Transcriptional regulation of heme oxygenases by HIF-1α in renal medullary interstitial cells

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Yang, Zhi-Zhang, and Ai-Ping Zou. Transcriptional regulation of heme oxygenases by HIF-1α in renal medullary interstitial cells. Am J Physiol Renal Physiol 281: F900–F908, 2001.—The present study was designed to test the hypothesis that hypoxia-inducible factor-1α (HIF-1α)-mediated transcriptional activation contributes to increased expression of heme oxygenase (HO) genes in renal medullary interstitial cells (RMICs). By Northern blot analysis, HO-1 mRNA expression was found to significantly increase in response to reduction of PO2 in culture medium. However, HO-2 mRNA was not altered by hypoxia. This hypoxia-induced upregulation of HO-1 mRNA was significantly blocked by HIF-1α inhibition with ferrous ammonium sulfate. To further determine the role of HIF-1α in the activation of HO-1, the inducers of HIF-1α were used to address whether induction of HIF-1α stimulates HO-1 mRNA expression. Both desferrioxamine and CoCl2 markedly increased HIF-1α mRNA and protein levels and resulted in the upregulation of HO-1 mRNA but not HO-2. Furthermore, inhibition of HIF-1α degradation by CBZ-LLL, an inhibitor of ubiquitin-proteasome, significantly increased HIF-1α protein and HO-1 mRNA but not HO-2 in these cells. Using cis-element oligodeoxynucleotide transfection to specifically decoy HIF-1α and block HIF-1α binding, increased mRNA expression of HO-1 in response to hypoxia and CoCl2 was attenuated. In vitro nuclear run-on assays further confirmed that hypoxia and alterations of HIF-1α mRNA or protein levels significantly affected the formation of HO-1 mRNA. Taken together, our results indicate that HO-1, but not HO-2, is transcriptionally activated by hypoxia through HIF-1α-mediated mechanism in RMICs. This hypoxia-induced transcriptional activation may be one of the important mechanisms mediating increased expression of HO-1 in the renal medulla.

HIF-1α; gene transcription; anoxia

RECENT STUDIES HAVE INDICATED that heme oxygenases (HOs) are involved in the cellular degradation of heme and the production of endogenous CO, biliverdin, and iron in a variety of mammalian tissues (3, 15). CO is one of the important autocrines or paracrines similar to nitric oxide, which may participate in the control of cardiovascular and renal functions and neural activity (11, 12, 14). Biliverdin can be converted to bilirubin, which is of importance in the antioxidant mechanism. It has been demonstrated that there are two catalytically active isozymes of the oxygenase, HO-1 and HO-2, which are encoded by two different genes. Recently, the isomorph HO-3 has been found to have 90% homology with HO-2 at the amino acid sequence level, but it displays very little heme-degrading activity (16). HO-1 is an inducible gene present at low levels in the tissues, and its expression can be rapidly and robustly accelerated in response to a variety of stimuli, including a number of physiological and pathological changes such as heat shock, ischemia, radiation, hypoxia, and cellular transformation. HO-2 has been considered as a constitutively expressed gene and relatively uninducible by stimuli (4, 6, 14, 15, 21, 22).

We have demonstrated that HO-1 and HO-2 were expressed more abundantly in the renal medulla compared with the renal cortex (33). The mechanism by which HO expression increased in this kidney region is poorly understood. Numerous studies have indicated that the renal medulla is characterized by a low PO2 level due to countercurrent exchange of O2 through vasa recta and a high metabolic rate in medullary thick ascending limb (5). Given the importance of hypoxia-induced adaptation in the gene expression, low PO2 in the renal medulla may importantly activate the expression of HOs. It has been reported that HO-1 expression, which is primarily regulated at the transcriptional level (3, 24), increased during ischemia or hypoxia in different tissues or organs. However, the mechanisms by which HO-1 is transcriptionally activated in the renal medullary cells are poorly understood.

More recently, a helix-loop-helix transcriptional factor, hypoxia-inducible factor-1 (HIF-1), which consists of HIF-1α and HIF-1β, has been cloned and characterized as a transcriptional activator of many oxygen-sensitive genes, such as erythropoietin, vascular endothelial growth factor, transferrin, and several glycolytic enzymes (25, 26, 28, 30). It has been indicated that HIF-1α is an inducible protein by a decrease in tissue or cellular O2. HIF-1β is not inducible, but it can be bound to HIF-1α to form a dimer to activate the transcription of many genes containing cis hypoxia-response element (HRE) in their promoter or enhancer regions. We have demonstrated that HIF-1α was more abundantly expressed in the renal medulla compared

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with the renal cortex (34). This raised a question of whether HIF-1α contributes to the increase in the expression of HO genes in this kidney region. Using the constructs of chloramphenicol acetyltransferase reporter gene with the HRE of mouse HO-1 genes, Lee et al. (13) have demonstrated that HIF-1 mediates transcriptional activation of the mouse HO-1 gene in response to hypoxia. The role of HIF-1 in the activation of HO-2 has not yet been studied. The present study was designed to test the hypothesis that HIF-1 mediates the transcription activation of HO in the renal medulla of rats. Using renal medullary interstitial cells (RMICs) isolated from the renal inner medulla of Wistar rats, we determined the effect of hypoxia and changes in HIF-1α levels by pharmacological and molecular interventions on expression of both HOs. These experiments provided evidence that HO-1, but not HO-2, is transcriptionally activated by HIF-1-mediated mechanism in RMICs.

MATERIALS AND METHODS

Isolation and culture of RMICs. RMICs were isolated and cultured as described previously (19, 20). Briefly, inbred male Wistar rats, weighing 300–350 g (Hanlan Sprague Dawley), were anesthetized with pentobarbital sodium (50 mg/kg body wt ip). Then, the left kidney was removed, and the renal papilla was dissected and finely minced. The minced tissue was resuspended in 3 ml of basal medium Eagle’s (BME; Sigma) and injected subcutaneously in two to four vertical tracks on the abdominal wall of a recipient rat (from the same litter). Four days after injection, many firm, yellow nodules located at the site of injections were carefully dissected. These nodules were minced, trypsinized in a 0.05% trypsin-EDTA solution at 37°C for 20–30 min, and then washed and centrifuged to obtain a cell pellet. The cell suspension was transferred to plastic tissue culture flasks and then incubated with BME containing fetal bovine serum (10%, vol/vol), amino acid mixtures (10%, vol/vol), lactalbumin hydrolysate (0.25%, wt/vol), yeast extracts (0.05%, wt/vol), and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) by using a 37°C incubator with a 95% air-5% CO2 environment. The culture medium was first replaced with fresh medium in 5 days and then was changed every 3 days. The cells formed a confluent monolayer in 18–21 days and were then trypsinized and subsequently replanted in flasks. The cells of passages 7 and 8 were used for hypoxia experiments.

A standard staining method as described previously (19, 20) was used to confirm the identity of these cells. The cells grown on coverslips were stained with oil-red O and Sudan black B, and lipid droplets within the cells were stained as a specific marker for these cells. Under a light microscope, we observed that >98% of cells contained lipid granules (dark black spots) (Fig. 1). These granules represent a unique and specific feature of RMICs, because other renal cells exhibit very little, if any, lipid staining in their cytoplasm. In addition, the cultured cells had long processes and round or irregularly shaped vacuoles. Nuclei were small and irregular in shape, with deep invaginations. Under an electron microscope, the cells were found to have lipid droplets and multiple vesicular bodies. They had elongated mitochondria, abundant rough endoplasmic reticula, and bundles of actin filaments under the plasma membrane. These light and electron microscopic features were different from those observed in renal tubular cells, lymphocytes, granulocytes, and endothelial cells (19, 20).

Western blot analysis. Nuclear extracts were prepared by a modification of the protocol described by Semenza and Wang (23). The cell pellet was washed with four packed-cell volumes of buffer A (10 mM Tris·HCl, pH 7.8, 1.5 mM MgCl2, 10 mM KCl) containing 0.5 mM dithiothreitol (DTT), 0.4 mM phenylmethanesulfonyl fluoride (PMSF), 2 µg/ml leupeptin, 2 µg/ml pepstatin, 2 µg/ml apronin, and 1 mM sodium vanadate (all obtained from Sigma), resuspended in buffer A, and incubated on ice for 10 min. Then, the cell suspension was homogenized, and the nuclei were pelleted by centrifugation at 3,000 rpm for 5 min, resuspended in three packed-cell volumes of buffer B (20 mM Tris·HCl, pH 7.8, 1.5 mM MgCl2, 0.42 M KCl, 20% glycerol) containing 0.5 mM DTT, 0.4 mM PMSF, 2 µg/ml leupeptin, 2 µg/ml pepstatin, 2 µg/ml apronin, and 1 mM sodium vanadate, and mixed on a rotator at 4°C for 30 min. Finally, nuclear extracts were collected by centrifugation of nuclei incubation mixtures in buffer B for 30 min at 13,500 rpm. Aliquots were frozen in liquid N2 and stored at −80°C. Protein concentrations were determined by a Bio-Rad assay with bovine serum albumin standards.

Western blotting was performed as we described previously (33). Briefly, 40 µg of the nuclear extracts were subjected to 8% SDS-PAGE and transferred onto a nitrocellulose membrane. Then, the membrane was washed and probed with 1:1,000 specific anti-HIF-1α serum and subsequently with 1:4,000 horseradish peroxidase-labeled goat anti-rabbit IgG. We made a polyclonal antibody against a 13-residue peptide from rat HIF-1α, which consisted of NH2-CIHVY-AJP-Renal Physiol • VOL 281 • NOVEMBER 2001 • www.ajprenal.org
DTSSNQPQ-COOH (amino acids 199–210 plus a cysteine end). This antibody was confirmed to be specific to HIF-1α with blockade by synthetic peptide before the commercial anti-HIF-1α antibodies were available and by removal of the signal in positive controls (Hela cell nuclear extracts) after immunoprecipitation using commercial anti-HIF-1α antibodies. To detect immunoblotting signal, 10 ml of enhanced chemiluminescence detection solution (Amersham Pharmacia) was added, and the membrane was wrapped and exposed to Kodak OMAT film.

RNA extraction and Northern blot analysis. The cDNA probes for HIF-1α, HO-1, and HO-2 were generated by RT-PCR with primer pairs designed and synthesized based on the sequences of rat HIF-1α, HO-1, and HO-2 cDNA in GenBank (accession nos. AF057308 for HIF-1α, M12129 for HO-1, and J05405 for HO-2) (33). The primer sequence is the following: for HIF-1α: 5'-CGGCCGAAGCAAGAGTCT-3' (sense) and 5'-TGAGTAGGTGTGACCTGTC-3' (antisense); for HO-1: 5'-GTCATGGCCGTTCTTCTCTC-3' (sense) and 5'-CTGACCCTTGCCCTAAG-3' (antisense); and for HO-2: 5'-GAATTCCGGGACCAAGGACCAT-3' (sense) and 5'-CTTACGACCTTGACCCGGCAAGA-3' (antisense). Total RNA was extracted using Trizol solution (Life Technologies), according to the manufacturer's protocol. Northern blot analyses of HIF-1α, HO-1, and HO-2 mRNAs were performed as we described previously (33). In brief, total RNA (10–20 μg) was fractionated on a 1% formaldehyde-agarose gel, stained with ethidium bromide (0.5 μg/ml), washed, photographed, transferred onto nylon membranes (Pierce), and cross-linked to the membrane by ultraviolet irradiation. The nylon membranes were first prehybridized with Rapid Hyb buffer (Amersham Pharmacia) and then probed with 32P-labeled rat HIF-1α, HO-1, or HO-2 cDNA, respectively, at 65°C for 2.5 h. After being washed once at room temperature and then twice at 65°C, the membranes were autoradiographed at −80°C for 24 or 36 h. The autoradiographed films were scanned with a laser densitometer (Hewlett-Packard ScanJet ADF) and then digitized by a UN-SCAN-IT software package (Silk Scientific). The densitometric values of those specific bands for corresponding gene expression were normalized to 28S rRNA (Life Technologies), according to the manufacturer's protocol.

Cell hypoxia. RMICs were plated in 100-mm2 tissue culture dishes in 5 ml of media 24 h before the experiments to form a subconfluence. To decrease PO2 in the culture medium, the dishes were transferred to a sealed, humidified modular chamber and flushed for 2 or 4 h with 5% CO2–95% N2. PO2 in the culture medium was measured as we described previously (35). PO2 in the culture medium, as measured by an electrode and oxygenmeter, was ~10 Torr after hypoxia for 2 h. After hypoxia, the culture medium was rapidly replaced by Trizol solution, and then total RNA was extracted.

Decoy of HIF-1α. It has been demonstrated that HIF-1α activates gene expression by binding to a promoter or an enhancer site, HRE. This cis element contains a CC-gt-consensus sequence. A standard fluorescein-attached HRE containing oligodeoxynucleotides (ODN) was synthesized with sequences of 5'-GCCCTAAGTCGTCTC-3' (sense) and 5'-TGAGACACAGCGAGGC-3' (antisense) and scrambled ODN with sequences of 5'-GCCCTTAACACGTGCTC-3' (sense) and 5'-GAGACAGTTGTAAGGCC-3' (antisense) (26, 28). The fluorescein attachment at the 5'-end was used as an indicator for transfection into the cells. To make double-strand ODN (dsODN), both sense and antisense ODNs (100 μM in TE, pH 8.0) were heated at 95°C for 5 min and then cooled slowly down to room temperature (17). These dsODNs were wrapped by using cationic liposomes (GenePORTER Transfection Reagent; GTS) and transfected into RMICs as described by the manufacturer. dsDNA (10 μg) was first mixed with 50 μl of liposome and then added to 5 ml of serum-free incubation medium. The transfection efficiency was evaluated by a fluorescence microscope (Olympus, Tokyo, Japan) 24 h after incubation of RMICs with the liposome–dsODN mixtures. Positively transfected cells (60–80% cells), indicated by a remarkable intracellular fluorescence, were used to determine the effects of the HIF-1α decoy on the gene expression of HO-1 and HO-2 induced by hypoxia or HIF-1α decoys and to prepare the nuclei for in vitro transcription and nuclear protein extraction.

Nuclear run-on assay. Nuclei were isolated from RMICs essentially according to a method previously described (1). Briefly, RMICs (2 × 107 cells/assay) were washed twice with ice-cold PBS, scraped, and collected in a 15-ml centrifuge tube by centrifugation at 500 g for 5 min at 4°C. Subsequent steps were performed at 4°C. The cells were resuspended in 4 ml of lysis buffer (10 mM Tris·HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl2, 0.5% Nonidet P-40), allowed to stand on ice for 5 min, and then centrifuged at 500 g for 4°C for 5 min. Nuclear debris was removed by centrifugation at 10,000 g for 5 min. Nuclear DNA was resuspended in 10 mM Tris·HCl, pH 8.3, 40% (vol/vol) glycerol, 5 mM MgCl2, 0.1 mM EDTA and frozen in liquid N2 until frozen. Frozen nuclei (200 μl) were thawed and mixed with 200 μl of 2× reaction buffer (10 mM Tris·HCl (pH 8.0), 5 mM MgCl2, 300 mM KCl), 1 mM each of ATP, CTP, and GTP and 100 μCi of [α-32P]UTP (3,000 Ci/mmol, Amersham). Samples were incubated at 30°C for 30 min with shaking and for 5 min in the presence of 20 units of DNase I (RNase free, Gibco). Extracted RNA was resuspended in diethylpyrocarbonate-H2O at 5 × 106 cpm/ml, where cpm is counts per minute. Target DNA membranes were prepared by denaturation, neutralization, and immobilization of 10 μg of linearized plasmid DNA containing a 396-bp fragment of rat HO-1, and a 356-bp fragment of rat β-actin served as a loading control. Prehybridization and hybridization were performed as described above in RNA extraction and Northern blot analysis.

Statistical analysis. Data are presented as means ± SE. The significance of the difference in mean values within and among multiple groups was examined with an ANOVA for repeated measures followed by a Duncan’s post hoc test. Student’s t-test was used to evaluate the significance of differences between two groups of experiments (SigmaStat, SPSS). A value of P < 0.05 was considered statistically significant.

RESULTS

Effect of hypoxia on HO and HIF-1α mRNA expression in RMICs. Figure 2 presents the results of Northern blot analysis of HIF-1α, HO-1, and HO-2 mRNA. Figure 2A shows a typical autoradiographic document of the membrane carrying RNAs extracted from RMICs under normoxic and hypoxic conditions, which were probed with HIF-1α, HO-1, and HO-2 cDNA probes, respectively. Exposure of RMICs to hypoxia produced a remarkable increase in HIF-1α and HO-1 mRNA levels. HO-2 mRNA expression was not altered by hypoxia. Figure 2B summarizes the data from these experiments. Densitometric analysis shows that reduction of PO2 in the culture medium for 2 h significantly increased mRNA levels of HIF-1α (n = 6) and HO-1 (n = 6) in RMICs, but it had no effects on HO-2 mRNA expression (n = 6).
Effect of HIF-1α induction on HO mRNA expression in RMICs. We performed the experiments to observe whether HIF-1α inducers desferrioxamine (DFX) and Cobalt chloride (CoCl2) increase the levels of HO-1 and HO-2 mRNA in RMICs. These cells were incubated with 260 μM DFX and 150 μM CoCl2 for 6 h, and then total RNA was extracted. Figure 3A shows a typical gel document of HIF-1α, HO-1, and HO-2 expression by Northern blot analysis. A significant increase in HIF-1α mRNA was found in DFX- or CoCl2-treated RMICs. In parallel, HO-1, but not HO-2, mRNA levels were increased in these cells. These results are summarized in Fig. 3B. HIF-1α inducers DFX and CoCl2 markedly increased mRNA expression of HIF-1α (n = 6) and HO-1 (n = 6). Both HIF-1α inducers were without effect on the levels of HO-2 mRNA.

Effect of HIF-1α expression inhibition on HO mRNA levels in RMICs. It has been reported that ferrous ammonium sulfate (FAS) is an inhibitor of HIF-1α expression, which blocked DFX-mediated induction of HIF-1α (27). The present study determined the effect of FAS on HIF-1α expression and HO mRNA levels during cell hypoxia. In these experiments, RMICs were incubated in a hypoxic chamber for 2 h in the presence or absence of 200 μM of FAS. As shown in Fig. 4A, RMICs subjected to hypoxia more abundantly expressed HIF-1α and HO-1 compared with control cells. In the presence of FAS, both HIF-1α and HO-1 mRNAs were not altered in response to cell hypoxia. These results are summarized in Fig. 4B. Hypoxia-induced upregulation of HIF-1α (n = 5) and HO-1 (n = 5) was inhibited by FAS. However, FAS was without effect on HO-2 mRNA levels.

Effect of ubiquitin-proteasome inhibition on HO mRNA expression in RMICs. Recent studies have indicated that HIF-1α is degraded via the ubiquitin-proteasome pathway (9, 10). Therefore, alteration of HIF-1α protein levels by blocking the aforementioned protease pathway may change HO-1 mRNA expression. In the present study, N-carbobenzoxy-L-leucinyl-L-leucinyl-L-norvalinal (CBZ-LLL) was used to specifically inhibit ubiquitin-proteasome (9), and then the
Effect of HO mRNA expression was observed. Pretreatment of RMICs with 10 μM CBZ-LLL for 6 h dramatically increased HO-1 mRNA levels in these cells, even under normoxic conditions. However, CBZ-LLL had no effect on the mRNA levels of HO-2 (Fig. 5A). Because CBZ-LLL primarily acted to block the degradation of HIF-1α protein, HIF-1α mRNA levels were not altered by this protease inhibition. The above results are summarized in Fig. 5B. CBZ-LLL significantly increased HO-1 mRNA expression (n = 6), but it had no effect on the mRNA levels of HO-2 and HIF-1α.

**Effect of hypoxia, CoCl2, and CBZ-LLL on HIF-1α protein expression in RMICs.** By Western blot analysis, HIF-1α protein expression was also found to increase significantly in RMICs exposed to hypoxia for 2 h, suggesting that increased transcripts during hypoxia are used to produce HIF-1α protein. Similarly, the treatment of RMICs with 150 μM CoCl2 for 6 h produced a significant increase in HIF-1α protein levels in these cells. Moreover, inhibition of proteasome by 10 μM CBZ-LLL markedly increased HIF-1α levels in RMICs. All of these results are summarized in Fig. 6. It should be noted that care was taken to calculate and load nuclear protein amount in each lane, because there is no positive control to confirm the loading in these nuclear extracts with very little β-actin.

**Effect of HIF-1α decoy on HO mRNA expression in RMICs.** As shown in Fig. 7, under a fluorescent microscope, introduction of fluorescein dsODN produced intensive fluorescence in RMICs. Using this liposome from GTS, 60–80% of the RMICs were transfected by fluorescein dsODN (Fig. 7C). These cells were used to determine the effects of dsOdns on HO mRNA expression in response to hypoxia or CoCl2. As shown in Fig. 8A, hypoxia induced HO-1 mRNA expression. However, the hypoxia-induced increase in HO-1 mRNA levels was depressed in the cells transfected with specific dsODN [dsODN(+) containing 5’-CGTG-3’]. The decoy of HIF-1 had no effect on the response of HO-2 mRNA levels to hypoxia in these cells. As a control, scrambled dsODN [dsODN(−)] without 5’-CGTG-3’ was used to transfet RMICs, but it had no effect on the increase in HO-1 mRNA levels induced by hypoxia (Fig. 8B). Figure 8C summarizes the results of these
experiments. Clearly, the decoy of HIF-1 by its specific binding dsODN blocked the induction of HO-1 mRNA.

Similarly, the decoy of HIF-1α was found to block the CoCl2-induced increase in HO-1 mRNA levels in these RMICs. As shown in Fig. 9A, CoCl2 increased HO-1 mRNA levels, but it had no effect on HO-2 mRNA in regular cells. In dsODN(+) -transfected cells, the increase of HO-1 mRNA induced by CoCl2 was markedly reduced. Figure 9B summarizes the results of these experiments. The decoy of HIF-1α, by introduction of its binding sequence, produced a remarkable blockade of CoCl2-induced HO-1 upregulation.

**HIF-1α-mediated HO-1 transcription induction in vitro.** Using the nuclei prepared from RMICs treated with hypoxia, HIF-1α inducer, inhibitor, or decoy oligodeoxynucleotides, we performed a nuclear run-on assay in vitro to determine whether HO-1 is transcriptionally activated by an HIF-1α-mediated mechanism. HO-1 mRNA formed during the nuclear run-on reaction was found to increase markedly in RMICs exposed to hypoxia for 2 h, but β-actin transcription was not...

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**Fig. 6.** Effects of hypoxia, CoCl2, and CBZ-LLL on HIF-1α protein expression in RMICs. A: typical autoradiographic documents of HIF-1α protein expression in RMICs exposed to hypoxia for 2 h or in the presence of 150 μM CoCl2 and 10 μM CBZ-LLL for 6 h. A band at 96 kDa is identified to be HIF-1α. B: summarized data showing the intensity of immunoreactive band of HIF-1α protein. *P < 0.05 compared with control.

**Fig. 7.** A: photomicrograph of RMICs under light microscope (×200). B: photomicrograph of RMICs without transfection of fluorescein-labeled double-strand oligodeoxynucleoside (dsODN) under fluorescence microscope. C: fluorescent photomicrograph of RMICs transfected with fluorescein-attached dsODN containing HIF-1α binding site under fluorescence microscope (×200).

**Fig. 8.** Effects of HIF-1α decoy by introduction of synthetic dsODN on HO-1 and HO-2 mRNA expression in response to hypoxia in RMICs. A: typical autoradiographic documents of Northern blot analysis using HO-1 and HO-2 cDNA probes and 28S rRNA. dsODN(+), 5′-CGTG-3′ containing dsODN. B: typical autoradiographic documents of Northern blot analysis using HO-1 and HO-2 cDNA probes and 28S rRNA. dsODN(−), Scrambled dsODN without 5′-CGTG-3′. C: summarized data showing intensity of HO-1 and HO-2 blots. *P < 0.05 compared with control. **P < 0.05 compared with values obtained from hypoxic cells.
altered. In the nuclei from the cells treated with 150 μM CoCl2 and 10 μM CBZ-LLL, newly formed HO-1 mRNA was also markedly increased (Fig. 10A). However, the nuclei from RMICs transfected with dsODN(-) lost the ability to produce HO-1 mRNA in response to hypoxia. In the cells transfected with dsODN(–), however, hypoxia still stimulated the production of HO-1 mRNA from their nuclei (Fig.10B).

**DISCUSSION**

In the present study, HO-1 and HO-2 mRNAs were detected in cultured RMICs. Under control conditions, the mRNA levels of HO-2 were higher than HO-1. When these cells were exposed to hypoxia for 2 or 4 h, HO-1 mRNA levels were markedly increased but HO-2 mRNA was not significantly altered. These results suggest that HO-1 and HO-2 are present in RMICs and HO-1 is a hypoxia-inducible gene in these cells, which is consistent with the view that HO-1 is inducible and HO-2 is constitutive.

In parallel to the upregulation of HO-1, the mRNA and protein levels of HIF-1α were also found to increase in response to hypoxia, suggesting that this transcription factor is probably involved in the transcriptional activation of HO-1. The increase in HIF-1α mRNA in RMICs in response to hypoxia indicates that the regulatory response of HIF-1α may occur at the mRNA level, which is consistent with the results obtained in in vivo experiments showing the high levels of HIF-1α mRNA in the renal medulla with low P(O2) (34). Although some studies have indicated that HIF-1α is regulated at the post-mRNA level (7, 8, 29), HIF-1α mRNA was also found to increase in response to hypoxia in vivo in mice or rats (2, 31, 32). It seems that HIF-1α mRNA is constitutively expressed in transformed cell lines (8, 29), but its expression can be induced by hypoxia or ischemia in vivo (2, 31, 32). The present study provides the first evidence that freshly-isolated and cultured RMICs behave more like cells in vivo, which exhibited elevation of mRNA in response to hypoxia.

To further determine the role of HIF-1α in the transcriptional activation of genes in RMICs, additional experiments were performed to examine the effects of HIF-1α inducers on the expression of HO genes. It was found that induction of HIF-1α by DFX and CoCl2 significantly increased the HIF-1α mRNA and protein levels and simultaneously increased HO-1 mRNA. In contrast, inhibition of HIF-1α production by an iron donor, FAS, substantially blocked the hypoxia-induced increase in HIF-1α and HO-1 mRNA in RMICs. Unlike HO-1, HO-2 mRNA levels were not altered by DFX, CoCl2, and FAS. It is HO-1 that is activated by pharmacologically increased or decreased HIF-1α levels in RMICs.

Recent studies have demonstrated that ubiquitin-proteasome is a primary protease system responsible...
for the degradation of HIF-1α, which may importantly contribute to the regulation of intracellular HIF-1α levels and thereby to the transcriptional activation of downstream genes (9, 10). In the present study, HO-1 transcripts were found to increase markedly even under control conditions, but HO-2 was not altered, when RMICs were incubated with a selective inhibitor of ubiquitin-proteasome, CBZ-LLL. This increase in HO-1 mRNA levels associated with the increase in HIF-1α protein suggests that transcriptional regulation of the HO-1 gene primarily requires a structural or functional integrity of HIF-1α protein in RMICs.

In addition to these pharmacological interventions, we also used a molecular decoy approach to determine the role of HIF-1α in the transcriptional regulation of HO genes. This anti-gene therapy strategy can decoy and thereby block the binding of transcription factors to their binding sites in promoter or enhancer regions by introducing a synthesized dsODN containing a binding cis element (18). We demonstrated that both hypoxia and HIF-1α induction by CoCl2 increased the mRNA levels of HO-1 to a much lesser extent in RMICs transfected with a dsODN containing an HIF-1 binding site, 5′-CGTG-3′, compared with control cells. These results provide evidence that specific blockade of HIF-1α binding decreases an increase in HO-1 mRNA in RMICs, which further supports our hypothesis that HIF-1 mediates the transcriptional activation of the HO-1 gene.

To further confirm that the actions of hypoxia and HIF-1α alterations on HO-1 mRNA expression are due to the changes in newly formed mRNA, in vitro nuclear run-on assays were performed. Using the nuclei prepared from RMICs treated with different compounds or hypoxia, newly formed HO-1 and β-actin mRNAs were detected. It was found that HO-1 mRNA formation remarkably increased in the nuclei prepared from RMICs exposed to hypoxia, the HIF-1α inducer CoCl2, and the ubiquitin-proteasome inhibitor CBZ-LLL, but β-actin mRNA did not change. Furthermore, the hypoxia-induced increase in HO-1 mRNA formation was not significantly attenuated in the nuclei from the cells with the decoy of HIF-1α by HRE dsODN. Taken together, these results further confirm that HO-1 is transcriptionally activated by an HIF-1α-mediated mechanism. A previous study has demonstrated a hypoxia-response element in the 5′-flanking region of the mouse HO-1 gene, which contains two potential binding sites for HIF-1 (13). Whether the same binding sites exist in the 5′-flanking region of the rat HO-1 gene remains to be further identified.

In summary, the present studies provided several lines of evidence indicating that HO-1 expression in RMICs is transcriptionally regulated via an HIF-1 pathway under normal and hypoxic conditions. First, alterations of HIF-1α formation or degradation by pharmacological interventions changed HO-1 mRNA levels. Second, the decoy of HIF-1α by transfection of synthesized dsODNs containing 5′-CGTG-3′ blocked the hypoxia- or CoCl2-induced increase in HO-1 mRNA levels. Finally, an in vitro transcription assay demonstrated that hypoxia or induction of HIF-1α increased, but the decoy of HIF-1α decreased, newly formed HO-1 mRNA in the nuclei of RMICs.

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