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Co-Regulation of Heme Oxygenase and Erythropoietin Genes

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Abstract The mechanism responsible for the accumulation of heme oxygenase and erythropoietin (epo) transcripts due to cobalt chloride ($CoCl_2$) administration was investigated in rat kidney using a rat heme oxygenase and mouse epo probes. We found an increase of heme oxygenase transcripts in kidney in response to $CoCl_2$. Quantitative evaluation of the heme oxygenase mRNA changes, by scanning densitometry, indicated that the levels of mRNA encoding heme oxygenase were increased by about fiftyfold in rat kidney after administration of $CoCl_2$. That the increase in heme oxygenase mRNA levels resulted from enhanced transcription of the heme oxygenase gene was confirmed by nuclear runoff using isolated rat kidney nuclei after $CoCl_2$ administration. Transcription of the heme oxygenase gene is greatly increased in rat kidney within 1 hr of administration of $CoCl_2$ as evidenced from the levels of ^{32}P-UTP incorporation into the specific transcript. Time course studies showed that stimulation of transcription was increased about fortyfold 3 hr after $CoCl_2$ administration. This stimulation is the most rapid transcriptional response to heavy metals yet described. In addition, Northern blot analysis demonstrated that epo mRNA was first detected 4 hr following $CoCl_2$ administration and reached a maximum at 5 hr. On the other hand, PCR analysis indicated that epo mRNA was increased as early as 1 hr following $CoCl_2$ administration. The fact that $CoCl_2$ caused increased transcription of both the epo and heme oxygenase genes suggests that a common mechanism may be involved in the regulation of these two genes by the heavy metal ion.

Key words: gene expression, nuclear runoff, gene activation, polymerase chain reaction, transcription, translation, parenchymal cells

The expression of the heme oxygenase gene shows marked variation in several cell types. Its transcript is present at very high levels in fetal liver, which consists mainly of erythropoietic cells, and at much lower levels in adult liver, which consists mainly of parenchymal cells [1]. A rapid decrease in heme oxygenase gene transcription in the liver occurs shortly after birth. In four weeks, the mRNA level decreases more than tenfold. Throughout fetal maturation, changes in the hepatic level of heme oxygenase mRNA are accompanied by corresponding changes in enzyme activity [1].

Erythropoietin (epo), the primary regulator of red cell formation, is synthesized in the liver during fetal life [2]. In the adult, the kidney is the major site of synthesis [3], although the liver may contribute to some extent [4].

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Expression of the heme oxygenase gene is also affected by environmental agents such as heavy metals [5–7]. Several heavy metals such as $CoCl_2$ have been shown to increase heme oxygenase activity by provoking de novo protein synthesis [for review, see 5]. It is believed that high heme oxygenase levels may result in decreased cellular heme [5]. However, in iron overload, elevation of heme oxygenase was accompanied by adaptive mechanisms to maintain normal cellular heme levels [5]. The gene for rat heme oxygenase has been isolated and sequenced by Muller et al. [8]. A heat shock element was identified in the 5′-flanking region of the gene. Its heat-responsive nature was demonstrated in a transient expression system and was independent of hemin regulation [9]. The heme oxygenase gene also contains a sequence encompassing core sequences of metal regulatory elements found in metallothionein genes [8]. Since $CoCl_2$ is a po-

tent stimulator of epo gene transcription [4], we examined the effect of $CoCl_2$ on both heme oxygenase and epo genes.

As part of the study of mechanisms by which heavy metals activate the heme oxygenase gene, we followed the time course of message accumulation after $CoCl_2$ treatment and compared the result with accumulation of epo mRNA. Using nuclear runoff analyses, we show here that the increased amount of heme oxygenase mRNA due to $CoCl_2$ was, at least in part, due to an increase in gene transcription and that activation of the heme oxygenase gene precedes that of the epo gene.

MATERIALS AND METHODS

Reagents

Guanidinium isothiocyanate, cesium chloride, and nick translation kit were purchased from Bethesda Research Laboratories (Gaithersburg, MD). SeaKem LE agarose and SeaPlaque low-gelling-temperature agarose were from FMC Corporation (Rockland, ME). Restriction endonucleases, Multiprime DNA labeling system, nitrocellulose, [α-^{32}P]dCTP, and [α-^{32}P]UTP were from Amersham Corporation (Arlington Heights, IL). RNAguard, a ribonuclease inhibitor, as well as nucleotide triphosphates were from Pharmacia (Piscataway, NJ). Reverse transcriptase and RNasin were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN), and Taq polymerase from Perkin Elmer Cetus (Norwalk, CT). Gene screen plus was purchased from EI DuPont de Nemours and Co., Inc. (Wilmington, DE). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Springfield, NJ).

Animals

Male Sprague-Dawley rats (150–175 g) from Charles River Laboratories (Wilmington, MA) were used throughout the study. The rats were housed in groups of three in plastic cages and given free access to food and water. A 12-hr light/dark cycle was maintained. We allowed at least three days for the animals to adjust to the new environment before subjecting them to any treatment. Rats were anesthetized by injection of pentobarbitone (40 mg/kg body wt), intraperitoneally, and sacrificed by decapitation. A portion of kidneys (0.5 g wet weight) was removed immediately, frozen in liquid nitrogen, and stored at −80°C for RNA extraction.

Preparation of Kidney Cell Nuclei

Nuclei were prepared from the remainder of the rat kidneys according to the method of McKnight and Palmiter [10]. Briefly, kidneys were homogenized in 0.3 M sucrose in buffer A containing 60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 15 mM 2-mercaptoethanol, 15 mM Tris (pH 7.4), 0.5 mM EGTA, 2.0 mM EDTA, 0.1% Triton X-100, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was then filtered through gauze and the crude nuclei pelleted at 600*g* at 2°C. The crude nuclei were resuspended in 1.37 M sucrose in buffer B containing 15 mM Hepes (pH 8.0), 0.15 mM spermine, 0.5 mM spermidine, 10 mM $MgCl_2$, 2 mM 2-mercaptoethanol, and 0.1 mM PMSF and pelleted through 2.4 M sucrose containing buffer A without Triton X-100 and centrifuged for 45 min at 75,000*g* using a Beckman SW25.1 rotor at 2°C. The nuclei were resuspended at 3.5×10^4/100 μl in a storage buffer containing 40% glycerol as described [10], and stored in liquid nitrogen.

In Vitro Transcription and RNA Isolation for Hybridization

Frozen nuclei were thawed, pelleted, and resuspended in 20 mM Tris (pH 7.9), 75 mM NaCl, 0.5 mM EDTA, 0.85 mM dithiothreitol (DTT), 0.125 mM PMSF, 40% glycerol, and 100 U/ml RNAguard by mixing with a cut Eppendorf pipet tip, and incubated in a 0.5 ml solution containing 16% glycerol, 20 mM Tris (pH 8.0), 5 mM $MgCl_2$, 150 mM KCl, 0.4 mM creatine phosphate, 0.4 mM DTT, 0.4 mM each of ATP, GTP, and CTP, and 100 μCi of [α-^{32}P]UTP (800 Ci/mmol) [11] for 20 min at 26°C. The reaction was stopped by mixing with 100 μg of tRNA and 10 μg of RNase-free DNase. Labeled RNAs were assessed as previously described [23].

Hybridization to Immobilized DNA

Plasmid DNAs were linearized with EcoRI as described [1], denatured by boiling in 0.5 M NaOH, and applied onto nitrocellulose filter strips with the aid of a Minifold II-dot blotter (Schleicher and Schull, Keene, NH) and light vacuum at a quantity of 3 μg per slot. After washes in 2 M NH_4-acetate and then in 2× Denhardt's solution (1× in 0.1% each of Ficoll, polyvinylpyrrolidone, and bovine serum albumin), the filters were baked and stored under

vacuum. Prehybridization and hybridization were performed as previously described [23].

RNA Blot Analysis

Total RNA was extracted from approximately 0.2 g of tissues by the guanidinium isothiocyanate/CsCl method of Chirgwin et al. [12]. Ten to thirty micrograms of total RNA from various tissue samples were denatured, electrophoresed in 1% agarose gels containing 1 M formaldehyde, transferred to nitrocellulose or gene screen plus filters by blotting, and hybridized with the ^{32}P-labeled rat heme oxygenase cDNA or mouse genomic epo probes. To ensure that an equivalent amount of RNA was compared among samples, we hybridized filters with cDNA for γ-actin which was labeled by the same method as described for heme oxygenase.

For the detection of heme oxygenase mRNA, the probe used was the 883 base-pair EcoRI/Hind III fragment of pRH01, a plasmid containing full-length cDNA for rat heme oxygenase [13] and labeled as previously described [1]. For the detection of epo mRNA, the probes used were two Pst I fragments of the mouse epo gene [4] subcloned into the plasmid pUC19. The two fragments between them encompass exons 2–4 and part of 5. The plasmids containing the fragments were labeled with ^{32}P-dCTP by nick translation to a specific activity of 1–2 × 10^8 cpm/μg. The probe was used at 5 × 10^5 cpm/ml in the hybridization bag. Heme oxygenase activity and epo serum levels were determined as previously described [16,23].

Detection of Epo RNA by Polymerase Chain Reaction (PCR)

For PCR, upstream (sense) and downstream (antisense) primers corresponding to sequences in exons 2 and 5 of the mouse epo gene were synthesized on a DNA synthesizer from Applied Biosystems Inc. (Foster City, CA). The seqeunce of the upstream primer was 5′ CTCTGGGCCTCCCAGTC3′ and that of the downstream primer was 5′ TGTTCGGAGTGGAGCAG3′. These primers would generate a 0.41 Kb fragment on cDNA. Amplification and cDNA synthesis were carried out essentially as described [14]. One microgram of RNA was reverse transcribed in 1× PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin) containing 200 ng of downstream primer, 20 U RNasin, 1 mM of each dNTP, and 60 U of reverse transcriptase. Reaction was for 10 min at 25°C and 60 min at 42°C. After heat-inactivation of reverse transcriptase and dissociation of the RNA/DNA hybrid at 95°C for 5 min, 80 μl 1× PCR buffer containing 200 ng of the upstream primer and 2.5 U of Taq polymerase were added. After layering with mineral oil, 50 PCR cycles were run with each cycle being 2 min at 94°C to denature, 2 min at 55°C to anneal, and 2 min at 72°C for the polymerization step. PCR products were analyzed on 1% agarose gels and, after blotting to Gene screen plus, hybridized to a ^{32}P-labeled epo probe.

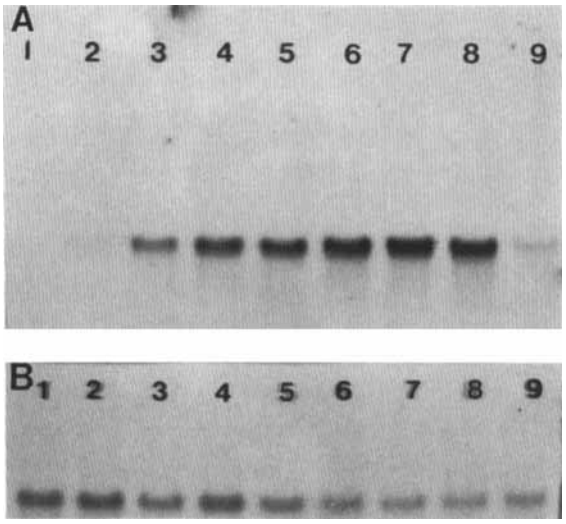

Fig. 1. **A:** Time course of heme oxygenase mRNA induction by $CoCl_2$. Total RNA (10 μg) was isolated from kidney of control rats, lane 1, and rats injected with $CoCl_2$, lane 2, 1 hr; lane 3, 2 hr; lane 4, 3 hr; lane 5, 4 hr; lane 6, 5 hr; lane 7, 6 hr; lane 8, 7 hr; and lane 9, 24 hr, and analyzed for heme oxygenase mRNA level by blot hybridization. Exposure time for the autoradiograms was 16 hr with two intensifying screens. **B:** Northern blot of a paired experiment probed with γ-actin in which RNA was obtained from the same rats. Exposure time for the autoradiogram was 4 hr with two intensifying screens.

RESULTS

Induction of Heme Oxygenase mRNA by $CoCl_2$ in Rat Kidney

In order to compare the rate of heme oxygenase induction by $CoCl_2$, we examined the changes in mRNA levels in kidneys from rats treated with $CoCl_2$ (250 μM/kg body weight) and sacrificed after various lengths of time. As can be seen in Figure 1, the transcripts were detectable at 1 hr after $CoCl_2$ administration (compare lane 2 to lane 1, untreated rats). The mRNA reached a maximum at about 5–6 hr (lane 7) but decreased greatly by 24 hr. Hybridization of the filters with the radiolabeled γ-actin confirmed

that similar amounts of total RNA were transferred to the filters in each lane of the paired experiments (Fig. 1B). Administration of 250 μM of $CoCl_2$ increased heme oxygenase activity by 30% as early as 2 hr. Heme oxygenase activity in the control was 0.65 ± 0.11 nmol bilirubin formed/mg/hr, which increased to 0.99 ± 0.23 nmol bilirubin formed after 3 hr, and by 600% after 8–9 hr (data not shown).

In contrast, after 3 hr, epo serum levels rose to a maximum of ninetyfold, from a control value of 4.7 ± 0.6 mU/ml to 416 ± 31 mU/ml, declining sharply after 9–10 hr (data not shown). At 50–125 μM, $CoCl_2$ induced epo and heme oxygenase synthesis, but at levels lower than those seen with 250 μM (unpublished work). The reasons for the differences in the magnitude of epo and heme oxygenase levels and the time of response after $CoCl_2$ administration are not clear.

PCR Analysis of Epo mRNA in Rat Kidney

The effect of $CoCl_2$ on epo mRNA induction in rat kidney was also studied by Northern blot analysis (Fig. 2A) and PCR (Fig. 2B). The Northern analysis shows that epo mRNA was first detected 4 hr (lane 6) following $CoCl_2$ administration and reached a maximum at about 5 hr (lane 7). This is consistent with an earlier finding using the Northern method which showed that epo mRNA first appeared between 3 and 6 hr following $CoCl_2$ injection [4]. As was seen for heme oxygenase, epo mRNA levels returned to control level by 24 hr (lane 10) after $CoCl_2$ injection.

Using the much more sensitive PCR method (Fig. 2B), not only were we able to demonstrate epo mRNA in control kidneys (lane 2), but we also found an increase over control 1 hr following $CoCl_2$ injection (Fig. 2B, lane 3). This method of analysis also showed agreement with the Northern analysis in that the epo mRNA level peaked at 5 hr (Fig. 2A, lane 7) and returned close to the control level 24 hr (Fig. 2A, lane 10) following $CoCl_2$ injection.

In Vitro Transcription of the Heme Oxygenase and Epo Genes

To determine whether the observed increase in heme oxygenase mRNA after $CoCl_2$ was a result of increased transcription of the heme oxygenase gene, we isolated nuclei from control and cobalt-stimulated rat kidneys and analyzed transcription by nuclear runoff assay. As seen in

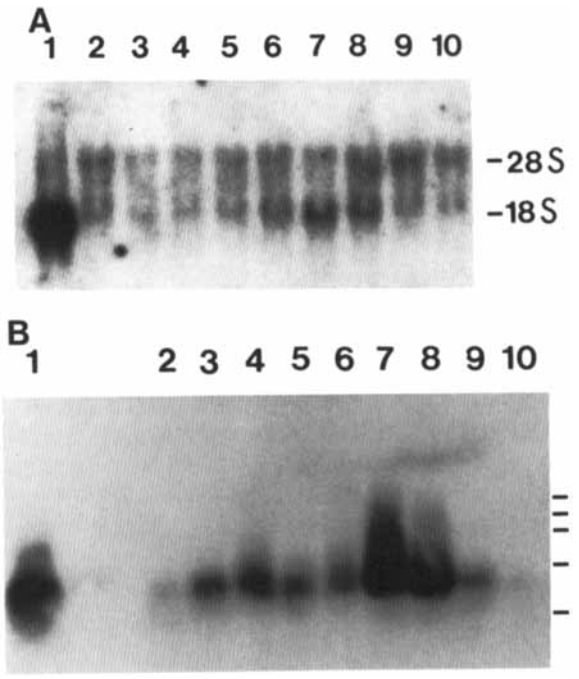

Fig. 2. Time course of the effects of $CoCl_2$ on epo mRNA transcription in rat kidney. **A:** Northern blot analysis. Thirty micrograms of total RNA from the kidneys of rats treated with $CoCl_2$ for 0, 1, 2, 3, 4, 5, 6, 7 and 24 hr (lanes 2–10, respectively) and mouse RNA (lane 1) used as a positive control were electrophoresed on agarose/formaldehyde gels, blotted onto gene screen plus filters, and probed with a ^{32}P-labeled mouse genomic epo probe. Lane 1 is a positive control in which 4 μg of poly (A)+ RNA from an epo-producing cell line (NN10) was used. The markers used are the 18S and 28S rRNA. **B:** PCR analysis of epo gene expression. One microgram of total RNA was used to make epo cDNA and amplify it using PCR as described in Methods. Following amplification, the PCR products were electrophoresed on 1% agarose gels and analyzed by Southern blotting using a mouse genomic epo probe. In lane 1, RNA from a hypoxic rat kidney was used as control. In lanes 2–10, the same RNA preparation as in A above—that is, RNA from rats exposed to $CoCl_2$ for 0, 1, 2, 3, 4, 5, 6, 7 and 24 hr, respectively, was used. The markers from top to bottom correspond to 1354, 1078, 872, 603, and 310 bp.

Figure 3, nuclei isolated from rats treated with $CoCl_2$ for 1 hr showed a striking increase in the transcription of the heme oxygenase gene (lane B). Such an increase was not due simply to an increase in the overall transcription efficiency, since transcription of the γ-actin gene was unaffected by $CoCl_2$ as compared to the other genes examined, including epo, heme oxygenase, and porphobilinogen deaminase (PBG-D) [E.C. 4.3.1.8]. The specificity of hybridization of the in vitro transcripts to the immobilized cDNAs was shown by their minimal binding to the cloning vector, Okayama and Berg [23]. To estimate the

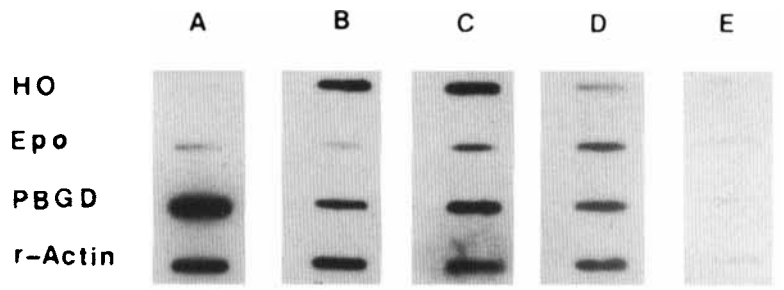

Fig. 3. Transcriptional runoff of heme oxygenase, epo, γ-actin, and PBG-D genes in rat kidney after administration of $CoCl_2$. Plasmids containing the appropriate probes were bound to nitrocellulose and hybridized with ^{32}P-labeled runoff transcripts from nuclei isolated at different times after $CoCl_2$ administration. ^{32}P-labeled transcripts hybridized with the rat heme oxygenase (HO), mouse epo, human porphobilinogen deaminase (PBG-D), and γ-actin probes. Column A represents ^{32}P-labeled transcripts obtained from control rat kidney; Column B, nuclei obtained from rat kidney treated for 1 hr with $CoCl_2$; Column C, nuclei obtained after 2 hr; Column D, nuclei obtained after 3 hr; Column E, nuclei obtained from rat kidney pretreated with $CoCl_2$ for 3 hr and labeled with ^{32}P-UTP in the presence of α-amanitin at 1 μg/ml. Filters were hybridized with ^{32}P-labeled RNAs (1.7×10^7 cpm). The autoradiogram corresponds to a 1-week exposure time.

relative activation of epo and heme oxygenase gene in control and $CoCl_2$-treated rats, we compared the amount of nascent epo and heme oxygenase transcripts in these nuclei. Nascent transcripts were radiolabeled in in vitro elongation reaction and hybridized to dot blots of both cDNA. Binding of the transcripts was quantified by autoradiography and densitometry. As seen in Figure 3, the amount of heme oxygenase transcripts present in nuclei from rats treated with $CoCl_2$ was elevated severalfold over those present in the control nuclei (Fig. 3b, lane B). On the other hand, a severalfold increase in epo transcripts was observed after 2–3 hr of $CoCl_2$ exposure. In a control experiment (lane E), the in vitro elongation reaction was carried out in the presence of the RNA polymerase II inhibitor, α-amanitin (1 μg/ml). As a result, the amounts of labeled heme oxygenase, PBG-D, and epo, as well as γ-actin transcripts generated by nuclei from rats treated with $CoCl_2$ were reduced to below the control levels. Therefore, an increase in the heme oxygenase and epo mRNA levels due to $CoCl_2$ seen in the Northern analysis is due to, at least in part, increased transcription.

DISCUSSION

Heavy metals have been shown to play an important role in kidney function and secretion of important hormones. $CoCl_2$ has been shown to be a potent inducer of epo in vivo [15,16]. Cobalt chloride–induced increase of epo was a result of the accumulation of epo mRNA [4] and an increased rate of transcription of the epo gene [17]. The effect of $CoCl_2$, nickel, and manganese on epo production extends to hepatoma cell lines [18,19], as well as fetal mouse liver cells [20].

We have demonstrated that the heavy metal related increase in heme oxygenase arises predominantly through the regulation of heme oxygenase transcription. An excellent correlation was found between heme oxygenase induction by the heavy metal, $CoCl_2$, and the related increase in the amount of mRNA levels in rat kidney (Fig. 1). Accumulation of heme oxygenase mRNA was decreased by co-administration of both cycloheximide and actinomycin D (data not shown). These studies demonstrate that transcription of heme oxygenase due to $CoCl_2$ requires de novo protein synthesis. It remains to be seen why heavy metal induction which requires binding of the metal to a cis element necessary for binding to a promotor region is dependent on protein synthesis.

One explanation for co-expression of the heme oxygenase and revised epo genes may be related to the fact that heme oxygenase is a heat shock or stress protein [9]. Several toxic compounds, including heavy metals and drugs, cause an increase in heme oxygenase [5]. Metal ion and heat shock transcriptional factors have been shown to increase heme oxygenase levels. The chimeric fusion genes, PRHO gpt 2 and 4, were constructed [21] which harbored the E. coli gene gpt, coding for xanthine-guanine phosphotransferase as a reporter gene, were used to show that

the 5′-flanking region of the rat heme oxygenase gene (−549 to −1) is sufficient to confer heat inducibility on the transient expression of gpt. Hemin treatment did not, however, increase gpt RNA production. Therefore, there are at least two mechanisms for the induction of heme oxygenase, namely, induction by a heat shock transcription factor and by a putative heme responsive transcription factor.

It is possible that $CoCl_2$ induces heme oxygenase by a different mechanism than that for metalloporphyrins, including heme [23]. $CoCl_2$ may involve heme oxygenase transcriptional factors (HOTF) on the promotor region, while heme involves other motifs. HOTF is also similar to the upstream promotor sequence (UPS) of the adenovirus 2 major late promoter or major late transcription factor previously identified in HeLa cells [22]. HOTF binds to the promotor region (−51/−35) of rat heme oxygenase and stimulates in vitro transcription of the heme oxygenase gene. This promotor element contains a TGACTCA motif which is known as the binding site for AP-1 or GCN4. Two such sequences with one mismatch (TGCCTCA at −45/−39 bp and TGTCTCA at −452/−446 bp) are found upstream of the cap site of the mouse epo gene.

Whether both genes carry common regulatory sequences is not known. It may be noted that there is a marked similarity in their organization in that both genes are composed of five exons and four introns. Indeed, a search for the heptanucleotide core sequence of the metal regulatory element (TGCPuCNC) in the 5′-flanking region of the epo gene yielded two such elements, 290 and 950 bp upstream of the cap site. A potential binding site for the metal-dependent transcription factor is also found 575 bp upstream of the cap site of the rat heme oxygenase gene [8]. These similarities are indicative of a potential common regulatory mechanism of $CoCl_2$-induced epo and heme oxygenase gene expression. A common regulatory mechanism for both heme oxygenase and epo remains a reasonable possibility and will be the subject of further studies.

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