0026-895X/03/6406-1485–1493$7.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 64:1485–1493, 2003

Vol. 64, No. 6
2638/1109664
Printed in U.S.A.

Hypoxia Inducible Factor-1α–Independent Suppression of Aryl Hydrocarbon Receptor-Regulated Genes by Nickel

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Received May 5, 2003; accepted August 28, 2003

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Aryl hydrocarbon receptor (AhR)-dependent enzymes are involved in the biotransformation of harmful xenobiotics into more easily excretable metabolites. Cross-talk between the AhR pathway and the hypoxia inducible factor-1α (HIF-1α) pathway has been demonstrated previously, although the mechanism remains unclear and quite controversial. Because nickel is known to mimic hypoxia, we investigated the effects of short-term nickel exposure on AhR-dependent gene expression. Gene-chip analysis identified several AhR-dependent genes that are suppressed by exposure to nickel. Using Northern blots, we then confirmed that nickel can down-regulate both the basal and benzo[*a*]pyrene-inducible expression of AhR-dependent genes in mouse and human cell lines. Using a HIF-1α knockout cell line and 3-[2-[4-(bis-(4-fluorophenyl) methylene]-1-piperidinyl)ethyl]-2,3-dihydro-2-thioxo-4(1H)-quinazolinone (R59949), which blocks HIF-1α protein accumulation, we show HIF-1α–independent suppression of AhR-dependent genes by nickel. Desferrioxamine and hypoxia were also able to suppress the basal and inducible expression levels of AhR-regulated genes. Finally, dimethyloxalylglycine, an inhibitor of Fe(II)- and 2-oxoglutarate–dependent dioxygenases also inhibited AhR-dependent expression in a HIF-1α–independent manner. Our data suggest that an Fe(II)-, oxoglutarate-, and oxygen-dependent enzyme may directly or indirectly be involved in the regulation of AhR-dependent transcriptional activity by nickel and other hypoxia-mimicking agents.

In response to xenobiotic insult, cells may attempt to biotransform foreign compounds into more easily excretable metabolites, thus reducing the exposure to harmful chemicals. Aromatic hydrocarbons, including benzo[*a*]pyrene, are known to be metabolized by a battery of cellular enzymes, some of which are activated in a ligand-dependent manner by the aryl hydrocarbon receptor (AhR). The AhR-regulated xenobiotic-metabolizing enzymes (XMEs) involved in the detoxification of harmful compounds include both phase I (CYP1A1, CYP1A2, and CYP1B1) and phase II enzymes [aldehyde dehydrogenase 3A1 (ALDH3), NADPH quinone reductase (NQO1), and glutathione *S*-transferase Ya] (Gu et al., 2000). The pathway involved in the activation of AhR-dependent XME expression has been the subject of extensive investigation. It was shown that unliganded cytoplasmic AhR exists in a complex with several cellular chaperone proteins (Petrulis and Perdew, 2002). After an exposure, ligand binds to the AhR protein, leading to the dissociation of the chaperone proteins from AhR and its translocation to the nucleus, in which it dimerizes with the aryl hydrocarbon nucleotranslocator (ARNT/HIF-1β) (Hankinson, 1995). Consequently, the binding of the AhR-ARNT complex to the promoter of target genes with the core sequence TNGCCTG activates the transcription of genes in the AhR gene battery (Bacsi et al., 1995; Swanson et al., 1995). The general subject matter of this model is well understood; however, the fine details are still being worked out.

ARNT/HIF-1β, the heterodimerization partner for AhR, is also known to be involved in several other signal transduction pathways. For example, it can heterodimerize with hypoxia-inducible factor-1α (HIF-1α). HIF-1α belongs to the basic helix loop helix/PAS protein family and is stabilized by hypoxic conditions. After binding with ARNT/HIF-1β, HIF-1α is involved in the regulation of the cellular response to low oxygen levels (hypoxic conditions) (Semenza, 2001). Nickel, cobalt, and desferrioxamine can mimic hypoxia, thus stabilizing the HIF-1α protein (Salnikow et al., 2000a). One likely explanation of the ability of nickel, cobalt, and desferrioxamine to stabilize HIF-1α protein comes from recent studies on protein hydroxylation. It was shown that the stability and transcriptional activation of the HIF-1α protein is

This work was supported by grants ES05512, ES00260, ES10344, and T32-ES07324 from the National Institute of Environmental Health Sciences and CA16087 from the National Cancer Institute.

ABBREVIATIONS: AhR, aryl hydrocarbon receptor; HIF-1α, hypoxia inducible factor-1α; XME, xenobiotic-metabolizing enzyme; ALDH3, aldehyde dehydrogenase 3A1; NQO1, NADPH quinone reductase; ARNT, aryl hydrocarbon receptor nuclear translocator; DMOG, dimethlyoxalylglycine; B(a)P, benzo[*a*]pyrene; XRE, xenobiotic-response element; R59949, 3-[2-[4-(bis-(4-fluorophenyl)methylene]piperidin-1-yl)ethyl]-2,3-dihydro-2-thioxo-4(1H)-quinazolinone; MTT, 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide; PCR, polymerase chain reaction; RT, reverse transcriptase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

regulated by at least two iron-dependent hydroxylases. Hydroxylation of proline 564 on HIF-1α allows von Hippel-Lindau binding, leading to rapid degradation of the protein (Ivan et al., 2001; Jaakkola et al., 2001), whereas a hydroxylated asparagine prevents p300 binding and the transactivation of HIF-1α–dependent genes (Lando et al., 2002). Both the prolyl and the asparagine hydroxylases are members of a dioxygenase superfamily that requires molecular oxygen, Fe(II), and 2-oxoglutarate to function (Kivirikko and Myllyharju, 1998; Jaakkola et al., 2001). It is conceivable that nickel, cobalt, and desferrioxamine interfere with iron metabolism or replace the nonheme iron in these enzymes. This may lead to a loss of hydroxylase activity, stabilization of the HIF-1α protein, and activation of HIF-1α–dependent genes.

Conditions that induce HIF-1α not only induce HIF-1α–dependent genes, but they have been shown to suppress AhR-regulated genes (Chan et al., 1999; Kim and Sheen, 2000). Because ARNT/HIF-1β is a common heterodimerization partner for both AhR and HIF-1α, it has been proposed that there could be a cross-talk between these two pathways. Although Pollenz et al. (1999) has shown that competition for the ARNT protein is not a likely cause of the cross-talk, other articles have suggested that ARNT or another protein might be a limiting factor between the hypoxia-response pathway and the AhR pathway (Gradin et al., 1996; Kim and Sheen, 2000). Indeed, several labs have shown that hypoxia, cobalt, and desferrioxamine can inhibit the induction of XRE-dependent reporter constructs, as well as the expression of endogenous AhR-dependent genes in vitro (Reisdorph and Lindahl, 1998; Nie et al., 2001). Additionally, the induction of the hypoxia-response pathway has been shown to inhibit the binding of the AhR-ARNT complex to XREs as measured by electromobility shift assays (Nie et al., 2001). Although some research has been done, it remains unclear what role, if any, HIF-1α plays in modulating AhR-dependent gene expression.

We have recently shown that nickel can induce the HIF-1α–dependent pathway. It has been hypothesized that the induction of the HIF-1α pathway may be involved in the carcinogenic process (Salnikow et al., 1999, 2000b). In this article, using HIF-1α knockout cells and an inhibitor of HIF-1α–dependent transcription, we show HIF-1α–independent down-regulation of both basal and inducible gene expression of AhR-regulated genes by nickel, cobalt, and the 2-oxoglutarate–dependent hydroxylase inhibitor dimethyloxalylglycine (DMOG). Because nickel, cobalt, and desferrioxamine may interfere with iron metabolism, we hypothesize that an iron-dependent enzyme might be involved in the regulation of AhR-dependent genes. Testing of this hypothesis could further elucidate connections between AhR and HIF-1α signaling pathways and provide understanding into possible mechanisms of toxicity for hypoxia and hypoxia-mimicking agents that act independently of the HIF-1α transcription factor.

TABLE 1
Cell survival

A549 cells were exposed to various concentrations of nickel, cobalt, chromium, cadmium, B(a)P, and DMOG. Doses with similar toxicities were listed in Table 1 and are representative of chemical doses used in this article. Toxicity was determined using the standard MTT assay. Values are presented as mean ± S.D.

Treatment	Survival ± S.D.
	%
Nickel chloride 1 mM	92.78 ± 7.9
Cobalt chloride 400 μM	87.66 ± 12.5
Potassium chromate 5 μM	89.93 ± 7.1
Cadmium chloride 5 μM	85.62 ± 0.8
B(a)P 5 μM	93.76 ± 9.6
B(a)P 5 μM + $NiCl_2$ 1 mM	90.63 ± 1.1
DMOG 1 mM	91.9 ± 5.4

Materials and Methods

Chemicals. Nickel chloride, cobalt chloride, cadmium chloride, 2-mercaptoethanol, and benzo[*a*]pyrene were obtained from Sigma-Aldrich (St. Louis, MO). Potassium chromate was obtained from Fisher Scientific Co. (Fair Lawn, NJ). Dimethyloxalylglycine was purchased from Frontier Scientific (Logan, UT). R59949 was purchase from BIOMOL Research Laboratories (Plymouth Meeting, PA). Other common stock chemicals were obtained from Invitrogen (Carlsbad, CA) and Sigma-Aldrich.

Cell Culture. HIF-1α knockout and wild-type cells were described previously (Salnikow et al., 2000a). Briefly, wild-type and HIF-1α knockout cells were derived from C57 B mice with either a normal or HIF-1α knockout genotype. A549 cells were obtained from the American Type Culture Collection (Manassas, VA). This cell line was maintained in Ham's F-12K medium (Invitrogen). All media were supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cell monolayers were incubated at 37°C in the presence of 5% CO_2 in air. Exposure to hypoxia has been described previously (Salnikow et al., 2000a). Briefly, cells were exposed to 1% oxygen to simulate hypoxic conditions. During coexposure of cells, chemicals were added either simultaneously or as indicated in the figure legends.

Determination of Cell Toxicity with the MTT Assay. A549 cells were seeded into a 96-well plate with a density of 15,000 cells per well and were allowed to attach for 24 h. Cells were then exposed in triplicate to the compounds listed in Table 1 for 24 h. After the exposure, the media were removed, and cells were rinsed with phosphate-buffered saline. Fresh media (200 μl) were then added to the wells. Added to each well was 20 μl of a 5 mg/ml phosphate-buffered saline solution of 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma). The 96-well plate was then incubated for an additional 4 h at 37°C. Media were then removed and, 150 μl of dimethyl sulfoxide was added to each well to dissolve formazan crystals. The absorbance was then read at 590 nm on an HTS7000 Bioassay Reader from PerkinElmer Life Sciences (Boston, MA).

RNA Isolation and Northern Blots. Total RNA was isolated from cells using TRIzol (Invitrogen). RNA was quantified by reading the UV absorbance at 260 nm on a spectrophotometer. Total RNA (15 μg) was electrophoresed on a 1.2% agarose/formaldehyde gel. RNA was then vacuum-transferred to a BrightStar-Plus positively charged nylon membrane (Ambion, Austin, TX). Probes were labeled with [α-^{32}P]dCTP using the Prime-a-Gene Labeling System (Promega, Madison, WI). The membranes were prehybridized in ULTRAhyb solution (Ambion) for 2 to 3 h and hybridized with the probe of interest overnight. Membranes were then washed and exposed to X-ray film (Eastman Kodak, Rochester, NY) or a phosphor screen. Northern blots were repeated three times, and representative blots were chosen for publication.

Gene Chip. HIF-1$\alpha^{+/+}$ and HIF-1$\alpha^{-/-}$ cells were exposed to 1 mM nickel chloride for 20 h, and labeled RNA was hybridized to murine Gene Chip U74Av2. Gene-chip probe preparation and analysis of the results was performed as described previously (Salnikow et al., 2002).

Northern Blot Hybridization Probes. For PCR-derived probes, one-step RT-PCR was performed using the LightCycler RNA Amplification Kit SYBR Green 1 (Roche Diagnostics, Indianapolis, IN). The following primers were used to amplify PCR products that were subsequently quantified and used as probes: murine CYP1B1: upper 5′-gcccctggcgacgatt-3′, lower 5′-aggttgggctggtcactcat-3′, annealing

temperature, 59°C; human NADPH quinone reductase: upper 5′-tggtttgagcgagtgttcatagg-3′, lower 5′-aggaaatccaggctaaggaatctc-3′, annealing temperature, 57°C; and murine CYP1A1: upper 5′-TTGCCCTTCATTGGTCACAT-3′, lower 5′-GAGCAGCTCTTGGTCATCAT-3′, annealing temperature, 56°C. A plasmid containing the murine NADPH quinone reductase was kindly provided by Dr. T. Kensler (Johns Hopkins University, Baltimore, MD). The probe was isolated as an 800-base pair NcoI/HindIII digestion product. The plasmid containing a probe for the human class-3 aldehyde dehydrogenase was graciously furnished by Dr. H. Weiner (Purdue University, West Lafayette, IN). The 1500-base pair insert used for the probe was cut from the plasmid using an XbaI/HindIII digest.

RT-PCR. RT-PCR reactions were performed using the Superscript One-Step RT-PCR reaction kit with Platinum *Taq* Polymerase (Invitrogen). RNA from A549 cells was reverse-transcribed at 50°C for 30 min. The PCR reaction for HIF-2α/endothelial PAS domain protein 1 was carried out using the following primers for 21 cycles at 60°C: 5′-CTTCTCAATCT ACATCAGGACG-3′ (HIF2F) and 5′-CTGCCCTCCTCACAATAGTC-3′ (HIF2R). For HIF-3α, the PCR reaction was performed with the following primers at 51°C for 27 cycles: 5′-TGCGCCGCAGGGGAGTGGAAC-3′ (HIF3-F1) and 5′-CGCCCGCGGC TGGTGAG-TGTA-3′ (HIF3-R1). PCR for GADPH was carried out at 60°C for 17 cycles with commercially available primers from BD Biosciences Clontech (Palo Alto, CA).

Results

Nickel Suppresses the Basal and Inducible Levels of AhR-Regulated Gene Expression. To identify genes affected by short-term exposure to nickel, HIF-1α knockouts and wild-type cells were exposed to 1 mM nickel chloride. Using the GeneChip (Affymetrix, Santa Clara, CA), we found that nickel suppresses the expression of AhR-regulated genes. The data showing the ability of nickel to affect gene expression in both a HIF-1α-dependent and –independent manner has been published previously (Salnikow et al., 2002, 2003a,b). Here, we report nickel-induced suppression of several genes involved in xenobiotic metabolism (Table 2). The table contains several AhR-regulated genes that were down-regulated, including CYP1B1, NQO1, UDP-glucuronyltransferase 1A6, and glutathione *S*-transferase Ya. All of the observed AhR-dependent genes were down-regulated in the HIF-1α knockout cells. One of the most highly suppressed genes was CYP1B1, which was suppressed more than 20-fold in both wild-type and HIF-1α knockout cells.

To confirm the data obtained from the gene chip, we used Northern blots to examine the effect of nickel on the suppression of AhR-dependent genes in mouse embryo fibroblasts. Nickel substantially suppressed the basal-level expression for CYP1B1 and NQO1 in both HIF-1α–proficient and –deficient mouse cells (Fig. 1, A and B). We also found that nickel suppressed the B(a)P-inducible expression levels of NQO1 (Fig. 1C). Because CYP1A1, a prototypical AhR-regulated gene, did not show suppression by nickel in our gene-chip results, we performed a Northern blot analysis to determine whether it was basally expressed in HIF-1$\alpha^{+/+}$ and HIF-1$\alpha^{-/-}$ cells. Our results show that there was no basal expression for CYP1A1 in either cell line, but that the B(a)P-inducible expression could be suppressed by nickel (Fig. 1D). To determine whether suppression of AhR-regulated genes by nickel also occurred in human cells, we performed Northern blots for these genes in A549 cells. Although ALDH3 was not identified by array analysis, it is an AhR-regulated gene known to be expressed in the A549 cell line. Both the basal and the B(a)P-inducible levels of transcription for ALDH3 and NQO1 were diminished by treatment with 1 mM nickel and 400 μM cobalt (Fig. 2). The B(a)P-inducible expression was even more significantly reduced when cells were pretreated for 2 h with nickel or cobalt (data not shown). In addition to ALDH3 and NQO1, we also checked the expression of CYP1A1 using RT-PCR. In agreement with previously published data, nickel was able to suppress the B(a)P-inducible expression of CYP1A1; however, low basal expression made it difficult to observe basal level suppression (data not shown). Next, we investigated the time course of this effect with the use of ALDH3 as an AhR-regulated gene (Fig. 3). When A549 cells were exposed to nickel alone, no major inhibition was seen until 16 h of treatment, and the basal expression was substantially reduced at 24 h. Similar results were obtained for B(a)P-inducible levels of expression for ALDH3.

The Down-Regulation of AhR-Dependent Genes by Nickel and Cobalt Is Not Caused by Oxidative Stress. Cobalt and, to a lesser extent, nickel are both known to cause oxidative stress (Salnikow et al., 2000b). To determine whether oxidative stress was involved in the down-regulation of AhR-dependent genes by nickel and cobalt, A549 cells were

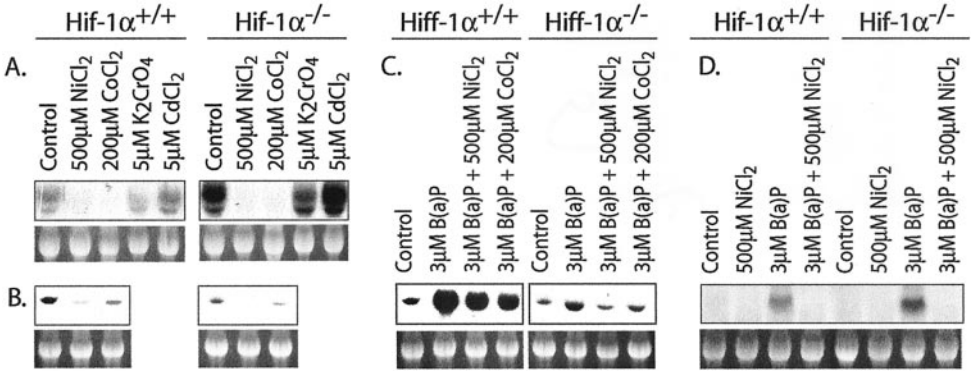

Fig. 1. Northern blot analysis of CYP1B1, NQO1, and CYP1A1 in HIF-1α–proficient cells and HIF-1α knockout cells (mouse embryo fibroblasts). RNA was isolated as described under *Materials and Methods*. Total RNA (15 μg) was subjected to Northern blot analysis and hybridized to either a mouse CYP1B1, NQO1, or CYP1A1 probe. The ethidium bromide staining of the lower ribosomal subunit is shown to ensure equal loading. The Northern blots shown come from one experiment but are similar to results that have been replicated in two additional experiments. A, suppression of basal CYP1B1 levels by nickel and cobalt in cells exposed to 500 μM $NiCl_2$, 200 μM $CoCl_2$, 5 μM K_2CrO_4, or 5 μM $CdCl_2$ for 24 h. B, suppression of basal levels of NQO1 by 500 μM $NiCl_2$ and 200 μM $CoCl_2$. C, suppression of B(a)P-inducible levels of NQO1 by 500 μM $NiCl_2$ and 200 μM $CoCl_2$. D, suppression of B(a)P-inducible levels of CYP1A1 by 500μM $NiCl_2$.

exposed to both nickel and B(a)P in the presence of vitamin E and 2-mercaptoethanol. Pretreatment with vitamin E could not substantially reverse the suppression of B(a)P-inducible gene expression caused by nickel and cobalt for both NQO1 and ALDH3 (Fig. 2, A and B). Additionally, pretreatment with 2-mercaptoethanol did not reverse the effects of nickel for ALDH3 (Fig. 2C).

Iron Is Involved in the Regulation of AhR-Regulated Genes by Nickel and Cobalt. Desferrioxamine induces the HIF-dependent pathway, probably by chelating iron from one or more of the iron-dependent hydroxylases involved in the regulation of HIF-1α. Exposure to desferrioxamine resulted in a notable reduction in the levels of basal and inducible transcription for NQO1 and ALDH3 (Fig. 4, C and D). The suppression of the basal level of ALDH3 and NQO1 by nickel and cobalt could be partially reversed by pretreatment with 500 μM iron citrate (data not shown). These data demonstrate that an iron-dependent enzyme may be involved in the regulation of AhR-dependent genes.

Hypoxia and Other Hypoxia-Mimicking Agents Suppressed AhR-Regulated Genes in both Murine and Human Cells. Because hypoxia is known to suppress AhR-regulated gene expression, we wanted to confirm the effect of hypoxia on AhR-regulated gene expression in our system. Hypoxia is hypothesized to activate the HIF-1α–dependent pathway by limiting oxygen, which is needed by the iron-dependent hydroxylases that control the level and transcriptional activity of HIF-1α (Kivirikko and Myllyla, 1980; Kivirikko and Myllyharju, 1998; Jaakkola et al., 2001). Treatment of A549 cells with hypoxia (1% oxygen) resulted in the down-regulation of both basal and B(a)P-inducible levels for ALDH3 (Fig. 4A). The inducible level of NQO1 was also down-regulated, whereas the basal level seemed unaffected by hypoxia treatment (Fig. 4B).

To confirm that the down-regulation of AhR-regulated genes was specific for agents that induce HIF-1α like nickel and cobalt, we exposed cells to several other metals. In both murine and human cells, compared with doses of cadmium and chromium with similar toxicities, nickel and cobalt produced a substantial down-regulation of AhR-dependent genes, whereas cadmium and chromium did not (Figs. 1A; 2, A and B; and 5, A and B; Table 1). Nickel and cobalt were also able to suppress the inducible levels of NQO1 and ALDH3 in A549 cells (Fig. 2, A and B).

DMOG, a competitive inhibitor of oxoglutarate-dependent enzymes, is known to induce HIF-1α–dependent genes. To confirm this, we show that it can induce CAP43/NDRG1 (Fig. 6B). Exposure to DMOG reduced the basal level expression of ALDH3 and NQO1 in a concentration-dependent manner (Fig. 6, C and D). DMOG was also able to suppress the inducible levels of expression for these genes to sub-basal levels (Fig. 6, C and D). When A549 cells were exposed to both nickel and DMOG, the observed suppression was greater than for nickel or DMOG alone (Fig. 6, C and D).

Nickel and DMOG Produce HIF-1α–Independent Suppression of AhR-Regulated Genes in Both Murine and Human Cells. It has been hypothesized previously that ARNT or another cofactor may be a limiting factor in the regulation of AhR-dependent genes when the HIF-1α pathway becomes induced (Gradin et al., 1996; Kim and Sheen, 2000). To investigate this possibility, we use a mouse embryo fibroblast cell line with a knockout of the HIF-1α gene. For both CYP1B1 and NQO1, nickel (500 μM) and cobalt (200 μM) suppressed AhR-regulated gene expression in both wild-type and HIF-1$\alpha^{-/-}$ cells (Fig. 1, A and B). Furthermore, using DMOG (1 mM), a competitive inhibitor of oxoglutarate-dependent enzymes, which includes the iron-dependent hydroxylases responsible for regulating HIF-1α, and R59949

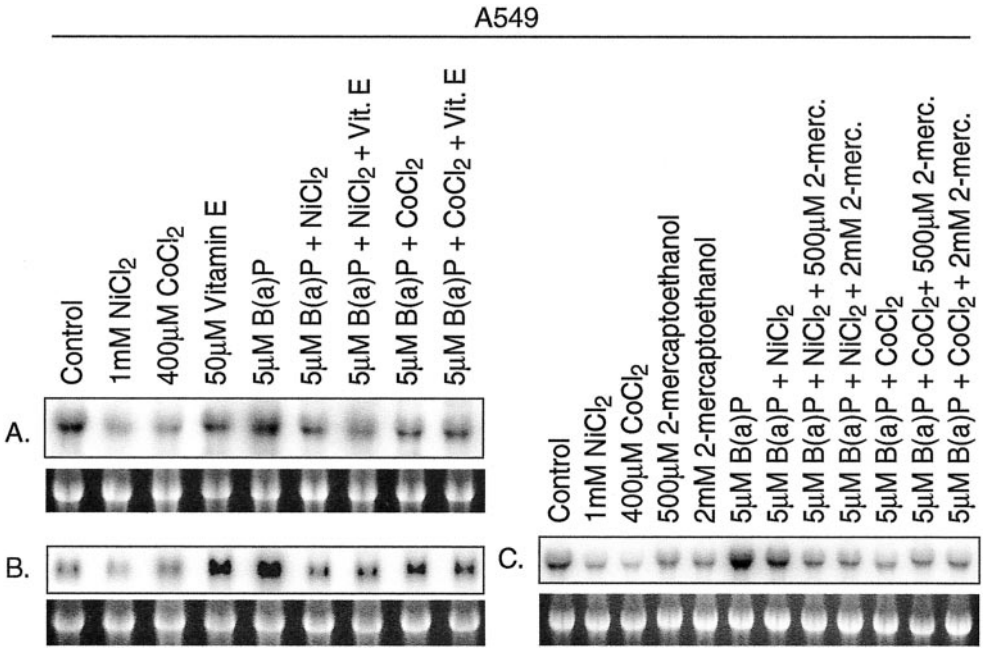

Fig. 2. Effects of pretreatment with 50 μM vitamin E or 2-mercaptoethanol on the inducible expression of ALDH3 or NQO1. A549 cells were pretreated with vitamin E or 2-mercaptoethanol for 2 h before exposure to B(a)P and $NiCl_2$ for 24 h. All other treatments times were 24 h. The Northern blots shown come from one experiment but are similar to results that have been replicated in two additional experiments. RNA was isolated as described under *Materials and Methods*. Total RNA (15 μg) was subjected to Northern blot analysis and hybridized to a probe for the gene of interest. Ethidium bromide of the lower subunit is shown to assess the RNA loading in each lane. A, hybridization to the human ALDH3 probe. B, hybridization to the human NQO1 probe. C, hybridization to the human ALDH3 probe.

(10 μM), an inhibitor of HIF-1α protein stabilization, we show that the basal and inducible expression of ALDH3 can be down-regulated by nickel and DMOG in an R59949-independent manner (Fig. 6A). These data indicate that in A549 cells, the down-regulation of AhR-regulated genes by nickel and DMOG is HIF-1α–independent and could involve an oxoglutarate-dependent enzyme.

Both HIF-2α and HIF-3α Are Expressed in A549 Cells. To determine whether HIF-2α and -3α were expressed in A549 cells, RT-PCR was performed. Both genes showed basal level expression, and no major changes were observed when cells were treated with 1 mM nickel chloride (Fig. 7). Treatment with cobalt chloride did not induce changes in HIF-3α, but a slight suppression of HIF-2α was observed.

Discussion

Activation of the AhR signaling pathway is involved in the adaptive and toxic responses to a variety of cellular insults, including dioxins and polycyclic aromatic hydrocarbons (Gu et al., 2000). XRE-containing phase I and phase II metabolic enzymes such as CYP1B1, NQO1, and ALDH3 are controlled by AhR signaling and play a role in the cellular response to a large variety of environmental contaminants, including benzo[*a*]pyrene. Although the induction of AhR-regulated genes has been the focus of extensive investigation, the suppression of both the basal and the inducible transcription of these genes has received less attention. Studies have suggested that the suppression of AhR-regulated genes results from cross-talk between the AhR and HIF pathways. These studies have shown that agents that induce the HIF-1α pathway, such as cobalt, desferrioxamine, and hypoxia, can suppress both AhR-dependent reporter genes and endogenous AhR-dependent genes (Chan et al., 1999; Kim and Sheen, 2000; Reisdorph and Lindahl, 2001). The mechanism of this cross-talk has not been fully elucidated, and some studies have presented contradicting arguments as to the mechanism. Here, we discuss a novel mechanism and possible toxicological implications.

To identify AhR-dependent genes affected by nickel, we performed a gene-chip analysis in which mouse embryo fibroblast cells were exposed to 1 mM nickel. Indeed, several AhR-regulated genes, including CYP1B1 and NQO1, were down-regulated by nickel treatment, supporting the idea that nickel treatment down-regulates AhR-dependent genes. Several AhR-dependent genes, such as CYP1A1, did not show nickel-induced suppression on our gene chip, but this is probably a result of low basal expression, making it difficult to observe suppression. We confirmed this hypothesis by conducting Northern blot analysis for murine CYP1A1 in HIF-1α–proficient and HIF-1α knockout cells (Fig. 1D). Although we focused our investigation on AhR-regulated genes, nickel also suppressed several non-AhR–regulated genes that have been discussed previously (Salnikow et al., 2002, 2003a,b). Interestingly, other genes involved in metabolism were suppressed by nickel as well, including other glutathione trans-

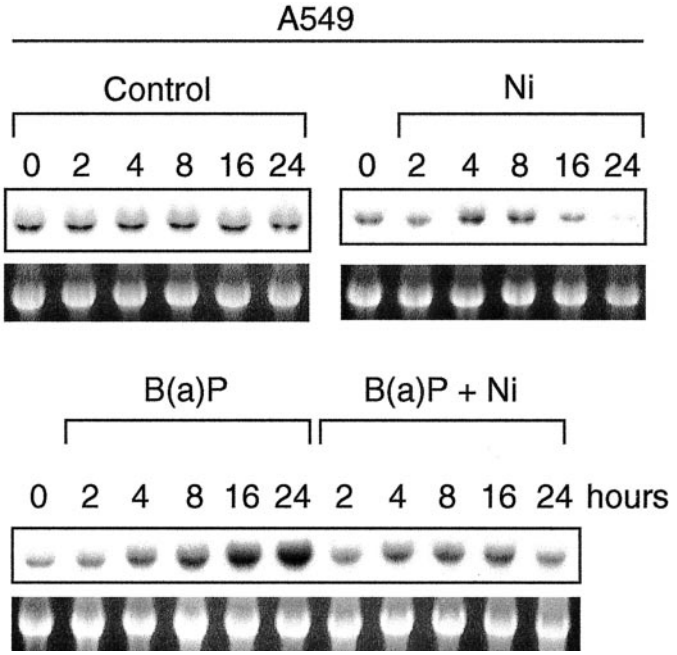

Fig. 3. Temporal changes of ALDH3 in human A549 cells. Cells were exposed for 2, 4, 8, 16, or 24 h to 1 mM $NiCl_2$, 5 μM B(a)P, or both. RNA was isolated from untreated cells at the numerous time points shown. RNA was isolated as described under *Materials and Methods*. Total RNA (15 μg) was subjected to Northern blot analysis by hybridization to the human ALDH3 probe. The ethidium bromide staining of the lower ribosomal subunit is shown to ensure equal loading. The Northern blots shown come from one experiment but are similar to results that have been replicated in two additional experiments.

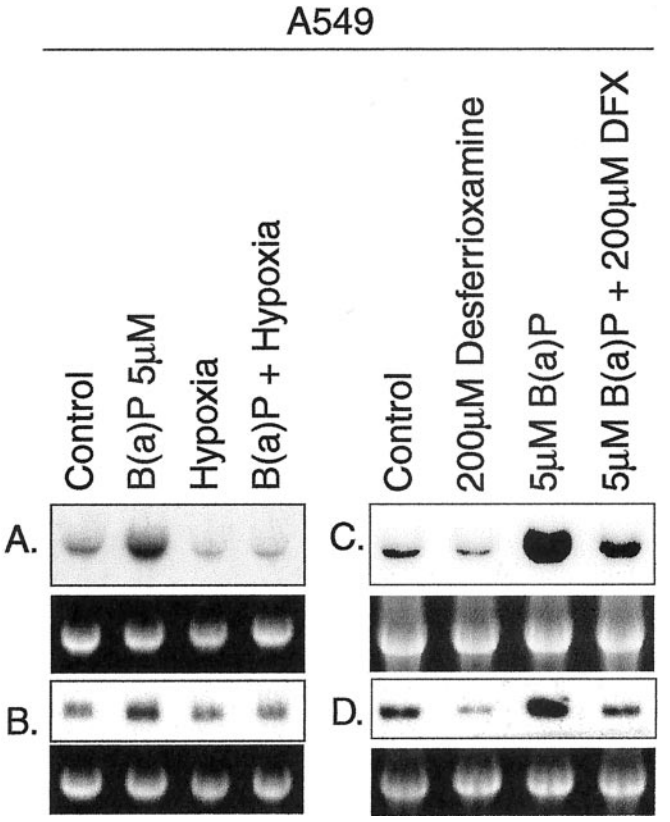

Fig. 4. Effects of hypoxia or desferrioxamine exposure on basal and B(a)P-inducible levels of ALDH3 and NQO1 expression. A549 cells were exposed either hypoxic conditions or to desferrioxamine, B(a)P, or both for 24 h. RNA was isolated as described under *Materials and Methods*. RNA (15 μg) was subjected to Northern blot analysis and hybridized to either the human ALDH3 probe (A and C) or human NQO1 probe (B and D). Ethidium bromide staining of the lower subunit is shown to ensure equal loading. The Northern blots shown come from one experiment but are similar to results that have been replicated in two additional experiments.

ferases and aldehyde dehydrogenase 2, a gene involved in alcohol metabolism. These data indicate that exposure to nickel could alter the balance of xenobiotic metabolism.

To make certain that we would see the same effect in human cells that we saw in murine cells, we exposed A549 cells to nickel and performed Northern blots on AhR-regulated genes. We chose A549 lung epithelial cells because the lung is a primary target of nickel exposure. Epidemiological studies have shown that exposure to nickel leads to lung injury and elevated risk for respiratory tract cancers (Shannon et al., 1984). Nickel suppressed both the basal and B(a)P-inducible expression of NQO1 and ALDH3 in these cells, as well as the B(a)P-inducible expression of CYP1A1. Although metabolites of B(a)P can alter gene expression independently of AhR through mechanisms involving oxidative stress, it has previously been shown that nickel, cobalt, desferrioxamine, and hypoxia all specifically suppress AhR-regulated genes,

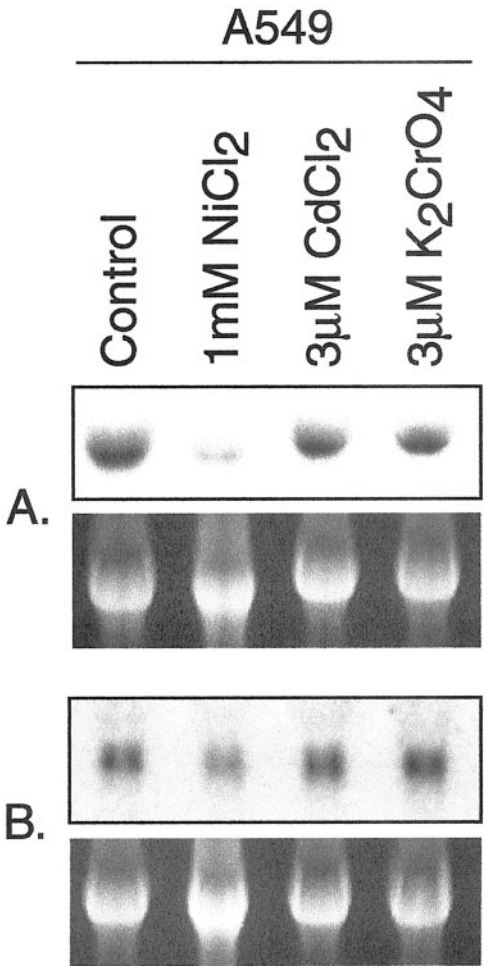

Fig. 5. Effect of various metals on the expression of ALDH3 and NQO1. A549 cells were exposed to either 1 mM $NiCl_2$, 3 μM K_2CrO_4, or 3 μM $CdCl_2$ for 24 h. RNA was isolated as described under *Materials and Methods*. RNA (15 μg) was subjected to Northern blot analysis and hybridized to either a human ALDH3 probe (A) or human NQO1 probe (B). Ethidium bromide staining of the lower RNA subunit is shown to ensure equal loading. The Northern blots shown come from one experiment but are similar to results that have been replicated in two additional experiments.

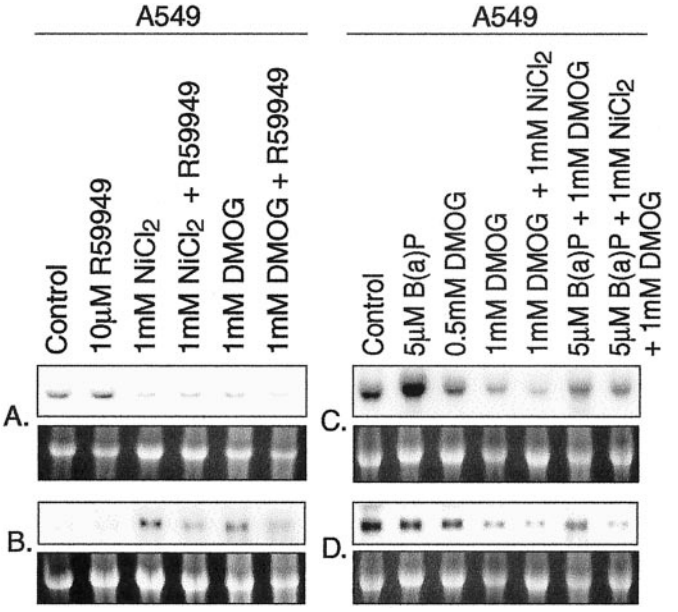

Fig. 6. The Effect of DMOG on the basal and inducible levels of ALDH3 and NQO1. RNA was isolated as described under *Materials and Methods*. RNA (15 μg) was subjected to Northern blot analysis and hybridized to either a human ALDH3 probe (A and C), human CAP43/NDRG1 probe (B), or human NQO1 probe (D). Ethidium bromide staining of the lower RNA subunit is shown to ensure equal loading. The Northern blots shown come from one experiment but are similar to results that have been replicated in two additional experiments. A and B, A549 cells were exposed to 1 mM DMOG, 10 μM R59949, or both for 24 h. Cells were pretreated with R59949 for 2 h in this treatment sequence. C and D, A549 cells were exposed to DMOG, B(a)P, and nickel separately and also in combinations with one another for 24 h. Treatment with more than one chemical was done simultaneously.

TABLE 2

Suppression by nickel

HIF-1α –proficient and HIF-1α–deficient mouse embryo fibroblasts were exposed to 1 mM nickel chloride, and transcriptional changes were analyzed with use of the GeneChip (Affymetrix). The listed genes are involved in xenobiotic metabolism.

Gene Name	-Fold Suppression		Accession Number
	Hif-1α $^{+/+}$	Hif-1α $^{-/-}$	
Cytochrome P450 1B1[a]	22.9	29.7	X78445
Prostaglandin-endoperoxide synthase 1	6.1	2.4	M34141
Aldehyde dehydrogenase II	6.0	9.0	M74570
NADPH quinone oxidoreductase[a]	3.4	3.1	U12961
Glutathione *S*-transferase θ1	2.6	N.S.	X98055
UDP glucoronyltransferase 1A6[a]	2.6	4.7	U16818
Glutathione *S*-transferase μ (GST 5–5)	2.5	1.6	J04696
Glutathione *S*-transferase α3	2.2	2.0	X65021
Glutathione *S*-transferase α Ya[a]	1.1	3.5	L06047

N.S., not suppressed.
[a] Known AhR-dependent gene.

such as ALDH3, through an AhR-mediated process (Reisdorph and Lindahl, 1998; Kim and Sheen, 2000; Nie et al., 2001).

Considering that nickel and cobalt have been known to produce oxidative stress, we wanted to determine whether vitamin E or 2-mercaptethanol could abrogate the down-regulation caused by these compounds. Because the suppression of AhR-dependent genes by nickel and cobalt could not be reversed by pretreatment with vitamin E or 2-mercaptoethanol, we concluded that oxidative stress is probably not involved in the observed suppression. If it were, we would probably see an increase in NQO1 expression, because this gene contains an electrophile response element. Surprisingly, we found that NQO1 expression was induced by vitamin E treatment. This may be because NQO1 plays a role in vitamin E metabolism (Ross et al., 2000).

Previous research has shown that hypoxia itself suppressed AhR-dependent gene expression in a cell-type–specific manner (Reisdorph and Lindahl, 1998). Here, we sought to confirm this occurrence in A549 cells. Hypoxia was able to affect the expression of both ALDH3 and NQO1, indicating that low oxygen levels can affect the regulation of AhR-regulated genes.

Several mechanisms explaining the cross talk between agents that induce the hypoxia pathway and AhR-dependent genes have previously been proposed. One hypothesis is that the down-regulation of AhR-regulated genes may be the result of a metabolic shift caused by general toxicity, in which nonessential genes are shut down. To examine this idea, we exposed A549 cells to doses of nickel, cobalt, chromium, and cadmium that had similar levels of toxicity. Our results show that the observed gene suppression only occurred during exposure to nickel and cobalt. This supports the idea that the suppression of AhR-dependent genes probably results not from a shift in the metabolic state of the cell but because of the inherent chemical attributes of nickel and cobalt.

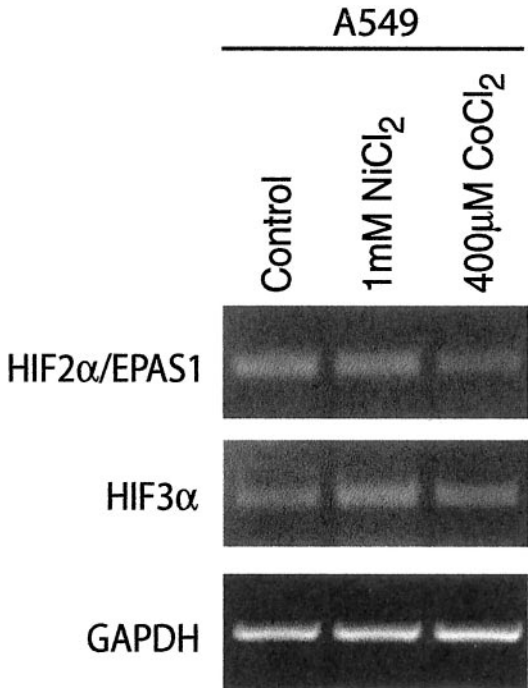

Fig. 7. RT-PCR for HIF-2α, HIF-3α, and GAPDH. Cells were exposed to nickel or cobalt for 24 h. RT-PCR reactions were done as described under *Materials and Methods*. GAPDH expression is shown to ensure equal loading.

Another explanation of the cross talk between agents that induce HIF-1α and the AhR pathway suggests that induction of the HIF-1α pathway recruits a limiting cellular factor, such as ARNT or a common coactivator, away from the AhR pathway. This is believable because ARNT and other factors are shared by both the HIF-1α and AhR pathway. Nie et al. (2001) suggested that the observed suppression by agents that induce HIF-1α might be because ARNT has a greater affinity for HIF-1α than AhR. However, quantitative Western blotting and immunostaining experiments showed that ARNT is probably not a limiting cofactor shared by these pathways in regard to AhR-regulated genes (Pollenz et al., 1999). This report did not rule out the possibility that AhR-regulated gene expression may be altered by another limiting factor shared by the two pathways. Using HIF-1α knockouts as a model, we have shown that nickel and cobalt can suppress the basal and inducible transcription levels of endogenous AhR-dependent genes in the absence of the HIF-1α pathway. We then looked at the ability of nickel and DMOG to suppress AhR-dependent genes via a HIF-1α–independent mechanism in a human cell line. To confirm reports that R59949 could block HIF-1α protein induction (Aragones et al., 2001), we showed that R59949 can block the HIF-1α inducible expression of CAP43/NDRG1. We then showed that nickel and DMOG can suppress ALDH3 independent of R59949, demonstrating HIF-1α–independent down-regulation. Interestingly, DMOG is an analog of 2-oxoglutarate and acts as an inhibitor of 2-oxoglutarate–dependent enzymes (Kivirikko and Myllyharju, 1998). This indicates that a member of the 2-oxoglutarate–dependent dioxygenase superfamily can regulate AhR-dependent genes in a HIF-1α–independent manner.

So far, the data presented in this article suggest that the suppression of AhR-regulated gene expression by nickel is not caused by a limiting factor between the AhR and HIF-1α pathways and that the observed down-regulation is not caused by oxidative stress. Nickel and cobalt have similar chemical properties, and we believe that they may be able to substitute for nonheme iron in cellular enzymes or alter iron transport. This idea is supported by studies on HIF-1α which show that exposure to nickel or cobalt results in the up-regulation of HIF-1α, which is regulated by two iron-dependent hydroxylases (Ivan et al., 2001; Jaakkola et al., 2001; Lando et al., 2002). In our experiments, the iron chelator desferrioxamine caused a down-regulation in the basal and inducible transcription of AhR-regulated genes. Because nickel and cobalt produce similar effects on AhR-regulated genes compared with the iron chelator desferrioxamine, it is likely that they can either replace nonheme iron or interfere with iron-dependent processes. This would alter the enzyme activity of the iron-dependent hydroxylases that regulate HIF-1α, resulting in its stabilization and in the transactivation of HIF-1α–dependent genes. Indeed, exposure to nickel or cobalt leads to both the stabilization of HIF-1α and activation of HIF-1α–dependent genes. Additional support for the interference of iron-dependent processes by nickel and cobalt is given, because iron citrate partially reversed the down-regulation of ALDH3 and NQO1.

Members of the dioxygenase superfamily, like the prolyl and asparagine hydroxylases that regulate HIF-1α, require

Fe(II), oxygen, and 2-oxoglutarate to be functional. Our data suggest that an iron-, oxygen-, and 2-oxoglutarate–dependent enzyme, which is probably a member of the 2-oxoglutarate–dependent dioxygenase superfamily, is involved in the transcriptional regulation of AhR-dependent genes. It remains unclear how AhR-regulated genes could be affected by a member of the 2-oxoglutarate–dependent dioxygenase superfamily, although several possibilities exist. It is possible that AhR itself, like HIF-1α, could be modified directly by a 2-oxoglutarate–dependent dioxygenase, although this remains unlikely because AhR does not have any domains known to be regulated in this manner (T. Davidson, unpublished observations). If AhR is not directly modified by an iron-dependent enzyme, one possibility is that HIF-2α or -3α, whose proteins are induced by hypoxia mimics via 2-oxoglutarate–dependent dioxygenases, may be involved in the suppression of AhR-regulated genes by nickel. We show that HIF-2α and -3α are expressed in A549 cells (Lando et al., 2003; Maynard et al., 2003). As a result, this article does not rule out the possibility that HIF-2α, -3α, or another member of the growing basic helix loop helix/PAS protein family may regulate AhR-dependent genes in an indirect manner. From the data presented, we hypothesize that an iron-dependent enzyme affects the regulation of AhR-dependent genes, but the exact mechanism remains unknown.

The down-regulation of AhR-regulated genes by nickel may have toxicological implications as well. Animal studies indicate that coexposure to nickel compounds and benzo[*a*]pyrene metabolites result in increased tumor occurrence (Maenza et al., 1971). Humans can be exposed to either nickel or cobalt and polycyclic aromatic hydrocarbons, such as benzo[*a*]pyrene, through both occupational and environmental exposure (Borm, 1997). Epidemiological studies have shown that exposure to both nickel and benzo[*a*]pyrene leads to an increase in lung and nasal cancers in humans (McEwan, 1976; Roberts et al., 1989). We have shown in vitro that when cells experience nickel or cobalt and benzo[*a*]pyrene exposure, the balance of metabolic phase I and phase II enzymes is altered. The induction of metabolic enzymes, particularly phase II enzymes, is believed to be very important in protection against cancer (Kwak et al., 2001). A reduction in these enzymes may lead to a decrease in toxicant removal and an increase in cancer susceptibility.

In summary, our results suggest that the down-regulation of AhR-regulated genes by agents that induce hypoxic-like conditions is independent of HIF-1α and that an Fe(II), oxygen, and 2-oxoglutarate–dependent enzyme is probably involved in the transcriptional regulation of these genes. We believe that this enzyme is a member of the Fe(II) and 2-oxoglutarate–dependent dioxygenase superfamily, although exactly how this enzyme regulates AhR-mediated gene expression is unclear. Furthermore, we suggest that the down-regulation of XMEs by nickel and cobalt may have toxicological ramifications. Further research should be done to determine the consequences of XME suppression, as well as the point in the AhR pathway in which a 2-oxoglutarate–dependent dioxygenase may play a role.

Acknowledgments

We thank Thomas Kluz for expert technical assistance. We are also grateful to Dr. T. Kensler (Johns Hopkins University, Baltimore, MD) for providing us with the murine NQO1 probe and Dr. H. Weiner (Purdue University, West Lafayette, IN) for giving us the human ALDH3 probe. We also acknowledge Haobin Chen for critical discussion of the project.

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