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# Carcinogenic Metals Induce Hypoxia-inducible Factor-stimulated Transcription by Reactive Oxygen Species-independent Mechanism[1]

**Konstantin Salnikow,[2] Weicheng Su, Mikhail V. Blagosklonny, and Max Costa**

*Nelson Institute of Environmental Medicine and Kaplan Comprehensive Cancer Center, New York University School of Medicine, New York, New York 10016 [K. S., W. S., M. C.], and Medicine Branch, National Cancer Institute, NIH, Bethesda, Maryland 20892 [M. V. B.]*

## Abstract

**Nickel ($Ni^{2+}$) and cobalt ($Co^{2+}$) mimic hypoxia and were used as a tool to study the role of oxygen sensing and signaling cascades in the regulation of hypoxia-inducible gene expression. These metals can produce oxidative stress; therefore, it was conceivable that reactive oxygen species (ROS) may trigger signaling pathways resulting in the activation of the hypoxia-inducible factor (HIF)-1 transcription factor and up-regulation of hypoxia-related genes. We found that the exposure of A549 cells to $Co^{2+}$ or $Ni^{2+}$ produced oxidative stress, and although $Co^{2+}$ was a more potent producer of ROS than $Ni^{2+}$, both metals equally increased the expression of *Cap43*, a hypoxia-regulated gene. The coadministration of hydrogen peroxide with metals induced more ROS; however, this did not further increase the expression of *Cap43* mRNA. The free radical scavenger 2-mercaptoethanol completely suppressed ROS generation by $CoCl_2$ and $NiCl_2$ but did not diminish the induced *Cap43* gene expression. The activity of the HIF-1 transcription factor as assessed in transient transfection assays was stimulated by $Ni^{2+}$, hypoxia, and desferrioxamine, but this activation was not diminished when oxidative stress was attenuated nor was HIF-dependent transcription enhanced by hydrogen peroxide. We conclude that ROS are produced during the exposure of cells to metals that mimic hypoxia, but the formation of ROS was not involved in the activation of HIF-1-dependent genes.**

## Introduction

Hypoxia results in the coordinated up-regulation of numerous genes involved in glucose transport, glycolysis, erythropoiesis, angiogenesis, and catecholamine metabolism that is mediated by the HIF[3]-1 transcription factor (1, 2). Whereas HIF-1$\alpha$ protein was expressed at low levels due to its rapid degradation, it was accumulated following hypoxic stress through inhibition of its proteasomal degradation (3).

The transition metals ($Co^{2+}$, $Ni^{2+}$, and $Mn^{2+}$) and iron chelater (DFX) also up-regulated HIF-1 and HIF-1-dependent transcription, but the mechanism involved in the metal activation is unknown (4–6). There are two groups of models that may explain HIF-1 activation (1, 7). First, that sensing of the low oxygen state (hypoxia) involves an iron-containing flavoheme protein. It is possible that transition metals by substituting for iron in this sensor activate a signaling cascade leading to HIF-1$\alpha$ stabilization (8, 9). Another model suggested that the modulation of endogenous $H_2O_2$ and $O_2^-$ levels while $O_2$ concentration declines provided a redox signal for HIF-1 induction (10–12).

$Co^{2+}$ and $Ni^{2+}$ increased the generation of oxidative stress in cells and increased the level of ROS (13–16). Although the increase of ROS under a state of hypoxic stress occurred after exposure to both metals and hypoxia, it was not clear whether this was the stimulus for a hypoxic gene response.

Recently, we have cloned a new human gene, *Cap43*, based on its high inducibility by $Ni^{2+}$ (17). This gene was found to be transcriptionally up-regulated by hypoxia, $Ni^{2+}$, or $Co^{2+}$ through HIF-1-dependent pathways (18). Because these transition metals generate ROS, we investigated whether ROS played a role in the activation of hypoxic genes by metals. 2-Mercaptoethanol, a free radical scavenger, attenuated ROS but did not prevent the induction of *Cap43* by $Ni^{2+}$, $Co^{2+}$, or hypoxia. The exposure of cells to $H_2O_2$ elevated ROS but did not induce *Cap43* gene expression. Additionally, the Ni or hypoxia-related enhanced expression of a HIF-1-dependent reporter plasmid, HRE-Luc, was not attenuated by 2-mercaptoethanol. We concluded that free radicals accumulation after $Ni^{2+}$ or $Co^{2+}$ exposure in A549 cells did not participate in HIF-1 activation or in the up-regulation of *Cap43* gene expression.

## Materials and Methods

**Cell Culture.** Human A549 lung cells (CCL 185) and human umbilical vascular endothelial cells (CRL 1730) were purchased from American Type Culture Collection (Manassas, VA). Cells were maintained in F-12K medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (equivalent to 100 units/ml and 100 $\mu$g/ml, respectively) at 37°C as monolayers in a humidified atmosphere containing 5% $CO_2$.

**Exposure of Cells to Metals and Other Agents.** $NiCl_2$ and $CoCl_2$ were purchased from Alfa Aesar (Ward Hill, MA). $H_2O_2$, 2-mercaptoethanol, and vitamin E were obtained from Sigma Chemical Co. (St. Louis, MO). All metals were dissolved in distilled water at high concentrations and then filtered through a sterile, pathogen-free nylon filter (pore size: 0.22 mm; MSI Inc., MA). A freshly prepared stock metal solution was mixed with F12K at various concentrations. Vitamin E was dissolved in DMSO. 2-Mercaptoethanol was dissolved directly into F12K medium.

**Northern Blot Analysis.** Total RNA was extracted from cells immediately following chemical/metal exposure using the TRIzol RNA isolation system (Life Technologies, Inc.), and 15 $\mu$g total RNA were separated by electrophoresis in 1.2% agarose/formaldehyde gels. *Cap43* and actin probes were labeled with [$^{32}$P]-$\alpha$-dCTP using a Random Primed DNA Labeling Kit (Boehringer Mannheim). The membrane was prehybridized for 2 h, hybridized with the probe of interest for 2 h, and then washed and exposed to film (Eastman-Kodak, Rochester, NY) or phosphorscreen.

**Measurement of Intracellular ROS Generation and Data Analysis.** The level of intracellular ROS was measured by the change in fluorescence resulting from oxidation of DCFH-DA (Molecular Probes, Eugene, OR). After the dye had entered cells, the acetate group on DCF-DA was cleaved by intracellular esterases, thereby trapping the nonfluorescent (DCFH). When DCFH was oxidized by ROS inside the cell it was converted into fluorescent DCF (19). DCFH-DA was dissolved in DMSO to a final concentration of 20 mM before use. For the measurement of ROS, cells were incubated with 10 $\mu$M DCF-DA at 37°C for 30 min. The excess DCFH-DA was washed with F12K media prior to metal exposure. The cells were subsequently plated at a density of $1 \times 10^4$ cells per well into Costar 96-well plates with a clear bottom (Costar Corp.,

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[2] To whom requests for reprints should be addressed, at New York University School of Medicine, Nelson Institute of Environmental Medicine, 57 Old Forge Road, Tuxedo, NY 10987. Phone: (914) 731-3516; Fax: (914) 351-2118; E-mail: salnikow@env.med.nyu.edu.

[3] The abbreviations used are: HIF, hypoxia-inducible factor; $Co^{2+}$, cobalt; $Ni^{2+}$, nickel; DFX, desferrioxamine; ROS, reactive oxygen species; $H_2O_2$, hydrogen peroxide; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DCF, dichlorofluorescein.

Cambridge, MA). The following day, metals or other chemicals were added to each well for a time period indicated in the figure or table legends. The intensity of fluorescence was recorded using a 37°C prewarmed fluorescent microplate reader, HTS 7000 (Perkin-Elmer Corp., Norwalk, CT), with an excitation filter of 485 nm and an emission filter 535 nm. The ROS level was calculated as a ratio: ROS = mean intensity of exposed cells:mean intensity of unexposed cells. The signals obtained from six separate wells were used to assess ROS for each treatment. The mean value for an individual group was obtained using the StatView or MiniTab software. Before performing the statistical analysis, the coefficient of variance for each treatment or time point was assessed to screen the distribution of data points within each treatment group. The data set was plotted with mean (ROS ratio) ± SE over time to compare the effects of different metals and chemicals. ANOVA was used to assess treatment effects, and a $P < 0.05$ was considered statistically significant for all tests.

**Transient Transfection Assay.** Cells ($5 \times 10^4$ per well) were plated in 24-well plates (Costar, Acton, MA). The next day, cells were transfected with HRE-Luc plasmid in the presence of Lipofectamine (Life Technologies, Inc.), according to the manufacturer's recommendations. Six h later, the medium was changed and cells were grown for an additional 24 h. The cells were lysed and analyzed for luciferase activity using a TopCount Luminometer (Packard Instrument, Meriden, CT).

## Results

**ROS in A549 Cells Exposed to $Ni^{2+}$ and $Co^{2+}$.** It has been previously shown that $Co^{2+}$ and $Ni^{2+}$ produced ROS in biological systems (13, 16, 20). However, comparative measurement of ROS levels resulting from exposure to each metal in the same cell type was not determined. To evaluate the induction of ROS in Ni- or Co-treated cells, A549 cells preloaded with DCFH-DA were exposed to either $NiCl_2$ or $CoCl_2$. DCFH-DA is commonly used to detect the generation of reactive oxygen intermediates in cells (19). DCFH-DA has been recently highlighted as an extremely useful reagent for assessing the overall oxidative stress phenomena during hypoxia (11). Fig. 1, *A* and *B*, shows the time- and concentration-dependent increase in ROS generation when A549 cells were incubated with various concentrations of $Ni^{2+}$ and $Co^{2+}$. A 5-fold increase of ROS above basal levels was found in cells exposed for 4 h to 300 $\mu$M Co (Fig. 2*A*). There was also a significant difference in ROS production between exposure to 100 $\mu$M or 300 $\mu$M Co, suggesting dose dependency. However, in $Ni^{2+}$-exposed cells, much lower amounts of ROS were detected (Figs. 1*B* and 2*B*). There were only small differences in ROS levels in cells treated with 250 $\mu$M, 500 $\mu$M, or 1000 $\mu$M $Ni^{2+}$ (Fig. 1*B*). It should be noted that the range of $Ni^{2+}$ and $Co^{2+}$ exposure conditions reflect equivalent levels of cytotoxicity for the A549 cells (data not shown).

We also assessed *Cap43* gene expression following exposure of A549 cells to 100 $\mu$M, 200 $\mu$M, and 300 $\mu$M $Co^{2+}$ or 250 $\mu$M, 500 $\mu$M, and 1000 $\mu$M $Ni^{2+}$ (Fig. 1, *C* and *D*). No association was found between the level of ROS in cells, as determined by DCF fluorescence shown in Fig. 1, *A* and *B*, and *Cap43* gene expression. For example, ROS levels were not different in cells treated with 500 $\mu$M or 1000 $\mu$M of $Ni^{2+}$, however, the expression of *Cap43* mRNA differed by 2.8-fold for these two Ni concentrations. In cells treated with 100 $\mu$M $Co^{2+}$ very few ROS were detected, however, the expression of *Cap43* increased 4-fold above basal levels. Additionally, exposure of A549 cells for 20 h to 300 $\mu$M $Co^{2+}$ produced much higher levels of ROS compared with 1000 $\mu$M $Ni^{2+}$ exposure (data not shown), yet the level of *Cap43* mRNA were comparable in both situations (Fig. 1, *C* and *D*).

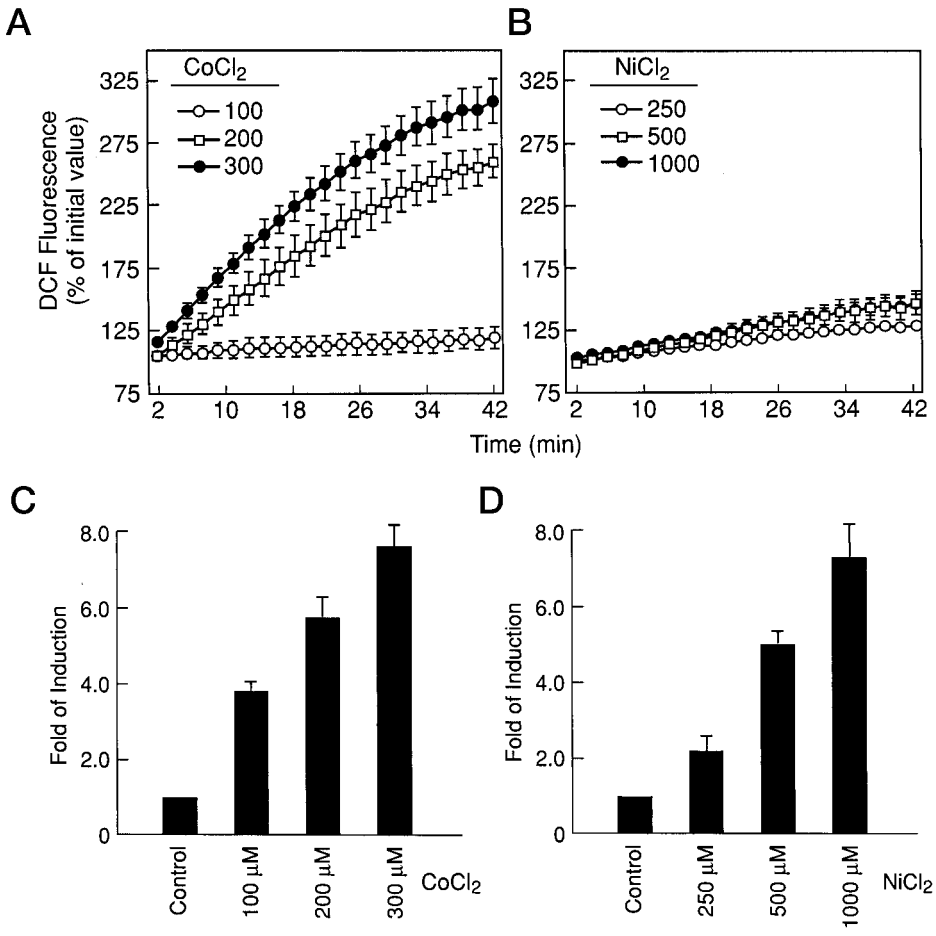


Fig. 1. The effect of $Co^{2+}$ and $Ni^{2+}$ on ROS generation and *Cap43* gene expression. *A*, intensity of DCF fluorescence in A549 cells exposed to different concentrations of $CoCl_2$. Cells were exposed to 100, 200, and 300 $\mu$M $CoCl_2$ for 45 min. The intensity of DFC fluorescence was measured every 2 min, and data represent mean values of eight independent measurements for the same time point ± single SD. *B*, intensity of DCF fluorescence in A549 cells exposed to various concentrations of $NiCl_2$. Cells were exposed to 250, 500, and 1000 $\mu$M $NiCl_2$ for 45 min. The intensity of DFC fluorescence was measured and evaluated as described in *A*. *C*, *Cap43* expression in A549 cells exposed to various concentrations of $CoCl_2$. Cells were treated with 100, 200, and 300 $\mu$M $CoCl_2$ for 20 h. Fifteen micrograms of total RNA were isolated and subjected to a Northern blot analysis. The level of *Cap43*-specific mRNA expression was measured by hybridization, and the mean ± SD for several experiments was quantitated using PhosphorImager Storm 860 and ImageQuant software. *D*, *Cap43* expression in A549 cells exposed to various concentrations of $NiCl_2$. Cells were treated with 250, 500, and 1000 $\mu$M $NiCl_2$ for 20 h. Fifteen micrograms of total RNA were isolated and subjected to a Northern blot analysis. The level of *Cap43*-specific mRNA expression was measured by hybridization, and the mean ± SD for several experiments was quantitated using PhosphorImager Storm 860 and ImageQuant software.

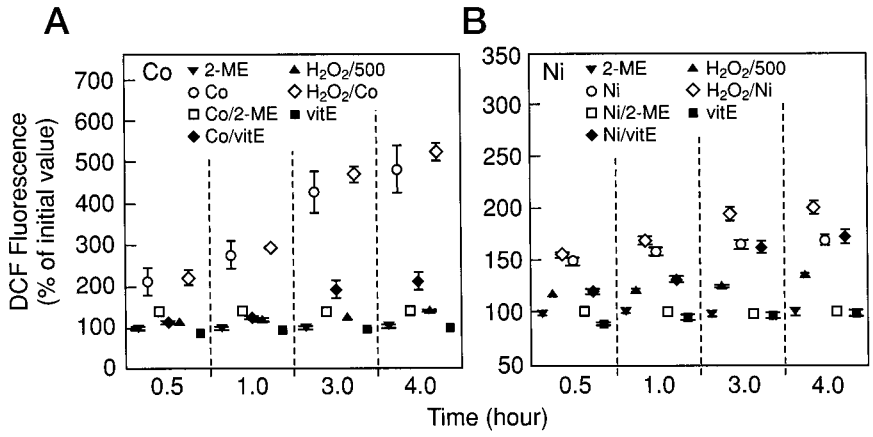


Fig. 2. The effect of 2-mercaptoethanol or $H_2O_2$ on metal-induced ROS generation. *A*, intensity of DCF fluorescence in A549 cells exposed to 300 $\mu$M $CoCl_2$ alone or in combination with 2-mercaptoethanol, vitamin E, or $H_2O_2$. ROS species was quantified by DCF fluorescence. The measurements were done at 0.5, 1, 3, and 4 h, and data represent mean values of six independent measurements for the same time point ± single SD, as described in "Materials and Methods." *B*, DCF fluorescence in A549 cells exposed to 1000 $\mu$M $NiCl_2$ alone or in combination with 2-mercaptoethanol, vitamin E, or $H_2O_2$. ROS species was quantified by DCF fluorescence. The measurements were done at 0.5, 1, 3, and 4 h, and data represent mean values of six independent measurements for the same time point ± single SD, as described in "Materials and Methods."

**The Effect of Free Radical Scavengers on ROS Production and *Cap43* Gene Expression.** To evaluate whether ROS participate in signaling pathways involved in the activation of hypoxia-inducible genes, we used two scavengers of free radicals, the monothiol-reducing agent 2-mercaptoethanol and vitamin E. 2-Mercaptoethanol seemed to be a more efficient scavenger of ROS produced by $Ni^{2+}$ or $Co^{2+}$ compared with vitamin E (Fig. 2, *a* and *b*). In fact, vitamin E was not very efficient in scavenging ROS in $Ni^{2+}$-exposed cells and was only partially effective in $Co^{2+}$-exposed cells. The addition of 2 mM 2-mercaptoethanol to $Ni^{2+}$- or $Co^{2+}$-exposed cells completely eliminated the DCF-detectable free radicals produced by the metals (Fig. 2, *A* and *B*). 2-Mercaptoethanol alone, or in combination with $Ni^{2+}$, $Co^{2+}$, or hypoxia did not affect *Cap43* gene expression but diminished detectable ROS levels (see above; Fig. 3). The addition of $H_2O_2$ alone or in combination with $Ni^{2+}$, $Co^{2+}$, or hypoxia similarly had no effect on *Cap43* gene expression (Fig. 3) despite somewhat augmenting the levels of ROS in Ni-exposed cells (Fig. 2, *A* and *B*). These data suggested that the up-regulation of *Cap43* gene expression by metals that induce hypoxia-related genes was not linked with enhanced ROS formation in A549 cells.

**The Effect of Free Radical Scavengers on HIF-1 Reporter Plasmids.** The augmentation of *Cap43* gene expression by hypoxia or metals was dependent on HIF-1 because this augmentation was absent in HIF-1$\alpha$-deficient cells (18). However, hypoxic conditions stabilized *Cap43* mRNA. To study whether there was a direct effect of ROS on HIF-1 activity, we used transient transfection assay and a HIF-1-dependent reporter plasmid, HRE-Luc. This plasmid contained three HIF-1-responsive elements of the iNOS promoter (6). Hypoxia or $Ni^{2+}$ enhanced the expression of the reporter plasmid, as did DFX, which also simulated hypoxic-related genes. The addition of 500 $\mu$M $H_2O_2$ or 2 mM 2-mercaptoethanol did not affect the enhancement in reporter plasmid activity attributed to either $Ni^{2+}$ or hypoxia (Fig. 4). Neither $H_2O_2$ nor 2-mercaptoethanol alone was effective in stimulating HRE-Luc expression (Fig. 4).

## Discussion

Current models of hypoxic response are based on changes in oxygen sensor or on the production of ROS during hypoxia. Although disputed, ROS are produced during hypoxia, but non-ROS-dependent oxygen sensing may also exist. $Ni^{2+}$ or $Co^{2+}$ produce ROS in cells, but they may also substitute for iron in oxygen sensing. In this situation, it is extremely difficult to differentiate between the two models. Therefore, we investigated the role of ROS in hypoxia-dependent gene induction triggered by $Ni^{2+}$ and $Co^{2+}$, as well as by hypoxic conditions. Recently, we have shown that the $Ni^{2+}$, $Co^{2+}$, or hypoxia induced expression of *Cap43* that was dependent on HIF-1 (18). The high inducibility of *Cap43* by hypoxia via a HIF-1-dependent pathway in many cell lines suggested that it might be a good marker of the activation of signals that lead to HIF-1 activation and enhanced expression of hypoxia-related genes.

Exposure of A549 cells to $Ni^{2+}$ or $Co^{2+}$ resulted in increased ROS production in cells, as detected by the DCF method. We have previously shown that exposure of mouse 3T3 cells to $NiCl_2$ caused increases of DCF fluorescence and that $Ni^{2+}$ was a better inducer of ROS than $H_2O_2$ (15). We also compared ROS production induced by $Ni^{2+}$ or $Co^{2+}$ in the same cell type. It was known that both $Co^{2+}$ and $Ni^{2+}$ produced oxidative stress in cells, perhaps by depletion of reduced glutathione (15, 21); however, a comparison of the intensity of oxidative stress by both metals in the same cell type had not been

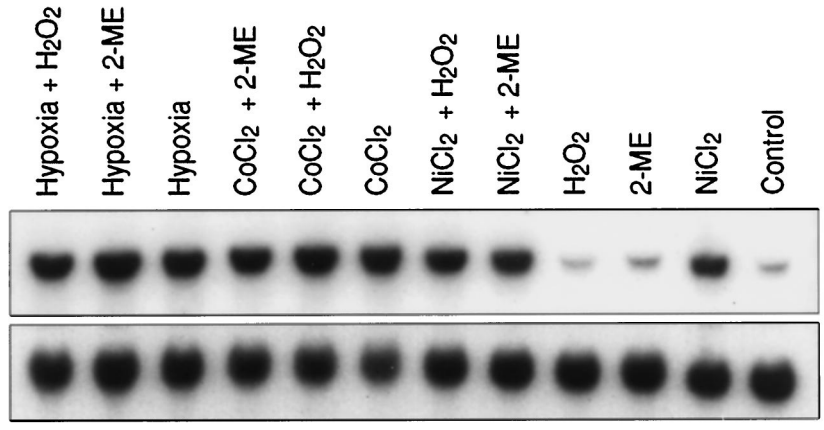


Fig. 3. The effect of 2-mercaptoethanol or $H_2O_2$ on metal- or hypoxia-driven *Cap43* gene expression. A549 cells were exposed to Ni, Co, or hypoxia alone or in combination with 2 mM 2-mercaptoethanol or 500 $\mu$M $H_2O_2$ for 20 h. Fifteen micrograms of total RNA were isolated and subjected to a Northern blot analysis. The *bottom lane* shows the expression of $\beta$-actin in the same sample.

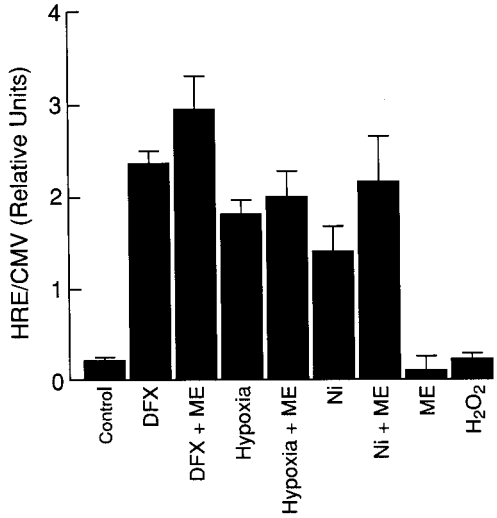


Fig. 4. HIF-1-dependent transcription in A549 cells exposed to Ni, DFX, or hypoxia. A549 cells were transfected with 1 $\mu$g of HRE-Luc plasmid. In some instances, transfected cells were also treated with 1 mM nickel chloride, 260 $\mu$M DFX, hypoxia (1% $O_2$), 2 mM 2-mercaptoethanol, or 500 $\mu$M $H_2O_2$ for 16 h. Results are presented as a ratio of HRE-Luc expression:CMV-Luc expression in the same cells.

previously examined. We have found that $Co^{2+}$ was more active in generating ROS than $Ni^{2+}$, but there was no correlation between the level of ROS in A549 cells and the degree of *Cap43* gene induction.

The free radical scavenger 2-mercaptoethanol efficiently attenuated ROS production by $Ni^{2+}$ or $Co^{2+}$ but failed to suppress the enhanced *Cap43* gene expression attributed to metals or hypoxia. We did not, however, show the level of ROS in A549 cells exposed to hypoxia, but it was conceivable that hypoxia might elevate ROS in these cells similarly to what has been found in the Hep3B cells (11). In our experiments, we found that hypoxia increased the level of ROS (data not shown), however, this may be due to reoxygenation when the microplate was rapidly transferred from a hypoxic chamber to the microplate reader for measurement of DCF fluorescence. Failure to suppress the *Cap43* gene expression by 2-mercaptoethanol was in agreement with the lack of activity of 2-mercaptoethanol on the expression of HIF-1-dependent plasmid alone or in combination with $Ni^{2+}$, $Co^{2+}$, DFX, or hypoxia.

Vitamin E seemed to be inefficient in suppressing ROS formation by both metals. ROS are considered to be important in Ni toxicity and mutagenicity, therefore, the observation that vitamin E did not suppress ROS produced by $NiCl_2$ was in line with our previous findings that vitamin E did not inhibit chromosomal aberrations or mutagenesis in cells exposed to soluble nickel chloride (22, 23). However, vitamin E was somewhat effective at inhibiting the effects of carcinogenic water insoluble nickel sulfide particles (23).

The role of ROS in the activation of HIF-1 is not clear (for review see Refs. 1 and 7). It was suggested that changes in cellular redox state signaled an activation of HIF-1 (24, 25). The fact that transition metals induced hypoxia-related genes and produced ROS in cells is not teleologically consistent with the observed induction of hypoxic genes by the free radical scavenger DFX (26). Our results suggested that the role of ROS related to HIF-1 activation may have been overstated. From the experiments shown here, we conclude that ROS were, indeed, generated in cells exposed to metals that induce hypoxic genes, but it was unlikely that they participated in the hypoxic signal transduction pathways. A similar observation was made by Hohler *et al.* (27), who found that ROS production was increased in PC12 cells during hypoxia but was not the cause of the hypoxia-driven tyrosine hydroxylase mRNA formation.

In summary, we concluded that ROS production was increased during exposure of A549 cells to Ni or Co, however, ROS were not involved in HIF-1 activation and did not cause up-regulation of *Cap43*. It is possible that these metals substituted for Fe in the oxygen sensor and thereby activated HIF and subsequently *Cap43*.

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