Cobalt Induces Heme Oxygenase-1 Expression by a Hypoxia-inducible Factor-independent Mechanism in Chinese Hamster Ovary Cells

REGULATION BY Nrf2 AND MafG TRANSCRIPTION FACTORS

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We have shown previously that activation of the heme oxygenase-1 (ho-1) gene by hypoxia in aortic smooth muscle cells is mediated by hypoxia-inducible factor-1 (HIF-1). In mutant (Ka13) Chinese hamster ovary cells lacking HIF activity, accumulation of ho-1 mRNA in response to hypoxia and the hypoxia-mimetic CoCl₂ was similar to that observed in wild type (K1) cells. These results support the existence of HIF-dependent and HIF-independent mechanisms for ho-1 gene activation by hypoxia and CoCl₂. In Ka13 cells, CoCl₂ stimulated expression of a luciferase reporter gene under the control of a 15-kilobase pair mouse ho-1 promoter (pHO15luc). Mutation analyses identified the cobalt-responsive sequences as the stress-response elements (StREs). In electrophoretic mobility shift assays, two specific StRE-protein complexes were observed using extracts from Ka13 cells. In response to cobalt, the level of the slower migrating complex X increased, whereas that of complex Y decreased, in a time-dependent manner. Members of the AP-1 superfamily of basic-leucine zipper factors bind to the StRE. Antibody supershift electrophoretic mobility shift assays did not detect Jun, Fos, or ATF/CREB proteins but identified Nrf2 and the small Maf protein, MafG, as components of complex X. Furthermore, dominant-negative mutants of Nrf2 and small Maf, but not of other bZIP factors, attenuated cobalt-mediated gene activation. Additional experiments demonstrated that induction by cobalt does not result from increased expression of MafG or regulated nuclear translocation of Nrf2 but is dependent on cellular oxidative stress. Unlike cobalt, hypoxia did not stimulate pHO15luc expression and did not increase StRE binding activity, indicating distinct mechanisms for ho-1 gene activation by cobalt and hypoxia in Chinese hamster ovary cells.

The adaptive response to low oxygen tension (hypoxia) includes increased production of a select set of proteins involved in oxygen homeostasis at the cellular and systemic levels. These proteins include: erythropoietin (EPO),¹ which stimulates erythropoiesis and the oxygen carrying capacity of the blood; vascular endothelial growth factor (VEGF) and one of its receptors Flt-1, which promote angiogenesis and the delivery of oxygen carrying blood to hypoxic sites; and glucose transporter-1 (Glut-1) and various glycolytic enzymes that provide for increased energy production through glycolysis during periods of reduced energy production via oxidative phosphorylation (reviewed in Ref. 1). Hypoxia-dependent expression of these and other proteins is regulated by both transcriptional and post-transcriptional mechanisms.

Transcriptional regulation of many hypoxia-responsive genes is critically dependent on a common cis-acting sequence, the hypoxia response element (HRE), and the trans-acting factor hypoxia-inducible factor-1 (HIF-1), a heterodimeric protein comprising α and β subunits. The α subunit is unique to HIF-1, whereas the β subunit is shared by other transcription factors, most notably the arylhydrocarbon receptor. In addition to hypoxia, certain transition metals such as cobalt, nickel, and manganese, and the iron chelator desferrioxamine (DFO) also stimulate expression of several hypoxia-responsive proteins including EPO, VEGF, phosphoglycerokinase 1, and Glut-1. These agents are considered to be hypoxia mimetics as they are proposed to exploit the cellular oxygen sensing mechanism or the hypoxia signal transduction pathway for gene activation. Consistent with this idea, cobalt and DFO stimulate HIF-1 activity and the HRE is required for target gene induction by these agents (reviewed in Refs. 1–3).

Another protein whose expression is stimulated by both hypoxia (4, 5) and cobalt (6, 7) is heme oxygenase-1 (HO-1). HO-1 catalyzes the first and rate-limiting reaction in heme catabolism, the oxidative cleavage of b-type heme molecules to yield

¹ The abbreviations used are: EPO, erythropoietin; heme, ferriprotoporphyrin IX; HO-1, heme oxygenase-1; CNC-Ki2IP, Cup/N’Collar/basic leucine zipper; Nrf, NF-E2-related factor; NF-E2; nuclear factor-erythroid 2; bZIP, basic region/leucine zipper; Gdbd, Gal4 DNA-binding domain; StRE, stress response element; ARE, antioxidant response element; HRE, hypoxia response element; HIF, hypoxia-inducible factor; EMSA, electrophoretic mobility shift assay; DFO, desferrioxamine; CHO, Chinese hamster ovary; VEGF, vascular endothelial growth factor; C/EBP, CAAT/enhancer-binding protein; kbp, kilobase pair(s); γ-GCS, γ-glutamylcysteine synthase.
equimolar quantities of biliverdin, carbon monoxide (CO), and iron. Biliverdin is subsequently converted to bilirubin by the action of biliverdin reductase. Both biliverdin and bilirubin are potent antioxidants, and CO has been shown to function as a neuronal messenger and a vasodilator (reviewed in Refs. 8 and 9). Recent studies indicate that CO modulates other cellular activities including signal transduction pathways (10) and apoptosis (11). In addition to hypoxia and cobalt, expression of HO-1 is widely induced by a variety of oxidative stress-associated agents including the substrate heme, inflammatory cytokines, heavy metals, hyperthermia, and UV irradiation (reviewed in Ref. 12). Because of its inducibility and the pleiotropic properties of the reaction products, HO-1 manifests potent antioxidant and anti-inflammatory activities and helps maintain cellular homeostasis in response to stress and injury.

Stimulation of HO-1 expression by most inducers is controlled primarily at the level of gene transcription, and we have previously identified two 5’ distal enhancer regions, E1 and E2, within the mouse ho-1 promoter that mediate gene activation by a variety of pro-oxidants (12). Both E1 and E2 contain multiple copies of a motif, termed the stress response element (StRE), that are sufficient and necessary for gene activation by multiple agents including heme, cadmium, and various xenobiotics (13, 14). Subsequent analysis of the mouse ho-1 promoter identified two sequence elements distinct from the StREs that resembled the consensus HRE. These elements bound HIF-1 and mediated hypoxia-dependent activation of a reporter gene in rat aortic vascular smooth muscle cells, indicating that HREs and HIF-1 regulate HO-1 expression in response to hypoxia (15). In contrast to these findings, Wood et al. (16) have developed mutants of Chinese hamster ovary (CHO) cells lacking the HIF-1α subunit and demonstrated that, although activation of certain hypoxia-responsive genes such as glut-1 is attenuated in these cells, induction of HO-1 mRNA by hypoxia and cobalt is not impaired. One explanation for the inducibility of HO-1 in these cells is the possibility of the more recently characterized HIF-2α (17) and HIF-3α (18) subunits to compensate for the HIF-1α deficiency. The mutant CHO cells, however, do not express HIF-2α and otherwise do not exhibit inducible HRE binding activity or HRE-dependent transcription activity (16, 17). Taken together, these results imply the existence of both HIF-dependent and HIF-independent mechanisms for ho-1 activation by hypoxia and hypoxia mimetics. We have used the mutant CHO cells to characterize the HIF-1-independent mechanism. Here we show that induction by cobalt, but not by hypoxia, is mediated by the StREs and the heterodimeric transcription factor Nrf2-MafG. Indirect evidence suggests that induction by hypoxia in CHO cells may not occur at the level of gene transcription.

**EXPERIMENTAL PROCEDURES**

**Materials**—Tissue culture media were from Life Technologies, Inc., and fetal bovine serum was obtained from Mediatech (Herndon, VA). Restriction endonucleases and other DNA modifying enzymes were purchased from either Life Technologies, Inc. or New England Biolabs (Beverly, MA). Oligonucleotides were synthesized by IDT, Inc. (Corvalle, IA). Radiolabeled nucleotides were obtained from PerkinElmer Life Sciences. Reagents for luciferase assays were purchased from Sigma-Aldrich. All antibodies except anti-mouse Nrf2 (kindly provided by M. Yamamoto, University of Tsukuba, Tsukuba, Japan) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were reagent-grade.

**Plasmids**—Construction of plasmids pH015uc, pH015ucαE1, pH015ucαE2, pH015ucαE1+α2, pE1uc, pE1uc+M379, pE2uc, and pE2ucM45 has been described previously (14, 19, 20). Plasmids pH01uc, pH04uc3, pH03.7uc, and pHO1.3uc were obtained by successively larger 5’ deletions of the ho-1 promoter in pHO15uc using appropriate restriction endonucleases. Plasmids pXSxRELuc, pXHRELuc, and pXSXHRELuc contain three copies of StRE3 (13) and the wild-type or mutant HIF-1 binding sites (15), respectively, of the mouse ho-1 gene cloned upstream of a minimal ho-1 promoter and the luciferase reporter gene. The dominant-negative mutants of Nrf2 and MaK (p18) have been described previously (21, 22). The cDNA clone for mouse JunD was obtained from American Type Culture Collection (ATCC; Manassas, VA). The dominant mutant of JunD (p52/JunD) was constructed by inserting a 0.5-kb fragment of Blunt-end cloning of the mouse JunD cDNA into the vector pCMV-Tag2B (Stratagene; La Jolla, CA). This manipulation deletes amino acid residues 1–169, resulting in a trans-activation domain-deficient mutant of JunD similar to one described previously (23). Dominant mutants of CAAT/enhancer-binding protein (C/EBP) and CREB have been developed and kindly provided by Dr. Charles W. Lang (NHLBI) (blunt-ended) fragment of the mouse JunD cDNA and the vector pCMV-Tag2B (Stratagene; La Jolla, CA). The Gal4 DNA binding domain (Gdbd) vector (pEG) was constructed by cloning the Gdbd (residues 1–147) into pEF/myc/mito (Invitrogen Corp., Carlsbad, CA). Full-length mouse Nrf2 or human MaFg cDNAs were subsequently cloned downstream of, and in frame with, the Gdbd to generate pEG/Nrf2 and pEG/MafG, respectively. Plasmid pFRluc, containing five tandem copies of the Gal4 binding site, was obtained from Stratagene. Plasmid pCMV-MafG encodes Escherichia coli β-galactosidase and was used to monitor transcription.

**Cell Culture, Transfection, and Enzyme Assays**—CHO cells were cultured in Ham’s F-12 medium supplemented with 10% fetal bovine serum and 50 μg/ml gentamicin in a humidified atmosphere of 5% CO2, 5% O2, and 90% air at 37 °C. Cells were subjected to hypoxia (Blummers-Rothenburg, Del Mar, CA) supplied with a constant flow of a hydrated 1% O2, 5% CO2, balanced N2 gas mixture. Hypoxic exposures were performed as cells approached 75–90% confluence. Transient transfections of luciferase reporter gene constructs were carried out by the calcium phosphate precipitation technique as described previously (26) or with FuGene6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer’s recommendations. For calcium phosphate transfections, cells were seeded (4 × 105 well) in six-well plates and in each well were transfected with a DNA mixture consisting of 1 μg of the luciferase plasmid, 0.5 μg of pCMVβ-gal, and 3.5 μg of pBlueScriptII SK- (Stratagene). For FuGene6 transfections, cells were seeded (2 × 105/well) in 12-well plates and cells in each well were transfected with a DNA mixture consisting of 50 ng of the luciferase plasmid, 10 ng of pCMVβ-gal, and, where indicated (see Figs. 6 and 7D), 50 ng of the dominant-negative mutant or Gdbd fusion construct. Additional details are provided in the figure legends. Prepararion of cell extract and measurement of luciferase and β-galactosidase activities was carried out as described previously (26).

**RNA Isolation and Analysis**—Total RNA was isolated by the procedure of Chomczynski and Sacchi (27). For RNA dot blot analysis, 5 μg of total RNA was transferred to Zeta-Probe (Bio-Rad) nylon membrane according to the manufacturer’s instructions. 32P–Radiolabeled hybridization probes were generated by random priming of cDNA fragments encoding rat HO-1 or ribosomal protein S3. Hybridization and washing conditions were identical to those described previously for Northern blots (28). HO-1 hybridization signals were quantified using a Molecular Dynamics (Sunnyvale, CA) BAS2000c Image Analyser. After signal quantification, the membranes were stripped and rehybridized to the S3 probe. Relative mRNA levels were calculated after correcting for RNA loading by normalizing the primary hybridization signal with the S3 signal.

**Electrophoretic Mobility Shift Assays (EMSAs)**—CHO cells were plated (4 × 105 cells/100-mm plastic dish) and cultured in complete medium for 40–48 h. Cells were then exposed to vehicle/normoxia, hypoxia (1% O2), or CoCl2 (100 μM) in fresh, complete medium. Whole cell extracts were prepared as described previously (29). Briefly, cells were scraped into phosphate-buffered saline and collected by centrifugation. Cells were re-suspended in three volumes of extraction buffer (20 mM HEPES-KOH (pH 7.9), 0.4 mM KC1, 1.5 mM MgCl2, 0.5 mM dithiothreitol, 50 mM glucose, 0.2 mM phenylmethylsulfonyl fluoride) and lysed by three freeze-thaw cycles. The lysate was centrifuged at 100,000 × g for 5 min, and the supernatant was diluted with an equal volume of extraction buffer lacking KC1 and stored frozen at −70 °C. The standard binding reaction mixture (12.5 μl) contained 18 mM HEPES-KOH (pH 7.9), 80 mM KC1, 2 mM MgCl2, 10 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml bovine serum albumin, 160 μg/ml poly(dI-dC), 20,000 cpm [32P]ATP, 5 mM dNTPs, and 5 μl of each antibody. Reaction mixtures were incubated at 25 °C for 20 min and analyzed by native 5% polyacrylamide gel electrophoresis and autoradiography as described previously (29, 30). A double-stranded oligonucleotide containing the sequence 5’-GATCCTTTTATGTCATGATGTGTGTT-3’ (core StRE underlined) was end-labeled by T4 polynucleotide kinase, purified on a 10% polyacrylamide gel, and used as probe in EMSA reactions.
Unlabeled, double-stranded oligonucleotides, either wild-type or those containing specific mutations within the core StRE, were used as competitors. In antibody supershift assays, 1 μl (2 μg) of pre-immune IgG or specific rabbit polyclonal antibodies were added to the reaction mixture and incubated for 20 min at room temperature prior to electrophoresis. In the case of MafG, 1 μl of whole serum (pre-immune and anti-MafG) was used.

Western Blot Analysis—Cell culture and treatment, preparation of whole cell extracts, and target protein detection were carried out as described previously (19, 21). Cyttoplasmic and nuclear fractions were prepared as described (31). Anti-mouse Nrf2 and anti-MafG antibodies were used at 1:1000 and 1:2000 dilutions, respectively. Anti-α tubulin and anti-histone H1 antibodies were used at concentrations recommended by the manufacturer.

RESULTS

Induction of ho-1 mRNA by Hypoxia or Cobalt Is Not Impaired in HIF-1α-deficient Cells—Wild-type (K1), clonal control (C4.5), and clonal HIF-1α-deficient (Ka13) CHO cells were exposed to normoxia, hypoxia (1% O2), or 100 μM CoCl2, NiCl2, or DFO for 18 h and then harvested for RNA isolation. RNA dot blot analysis, quantization, and signal normalization were carried out as described under "Experimental Procedures." -Fold induction relative to control is presented, and each bar represents the average ± S.E. of three or four independent experiments.

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Together with the reporter gene transfection data, these results clearly point to divergent mechanisms for ho-1 gene activation by cobalt and hypoxia.

**EMSA Complex X Contains Nrf2 and MafG Transcription Factors**—The consensus StRE (13) resembles the binding sites for AP-1 (Fos/Jun), ATF/CREB, Maf, and CNC-bZIP families of transcription factors. In order to determine which, if any, member(s) of these families of proteins could function as the cobalt-responsive transcription factor(s), antibody “supershift” EMSA reactions were carried out to identify specific StRE-binding proteins in Ka13 cells. As shown in Fig. 6, commercially available antibodies directed against individual ATF/CREB, Jun, Fos, and Maf proteins did not significantly or consistently alter formation or migration of either complex X or Y. In contrast, incubation with anti-Nrf2 completely supershifted (and also possibly inhibited formation of) DNA-protein complex X. We have recently generated antibodies to MafG that do not, or only poorly, cross-react with MafF and MafK, respectively (Ref. 32; data not shown). Reduced migration, or supershifting, of complex X was also observed with anti-MafG. The nearly complete elimination or supershifting of complex X by antibodies against Nrf2 and MafG strongly suggest that Nrf2-MafG heterodimers are the primary protein components of complex X.

**Dominant-negative Mutants of Nrf2 and Small Maf Inhibit**

**Fig. 2. ho-1 promoter constructs.** Plasmid pH015luc contains ~15 kilobase pairs (kb) of the 5' flanking region of the mouse ho-1 gene fused to the firefly luciferase (Luc) reporter gene. The relative locations of the previously identified E1 and E2 enhancers and the HIF-1 binding site/hypoxia response element (Hif) are indicated. Derivatives of pH015luc, which lack 1, 2, or all 3 of these cis-acting regions, are diagrammed.

**Fig. 3. Cobalt activates the ho-1 promoter via the StRE.** Ka13 cells were plated and transfected with pH015luc (A) or with the indicated luciferase constructs (B–D) by the CaPO4 (A–C) or Fugene6 (D) methods as described under “Experimental Procedures.” Transfected cells were treated with vehicle, the indicated agent (A), or 100 μM CoCl2 (B–D) for 24 h, and cell lysates were assayed for reporter enzyme activities. Background luciferase activity (from mock-transfected cells) was subtracted from each experimental measurement, and the resulting value was corrected for variation in transfection efficiency by normalization with background-subtracted β-galactosidase activity in the same cell extract. -Fold change in normalized luciferase activity (relative to that in vehicle-treated cells) is presented; each data point (A) represents the average of two, and each bar (B–D) represents the average ± S.E. from three to six independent experiments.
Cobalt-mediated Activation of StRE—The role of Nrf2 and MafG in cobalt-mediated hO-1 gene activation was further examined by using dominant-negative mutants of Nrf2 and small Maf proteins in transient transfection assays. As shown in Fig. 7, overexpression of Nrf2 and MafK mutants completely inhibited induction of p3XStREluc by CoCl2 in Ka13 cells. The MafK mutant, generated by sequence substitutions within the DNA-binding domain, is capable of forming homodimers with wild-type MafK and heterodimers with MafK partners (e.g. Nrf2), but the resultant dimers cannot bind to their recognition sequences. Because of the high degree of sequence similarity within the DNA-binding and dimerization domains of small Maf proteins, the dominant mutant of MafK would be expected to similarly inhibit MafG and MafF activities. Dominant-negative mutants of other bZIP proteins including C/EBP, CREB, and JunD did not significantly affect cobalt-mediated reporter gene induction, providing additional support for the specificity of Nrf2 and MafG in this process.

N-Acetylcysteine Inhibits Cobalt-dependent Gene Activation—Recent studies have implicated Nrf2 (21, 33–36) and, in some cases, Nrf2-small Maf dimers in induction of various genes by xenobiotics. Models resulting from some of these studies have suggested that 1) gene induction results as a consequence of cellular oxidative stress and Nrf2 functions as a sensor for oxidative stress, 2) oxidants stimulate Nrf2 activity at least in part by promoting translocation of Nrf2 from the cytoplasm to the nucleus, and 3) inducer-dependent increase in Nrf2-MafG DNA binding activity results in part from increased expression of MafG. We have examined these and other parameters to better characterize the mechanism of hO-1 gene activation by cobalt. As shown in Fig. 8A, induction of the StRE-regulated luciferase gene by cobalt is dependent on oxidative stress as this stimulation was completely abrogated by N-acetylcysteine, a glutathione precursor, and an antioxidant, in a dose-dependent manner. Treatment of K13 cells with 100 μM CoCl2 from 0 to 24 h (Fig. 8B, lanes a–f), however, did not enhance expression of either Nrf2 or MafG protein as judged by Western blot analysis. In a similar analysis, Nrf2 was detected in both cytoplasmic and nuclear (Fig. 8C and N) fractions, but nuclear accumulation did not increase upon exposure of K13 cells to cobalt. The integrity of the cytoplasmic and nuclear fractions were examined by monitoring the cytosolic protein α-tubulin (Tub) and the nuclear protein histone H1. Nrf2 function may also be affected by modulation of its trans-activation capacity. This possibility was tested using a yeast Gal4-based system to assay for Nrf2 trans-activation potential. As shown in Fig.
8D, a Gal4 DNA binding domain/Nrf2 fusion (Gdbd-Nrf2) potently induced expression of the luciferase reporter gene under the control of Gal4 binding sites. Cobalt, however, did not stimulate Nrf2 transcription activity. Because small Maf proteins lack transcription activation domains, as expected, the Gdbd-MafG fusion did not increase luciferase activity beyond that observed with Gdbd alone (data not shown for Gdbd).

**DISCUSSION**

With respect to mammalian gene regulation, cobalt has been most commonly studied as a non-physiological inducer of the heavy metal-responsive metallothionein genes and of several hypoxia-responsive genes. In this capacity, cobalt is able to activate the metal-responsive element/metal transcription factor-1 (37) and HRE/HIF-1 pathways. In this report we show that cobalt utilizes a different mechanism, activation of the StRE/Nrf2 pathway, for induction of HO-1 expression in CHO cells.

Accumulating data implicate Nrf2 as a key regulator of the adaptive response to oxidative stress (21, 33, 35, 36, 38–40). In response to pro-oxidants and xenobiotics, Nrf2 coordinately activates transcription of a select set of target genes by binding to distinct but very similar DNA elements, individually or alternatively referred to as the NF-E2 binding site, the Maf recognition element, the stress-response element, or the antioxidant response element (ARE). Many of the Nrf2 target genes (21, 33, 35, 36, 41–43) encode proteins that individually and collectively manifest anti-oxidant activity by one of several mechanisms: 1) metabolism or detoxification of xenobiotics and oxidants, 2) reduction of oxidized proteins, or 3) production of antioxidants. Proteins in each of these categories include 1) NAD(P)H:quinone oxidoreductase, which catalyzes two-electron reduction of quinones, preventing the participation of such compounds in redox cycling and oxidative stress and glutathione S-transferase, which conjugates hydrophobic electrophiles and reactive oxygen species with glutathione; 2) thioredoxin, an ubiquitous dithiol hydrogen donor for a variety of proteins including transcription factors; and 3) HO-1 and \( \gamma \)-glutamylcysteine synthase (\( \gamma \)-GCS), which catalyzes the rate-limiting reaction in biosynthesis of glutathione, the primary non-protein thiol in cells.

Nrf2 is one of several members of the CNC-bZIP subfamily of basic region-leucine zipper (bZIP) transcription factors that function as obligate homo- or heterodimers and are characterized by a bipartite structure: a region enriched in basic residues necessary for DNA binding and an adjacent protein dimerization domain in which leucine (or equivalent) residues are present at every 7th position (i.e. the “leucine zipper”). The CNC-bZIP subfamily, along with the Fos, Jun, ATF/CREB, and Maf subfamilies, can be categorized into the larger “AP-1” superfamily of bZIP proteins because of similarities between the

**FIG. 6.** StRE-protein complex A contains Nrf2 and MafG. Antibody “super-shift” EMSA reactions were carried out as described under “Experimental Procedures” using whole cell extracts from Ka13 cells treated with 100 \( \mu \)M CoCl\(_2\) for 2 h, a time point at which complexes A and B are present at roughly equivalent levels. Antibodies (Ab) to individual transcription factors are indicated. “Super-shifted” complexes are marked by asterisks. Lane 1 (left) corresponds to EMSA reaction without cell extract. CIgG, control IgG; CS, control serum.

**FIG. 7.** Dominant-negative mutants of Nrf2 and small Maf inhibit cobalt-mediated activation of the StRE. Ka13 cells were transfected with p3XStREluc and plasmids encoding the indicated dominant mutant transcription factor, treated with vehicle or 100 \( \mu \)M CoCl\(_2\), and analyzed as described in the legend to Fig. 3D and under “Experimental Procedures.” Each bar represents the average of two or the average ± S.E. of three independent experiments.
consensus binding sites, sequence conservation within the bZIP domains, and the tendency for individual members to form cross-family heterodimers.

As is the case for Fos proteins, the sequence of the leucine zipper domain of Nrf2 precludes self-dimerization (44) and thus Nrf2 functions as an obligate heterodimer. In accordance with the paradigm established by NF-E2 (45), the first CNC-bZIP containing mammalian transcription factor isolated, the most prominent dimerization partners of Nrf2 are the small Maf proteins, MafF, MafG, and MafK (46). The precise function of such Nrf2-Maf dimers, however, is somewhat controversial. For instance, Nrf2-small Maf dimers have been proposed to function as positive regulators of genes encoding NAD(P)H:quinone oxidoreductase and various glutathione S-transferase subunits in response to butylated hydroxyanisole (33). Similarly, the Nrf2-Maf G complex (and possibly other Nrf2-bZIP dimers) may mediate ARE-dependent induction of the γ-GCS subunit genes by β-naphthoflavone and pyrrolidine dithiocarbamate (36). On the other hand, based in part on transfection studies that consistently show attenuation of Nrf2-mediated trans-activation of the ARE by co-expression of small Maf proteins, others have suggested that Nrf2-small Maf dimers do not function as positive regulators of the ARE and may indeed have repressor activity (47, 48). In addition to Nrf2 and small Maf factors, Jun and Fos subfamily members have also been identified as ARE-binding proteins (36, 41, 49). Indeed, Nrf2-Jun complexes have been implicated as positive effectors of ARE-dependent genes (41). The supershift EMSA data presented here support a role for Nrf2-MafG but not of Nrf2-Jun dimers in ho-1 gene regulation by cobalt. The increase in the level of the Nrf2-MafG/StIRE complex in response to cobalt and inhibition of gene activation by MafK and Nrf2 dominant-negative mutants further suggest that the Nrf2-MafG heterodimer functions as a positive regulator in this process.

Our results indicate that activation of the ho-1 gene by cobalt occurs, at least in part, as a consequence of increased binding of Nrf2-MafG to the StIREs, a stimulation that could result from increased expression of MafG, a stress-responsive protein in multiple cell types. Agents known to stimulate MafG mRNA accumulation include hydrogen peroxide (50); arsenite, heavy metals, and hyperthermia (51); and β-naphthoflavone and pyrrolidine dithiocarbamate (36). In the latter case, increased MafG production may contribute to the ARE-dependent induction and even subsequent repression of the γ-GCS subunit genes. Our data show that cobalt does not stimulate MafG synthesis in CHO cells; thus, such an induction is not necessary for increased StIRE binding activity or ho-1 gene activation.

Accumulating evidence (34, 43, 52, 53) suggests that, under normal conditions, Nrf2 exists in an inactive, cytoplasm-localized state, in part or fully as a consequence of binding to the cytoskeleton-associated protein Keap1. After exposure of cells to electrophiles or oxidative-stress generating agents, such as diethylmaleate, 12-O-tetradecanoylphorbol-13-acetate, or tetrabutylhydroquinone, the cytoplasmic retention mechanism is inactivated and Nrf2 is transported to the nucleus by an as yet uncharacterized mechanism(s) but one that may involve protein kinase C-mediated phosphorylation of Nrf2 (52). We have been unable to obtain any evidence for such a process in cobalt-stimulated CHO cells, either by subcellular fractionation (Fig. 8) or by immunocytochemistry (data not shown). These results suggest that regulation of Nrf2 activity by nuclear transport is an inducer-specific and/or a cell-specific mechanism. Certainly, at least in the latter case, this specificity could easily arise because of cell-dependent variations in the ratio of Nrf2 and Keap1. Indeed, variations in the level of Keap1 have been noted and fibroblasts appear to express lower amounts of this protein than, for instance, hematopoietic cell lines (34). The inability of cobalt to affect nuclear translocation of Nrf2, MafG, and Nrf2 expression or Nrf2 transcription activity suggests that regulation of Nrf2 function in response to cobalt occurs primarily at the level of DNA binding affinity (and of necessity, protein dimerization). The post-translation processes responsible for modulation of Nrf2 DNA binding activity are not known but are likely to involve phosphorylation/dephosphorylation reactions that are known to regulate the various activities of most transcription factors.

One interesting result of this study is that the 15-kbp ho-1 promoter, which contains the previously characterized HIF-1 binding sites observed to be active in aortic smooth muscle cells (15), is unresponsive to hypoxia in wild-type CHO cells. That the HIF-1 pathway is operative in these cells is evident from the fact that the same elements, when placed out of context and in multiple copies, are capable of supporting hypoxia-dependent reporter gene induction. These observations certainly point to cell-specific variations in the mechanism of HO-1 induction by hypoxia in vascular smooth muscle and CHO cells but do not explain why the HIF-1 binding sites would be unresponsive in their normal environment. Perhaps, in CHO cells, these elements function only in concert with other sequences (and their cognate binding factors) located outside of the region tested. We note, however, that sequences necessary for gene activation by all HIF-1 inducers thus far tested reside within the 15-kbp segment (12). Given this fact, one must consider...
the possibility that hypoxic induction of HO-1 in CHO cells occurs not because of gene activation but by a post-transcriptional mechanism such as mRNA stabilization. The expression of several hypoxia-responsive proteins, including EPO, tyrosine hydroxylase, and VEGF, is in part regulated in this manner (54–58), and Panchenko et al. (59) have recently provided evidence for hypoxia-dependent stabilization of HO-1 mRNA in human dermal fibroblasts. Furthermore, the results presented here provide interesting parallels with the latter study. For instance, desferrioxamine does not stimulate HO-1 expression in either CHO or skin fibroblast cells. Similar to our findings with cobalt, hydrogen peroxide induction of HO-1 mRNA in dermal fibroblasts is sensitive to antioxidants and appears to be regulated at the level of gene transcription. In contrast, hypoxic skin fibroblasts do not exhibit increased free radical production and HO-1 mRNA accumulation is not inhibited by free radical scavengers. Although we have not directly tested the effect of hypoxia on free radical levels in CHO cells, the lack of stimulation of Nrf2 DNA binding activity by hypoxia indirectly suggests that such an effect would be minimal.

In summary, cobalt and hypoxia utilize divergent mechanisms for stimulation of HO-1 expression in CHO cells. Induction by cobalt is regulated primarily at the level of gene transcription, is oxidative stress-dependent, and is mediated by the StRE/Nrf2 transcription factor pathway. On the other hand, induction by hypoxia does not involve the StRE/Nrf2 pathway, is apparently independent of oxidative stress, and may be regulated by a post-transcriptional mechanism.

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