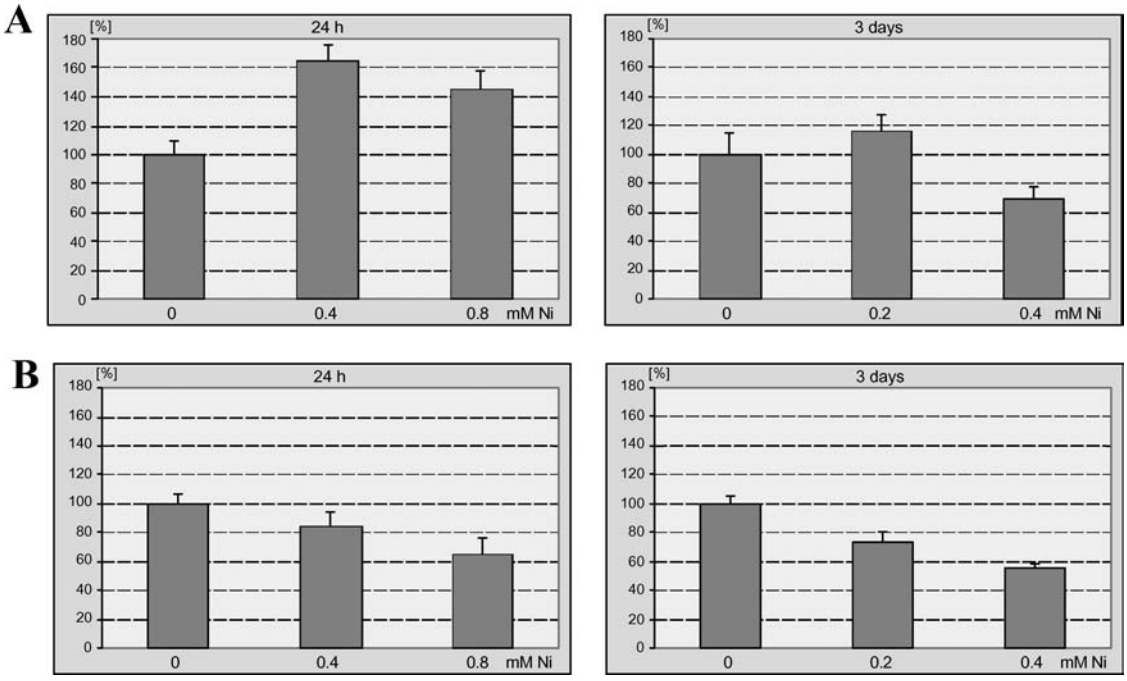

Fig. 3. Newly transcribed RNA assay. The effect is expressed as a ratio of [^{3}H]-uridine incorporation per microgram RNA in Ni(II)-treated cells *versus* the incorporation in untreated cells. (A) HAE cells. (B) NRK cells.

inhibition of global histone acetylation observed in these cells (above).

Discussion

Acetylation of the N-terminal tails of core histones is likely to be one of the most important factors involved in regulation of gene transcription. Generally, it has been thought that acetylation, by reducing positive charge on the histone tail, weakens its interaction with DNA and thus facilitates opening of the chromatin for gene expression. However, more recent studies reveal a much more complex situation in which gene expression may be up-regulated through both increase and decrease of acetylation of particular histone sites [14]. So, it is a unique combination of hypo- and hyperacetylation of specific lysine residues of different histones (together with other modifications, e.g., methylation and phosphorylation), rather than global up- or down-regulation of histone acetylation that is a part of the regulatory "histone code" for gene expression.

Nickel compounds were found before to uniformly decrease acetylation of histones H3 and H4 that in concert of increased DNA methylation should down-regulate gene expression [15–17]. However, many reports describe both, increased and decreased activity of various genes in response to nickel treatment under the same conditions [1, 18], a situation which cannot be explained by the above mentioned deacetylation of histones H3 and H4, or global decrease of histone acetylation due to the reported inhibition of histone acetyl transferase, HAT [15, 16]. Perhaps, as it was found in yeast [14] particular gene expression in mammalian cells depends more on individual histone/site acetylation status than on global hypo- or hyperacetylation of core histones. To answer this question, we had to know the effects of nickel on acetylation of all four core histones and, eventually, all known acetylation sites. A challenging task, which we decided first to tackle by measuring the acetylation levels of histones H2B and H2A and comparing them with nickel effects on histones H3 and H4 under the same experimental conditions. The results for acetylation of individual lysines in the N-terminal tail of histone H2B constitute the initial step in our more detailed investigations of nickel effects on histone acetylation. We also tested nickel influence on global RNA transcription as a balance between activation and silencing of various genes. At shorter, 12- and 24-h exposures, Ni(II) at concentration up to 1 mM was not toxic to the cells, as determined by the Trypane Blue exclusion; but concentrations exceeding 0.3 mM visibly slowed cell growth during longer expoures (3–5 days). Therefore, for the longer experiments, lower Ni(II) concentrations were tested. In addition, only viable cells attached to the bottom of the dish were collected for analysis in every case.

The overall picture emerging from the present study is consistent with the more complex interdependence between histone acetylation and global gene expression patterns, as

observed in yeast [14]. In both cell lines, the global acetylation of all four histones tended to decrease with increasing Ni(II) dose and exposure time. At the same time, however, in HAE cells the *de novo* synthesis of RNA was markedly suppressed only after a longer, 3-day exposure, and only at higher Ni(II) dose (Fig. 3). At shorter times and lower Ni(II) concentrations, RNA synthesis was clearly enhanced (up to 60%). This was happening without any detectable increase in global histone tail acetylation as could be expected, but rather concurrently with a conspicuous decrease in acetylated-H2B level and no practical changes among the other histones. Interestingly enough, however, the observed enhancement of RNA synthesis in HAE cells exposed to Ni(II) for 24 h, is consistent with a marked increase in acetylation of lysine 5 in histone H2B as compared with that in the untreated cells (Fig. 2A). At the same time, acetylation of the other lysines is clearly down, or unchanged (K15). This result strongly indicates a selective character of Ni(II) effect on histone acetylation that seems to exclude a common direct mechanism of action as the only factor, e.g., through inhibition of HAT. Also interesting is the lack of such transient increase in RNA synthesis and histone acetylation in NRK cells that may reflect differences in sensitivity to nickel between cells. It was shown before in yeast that acetylation of lysines 11 and 16 of histone H2B was negatively correlated with gene expression, but the other sites were not tested [14]. On the other hand, increased acetylation of histone H2B was also suggested to be associated with only the most active genes/loci in chicken embryo erythrocytes [20]. This apparent contradiction may be explained by different regulatory mechanisms in yeast and higher eukaryotic species or just by data limited to one loci in the second of the mentioned studies. Otherwise, our findings are consistend with previous reports, where nickel inhibited in time- and concentration-dependent manner the acetylation of histone H4 in hepatoma cells treated with up to 1 mM nickel for up to 24 h [15]. Another report shows lack of deacetylation of histone H4 in lung carcinoma cells treated with soluble nickel, but presence of such inhibition after the treatment with insoluble nickel compound (Ni_3S_2) [16]. All these findings indicate that acetylation status of histone H4 as well as of other histones may depend on many factors such as cell lines, exposure time, nickel concentration and physical properties of the compound used (e.g., water solubility). Nickel was proposed to mediate HAT inhibition through reactive oxygen species [15] or by binding to the target histone, e.g., to histidine 18 of histone H4 [16].

In conclusion, our investigations show a relatively more pronounced deacetylation of histone H2B than the other histones in nickel-exposed cells, with acetylation of histone H3 being least affected. Detailed analysis of the four N-terminal H2B lysine residues revealed some novel differences between their acetylation states: in both cell lines, lysines 5 and 15 were deacetylated by nickel to a lesser degree than lysines 12 and 20. The decrease in histone acetylation with Ni(II) dose and time was followed by eventual decrease in *de novo* RNA synthesis. However, in HAE cells, but not NRK cells, nickel caused transient increase in acetylation of lysine 5 in histone H2B that was accompanied by a concurrent increase in RNA synthesis; at the same time, the acetylation of lysine 15 remained unchanged and that of lysines 12 and 20 was clearly down-regulated. This result indicates a selective character of Ni(II) effect on histone acetylation. Further investigations are necessary to relate the present results with the expression of specific genes or loci relevant to nickel toxicity and carcinogenesis.

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