Journal of Cellular Biochemistry 85:410–421 (2002)

Functional Expression of Human Heme Oxygenase-1 (HO-1) Driven by HO-1 Promoter In Vitro and In Vivo

Shuo Quan,[1] Liming Yang,[1] Sylvia Shenouda,[1] Houli Jiang,[1] Michael Balazy,[1] Michal L. Schwartzman,[1] Ichiyo Shibahara,[2] Kousei Shinohara,[2] and Nader G. Abraham[1]*

[1]Department of Pharmacology, New York Medical College, Valhalla, New York
[2]Department of Molecular Biology and Applied Physiology, Tohoku University School of Medicine, Japan

Abstract We developed a retrovirus-mediated human *heme oxygenase-1* (*HO-1*) gene expression system and assessed the impact of heme on the inducibility of the *HO-1* gene in rat lung microvessel (RLMV) endothelial cells and in newborn Sprague-Dawley (SD) rats. Overexpression of the *HO-1* gene driven by HO-1 promoter (HOP) resulted in an increase in HO-1 protein and HO activity by 4.8- and 1.3-fold, respectively, compared to the viral LTR promoter. The increased *HO-1* gene expression was associated with the enhancement of CO production. In cells transduced by HOP-driven *HO-1* gene, there was a decrease in basal cyclooxygenase (COX) activity as measured by PGE_2. The degree of HO-1 expression and, consequently, the levels of cellular heme were directly related to COX activity. Supplementation with heme markedly increased PGE_2 and cGMP synthesis. In all (6/6) of newborn SD rats injected with retrovirus LSN-HOP-HO-1, both HO-1 and neo^r transcripts were expressed in tissues. We hypothesize that degree of *HO-1* gene expression resulted in a differential rate of cellular heme-dependent enzyme gene expression, which may play a vital role in maintaining cellular homeostasis. J. Cell. Biochem. 85: 410–421, 2002. © 2002 Wiley-Liss, Inc.

Key words: carbon monoxide; retrovirus; cyclooxygenase; cyclic GMP

Endothelium modulates the response of vascular smooth muscle to hormones [Kanse et al., 1991], neurotransmitters [Zanetti et al., 2000], and vascular relaxing/contracting factors, such as endothelin-1 [Taddei et al., 2000], angiotensin II [Haugen et al., 2000], nitric oxide (NO) [Prabhakar, 1999; Polte et al., 2000], and carbon monoxide (CO) [Motterlini et al., 1998; Togane et al., 2000]. CO is an end metabolic product of heme catabolized by heme oxygenase (HO) [Motterlini et al., 1998; Elbirt and Bonkovsky, 1999]. There are two major isoforms of HO, inducible HO-1 and constitutive HO-2 [Cao et al., 2000]. HO-1 expression is activated in virtually all cell types by stress-related factors and reagents such as heat shock [Taketani et al., 1988], heme and heavy metals [Taketani et al., 1988], endotoxin [Shi et al., 2000], UV irradiation [Reeve and Tyrrell, 1999], inflammatory cytokines [Rizzardini et al., 1993; Terry et al., 1998] and hypoxia [Christou et al., 2000], and it is found to be upregulated in disease models such as endotoxemia and ischemia [Takeda et al., 1994] in rodents and in human Alzheimer's disease [Takeda et al., 2000]. This response is explained by the multitude of stress-activated recognition sites contained within the HO-1 promoter, including activator protein 1 sites, CCAAT/enhancer-binding protein sites, phorbol ester response elements, heme response elements and antioxidant response elements [Muraosa and Shibahara, 1993; Hartsfield et al., 1998; Hartsfield et al., 1999].

CO has been implicated in the control of vascular tone [Coceani, 1993; Kozma et al., 1999], which is mediated by a cGMP-signaling pathway and by calcium-activated potassium channels [Furchgott and Jothianandan, 1991; Christodoulides et al., 1995; Coceani et al.,

Abbreviations used: HO-1, Heme oxygenase-1; CO, Carbon monoxide; HOP, HO-1 transcriptional-regulatory sequence; PGE_2, Prostaglandin E2; RLMV, (rat lung microvessel) endothelial cells.

Grant sponsor: NIH; Grant numbers: RO1 DK56601, HL34300; Grant sponsor: American Heart Association; Grant numbers: AHA 50948T, 20285T.

*Correspondence to: Nader G. Abraham, Department of Pharmacology, New York Medical College, Valhalla, NY 10595. E-mail: nader_Abraham@nymc.edu

Received 27 November 2001; Accepted 18 January 2002
DOI 10.1002/jcb.10147

1996; Wang et al., 1997]. Exogenous administration of CO relaxes isolated blood vessels and the addition of HO-1 inducers causes a marked decrease in blood pressure in hypertensive rats [Vedernikov et al., 1989; Graser et al., 1990], whereas, the administration of HO-1 inhibitors increases peripheral vascular resistance and blood pressure [Johnson et al., 1995]. Moreover, the first human case of HO-1 deficiency, which has been reported to be due to a genetic disorder, shows severe persistent endothelial damage and increased tissue vulnerability to oxidant injury besides growth retardation and anemia [Yachie et al., 1999; Ohta et al., 2000].

Experimental evidence from our laboratory demonstrated that overexpression of HO led to the reduction of cellular unmetabolized heme, which might affect the production of other heme enzymes and their metabolic products, such as PGE_2 and cGMP. Several investigators have proposed that upregulation of HO-1 during stress and hypertension is an adaptive mechanism that may protect cells from oxidative damage [Deramaudt et al., 1999] and lower blood pressure [Lavrovsky et al., 1993; Lavrovsky et al., 1994; Lavrovsky et al., 1996]. Our laboratory has shown that overexpression of HO-1 via retrovirus and/or adenoviral-mediated gene transfer, substantially enhanced cellular protection against subsequent oxidative challenges [Abraham et al., 1995; Yang et al., 1999] and blocked the development of hypertension in newborn hypertensive rats [Lee et al., 1996; Abraham et al., 2000]. However, uninducible and low-efficient viral promoters (such as LTR or CMV) limit their further application in studies of in vivo gene therapy.

The present study was designed to evaluate the feasibility of utilizing a human HO-1 promoter to control *HO-1* gene expression in rat lung microvessel (RLMV) endothelial cells and in newborn Sprague Dawley (SD) rats and explore the relationship between endogenous *HO-1* gene expression and the products of other heme proteins. Our results demonstrated that the human HO-1 promoter mediated stronger inducible *HO-1* gene expression compared to the viral LTR promoter. Our results also demonstrated that overexpression of HO-1 led to an increase in cellular CO and cGMP production and a reduction in the COX product, PGE_2.

METERIALS AND METHODS

Cell Culture

The amphotropic retroviral packaging cell lines PA317 (ATCC, Manassas, VA) or PT67 (Clontech, Palo Alto, CA) were used for the generation of replication-deficient recombinant retroviruses. PA317 and PT67 cells were grown in DMEM medium (GIBCO-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum. NIH3T3 fibroblasts were cultured in DMEM with 10% calf serum. RLMV cells were cultured in MCDB131 medium with 10% FBS, 10 ng/ml EGF, 1 μl/ml hydrocortisone, 0.1 mg/ml ENDO GRO (VEC Technologies, Inc., Rensselaer, NY), and 90 μg/ml heparin (Sigma, St. Louis, MO). All cells were incubated at 37°C in a 5% CO_2 humidified atmosphere, and maintained at subconfluency by passaging with trypsin-EDTA (GIBCO-BRL).

Development of Recombinant Retroviral Vectors

LSN-HHO-1: The HHO-1 expressing replication-deficient retrovirus vector LSN-HHO-1 was previously constructed in our laboratory [Yang et al., 1999]. In this vector, HHO-1 cDNA, a kind gift from Dr. Shigeki Shibahara (Tohoku University School of Medicine, Japan) [Shibahara et al., 1987; Shibahara et al., 1989], is under the control of the viral internal LTR promoter.

LSN-HOP-HHO-1: This retroviral vector, in which HHO-1 cDNA is driven by the HHO-1 promoter, was constructed as follows: (1) a 1,519-bp (+19 to −1500) HHO-1 transcriptional-regulated sequence (HOP) was released from the plasmid A-CAT and inserted at the XhoI and HindIII sites of the plasmid pGEM-7zf(+) (Promega, Madison, WI). The resulting plasmid was named pGEM-HOP; (2) the retroviral vector, LSN-HOP, was constructed by cloning the XhoI-EcoRI HOP sequence of the pGEM-HOP at the EcoRI and XhoI sites of the retroviral vector LXSN; (3) the 987 bp (−63 to +924 bp) HindIII HHO-1 cDNA fragment from the pRc-CMV-HHO-1 was end-blunted and was inserted at the end-blunted BamHI site of the LSN-HOP. After clone selection, the transcription-oriented construct was designated as LSN-HOP-HHO-1.

Development of Retrovirus-Mediated Expression of Human *HO-1* Gene

PA317 retroviral packaging cells were stably transfected with retroviral vector LSN-HHO-1 and control retroviral vector LXSN. G418-resistant clones (PA317/HHO-1 and PA317/LXSN) were isolated and tested for their ability to produce a retrovirus in a culture medium. The results showed that the highest retroviral titers could reach 1.4×10^6 and 1.2×10^6 cfu/ml in PA317/HHO-1 and PA317/LXSN cell clones, respectively. The neomycin resistant (*neo*^{r}) gene was expressed in both PA317/HHO-1 and PA317/LXSN cells, whereas only *HHO-1* gene expression was detected in PA317/HHO-1 cells as confirmed by RT–PCR and Northern blot analysis [Yang et al., 1999].

To develop the HO-1 promoter-controlled HHO-1, we used a genetically modified packaging cell line (PT67 cells) to produce a higher titer of retrovirus. PT67 retroviral packaging cells were transfected with retroviral vectors (LSN-HOP-HHO-1) and control retroviral vectors (LSN-HOP). G418-resistant clones (PT67/HOP-HHO-1 and PT67/HOP) were isolated and tested for their potential to produce a retrovirus in a culture medium. The results showed that the use of the PT67 packaging cell line yielded higher retroviral titers, reaching 1.5×10^7 cfu/ml. The neo^r was expressed in both PT67/HOP-HHO-1 and control cells (PT67/HOP). Non-transduced PT67 packaging cells did not yield RT/PCR products with either neo^r or HHO-1 primers. PT67 packaging cells transduced with retrovirus vectors containing HHO-1 resulted in the generation of RT/PCR products using HHO-1 primers corresponding to the expected size of the *HHO-1* gene [Quan et al., 2001].

RLMV endothelial cells were infected using the supernatants of the above retroviral packaging cells. After selection with G418, stable-transfected cell lines, RLMV cells expressing HHO-1 were obtained [Yang et al., 1999].

Measurement of HO Activity and CO Levels

Microsomal HO activity was assayed by the method of Abraham et al. [1995] in which bilirubin, the product of HO degradation, was extracted with chloroform and its concentration determined spectrophotometrically (model DW-2C, Aminco, Urbana, IL) using the difference in absorbance at wavelength from λ 460 to λ 530 nm with an absorption coefficient of 40 $mM^{-1}cm^{-1}$ [Yang et al., 1999].

CO was analyzed using a HP5989A mass spectrometer interfaced to a HP5890 gas chromatograph. The separation of CO from other gases was carried out on a GS-Molesieve capillary column (30 m; 0.53 mm ID; J & W Scientific Inc., Folsom, CA) kept at 40°C. Helium, with a linear velocity of 0.3 m/s, was used as the carrier gas. CO, eluted at 3.6 min, was fully separated from N_2, O_2, H_2O, and CO_2. The mass spectrometer parameters were as follows: ion source temperature, 120°C; electron energy, 31 eV; transfer line temperature, 120°C. Using a gas-tight syringe, 100 μl aliquots of the headspace gas of either standard solutions or experimental samples were injected into the spitless injector at a temperature of 120°C. Abundance of ions at m/z 28, 29, and 31 corresponding to $^{12}C^{16}O$, $^{13}C^{16}O$, and $^{13}C^{18}O$, respectively, were acquired via a selected ion monitoring. For the measurement of CO concentration, the sample in 1 ml solution was prepared in an amber glass vial (2 ml), and then capped tightly with Teflon/Silicone septum. A 1 μl of the ^{13}C-carbon monoxide saturated solution (1 mM) was added to the sample, resulting in the internal standard concentration of 1 μM. After sample equilibration, 100 μl of the headspace gas was taken from the vial and injected into the GC. The amount of CO in cell culture samples was calculated from standard curves constructed with abundance of ions at m/z 28, 29, or m/z 31 as previously described. Both standard curves were linear over the range 0.05–5.0 μmol/L and both yielded comparable results when used for determining the concentration of endogenous CO [Zhang et al., 2001].

RT–PCR and PCR Analysis

Reverse transcription (RT) was carried out using the Advantage^{TM} RT-for-PCR Kit (Clontech). Poly-d(T)n was used as the reverse transcription primer. Specific primers for amplifying HHO-1, neomycin resistant (neo^r), and *LXSN* vector gene fragments are listed in Table I. PCR was performed using a Taq PCR kit (Roche, Indianapolis, IN). For each RT–PCR, a sample without reverse transcriptase was processed in parallel and served as a negative control. Cycling parameters for amplifying RT products were as follows: 95°C, 1′; 60°C, 1′; 72°C, 1–3′, for 30 cycles, and then extended at 72°C for another 5′. After amplification, PCR products

TABLE I. Synthetic Primers for PCR or RT–PCR

Amplified DNA fragment	Primer sequence	Symbol
LXSN vector (139 bp)	S: 5′-CCCGGGAACCTCCTCGTTCGACC-3′ AS: 5′-GAGCCTGGGGACTTTCCACACCC-3′	P1
HHO-1 cDNA (555 bp)	S: 5′-CAGGCAGAGAATGCTGAGTTC-3′ AS: 5′-GATGTTGAGCAGGAACGCAGT-3′	P2 P4
Neo^r gene (313 bp)	S: 5′-AAGATGGATTGCACGCAGG-3′ AS: 5′-GCAAGGTGAGATGACAGGAG-3′	P3 P5

were electrophoresed on 1.2% agarose gel, stained with ethidene bromide, and visualized under UV light.

Genomic DNA was extracted from cells using the DNeasy Tissue Kit (QIAGEN Inc., Valencia, CA). Different combinations of primers (Table I and Fig. 1) were used to amplify the integrated DNA fragments via the Expand High Fidelity PCR System (Roche). Cycling parameters are described as above.

Western Blot Analysis

Cells were harvested using cell lysis buffer as described previously [Yang et al., 1999]. The lysate was collected for Western blot analysis. Protein levels were visualized by immunoblotting with antibodies against human HO-1, total immunoreaction HO-1 (rat and human) or HO-2 (Stressgen Biotechnologies Corp., Victoria, BC, Canada). Briefly, 30 μg of lysate supernatant was separated by SDS/polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Amersham, Piscataway, NJ) using a semidry transfer apparatus (Bio-Rad, Hercules, CA). The membranes were incubated with 5% milk in 10 mM Tris-HCl (pH 7.4) 150 mM NaCl, 0.05% Tween 20 (TBST) buffer at 4°C overnight. After washing with TBST, the membranes were incubated with a 1:2,000 dilution of anti-HO-1 or anti-HO-2 antibodies for 1 h at room temperature, while undergoing constant shaking. Then, filters were washed and subsequently probed with horseradish peroxidase-conjugated donkey anti-rabbit IgG (HO-2 and total HO-1 or anti-mouse IgG (hHO-1) (Amersham) at a dilution of 1:2,000. Chemiluminescence detection was performed with the Amersham ECL detection kit according to the manufacturer's instructions.

Measurement of cGMP and PGE_2 Levels

Cellular cGMP content was determined with a commercial ELISA kit (BIOMOL, Plymouth Meeting, PA) following instructions provided

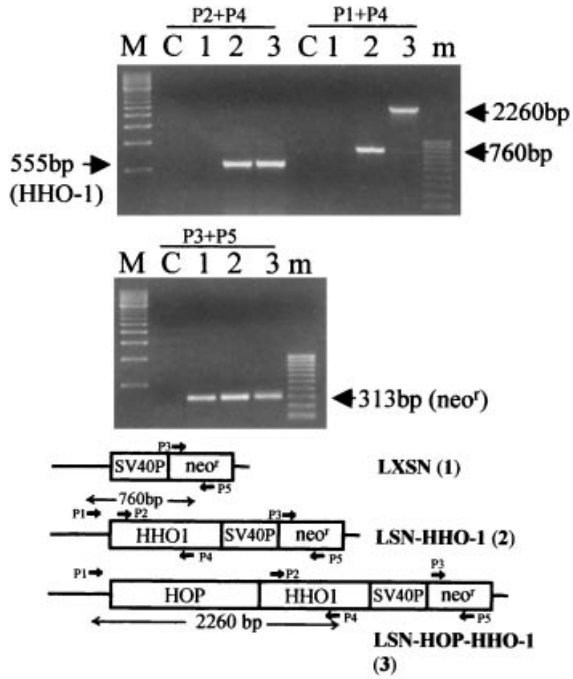

Fig. 1. PCR analysis of genomic DNA from RLMV endothelial cells untransduced or transduced with retroviral vectors (LSN-HOP-HHO-1, LSN-HHO-1, and LXSN). C, control RLMV cells; 1, RLMV cells transduced with the retroviral vector LXSN (RLMV/LXSN); 2, RLMV cells transduced with LSN-HHO-1 (RLMV/HHO-1); 3, RLMV cells transduced with LSN-HOP-HHO-1 (RLMV/HOP-HHO-1). Different combinations of primers were used to amplify various lengths of DNA fragments in the three kinds of RLMV cells. Primer sequences and their symbols are listed in Table I. P1, primer for LXSN vector (sense); P2, primer for HHO-1 (sense); P3, primer for *neo*^{r} gene (sense); P4, primer for HHO-1 (antisense); P5, primer for *neo*^{r} gene (antisense). The primer set of P1 and P4 detected a 2,260 bp DNA fragment (containing partial vector sequence, HOP and partial HHO-1 sequence) in RLMV/HOP-HHO-1 cells, and a 760 bp DNA fragment (containing partial vector and partial HHO-1 sequence) in RLMV/HHO-1 cells. The primer set of P2 and P4 amplified a 555 bp DNA fragment (*HHO-1* gene fragment) in both RLMV/HHO-1 and RLMV/HOP-HHO-1 cells. The 313 bp PCR products (*neo*^{r} gene fragment) were found in all three of the retroviral vector transduced RLMV cells, when the primer set of P2 and P4 was used. These results indicate that different DNA fragments have been integrated into the genomic DNA of RLMV endothelial cells.

by the manufacturer. Briefly, the cells were washed with ice-cold PBS once and then incubated with 0.1 M HCl for 10 min. The cells were collected using a scraper and were centrifuged at 8,000*g* for 5 min at 4°C. Supernatants were stored at −80°C until used. cGMP concentration was normalized to protein content as determined by a dye-binding assay (Bio-Rad).

For measurement of PGE_2, RLMV cells untransduced or transduced with either viral LTR promoter-driven HHO-1 or HHO-1 promoter-driven HHO-1 were incubated with heme (10 μM) for 24 h. The cell-free media were assayed for PGE_2 by ELISA (Neogen Corporation, Lexington, KY). Briefly, 50 μl of diluted medium and 50 μl of HRP-conjugated PGE_2 were placed for 1 h, into wells of a 96-well plate that had previously been coated with anti-PGE_2 antibody. Following incubation, the HRP substrate was added to each well for 30 min. The reaction was stopped by the addition of 1 N HCl. Quantitation was achieved by measuring absorbance at 650 nm.

Microsomal Heme Determination

Microsomal heme was determined as the pyridine hemochromogen by using the reduced minus oxidized difference in absorbance at λ 400 and λ 600 nm with an absorption coefficient of 32.4 $mM^{-1}cm^{-1}$ [Fuhrop and Smith, 1975].

Concentration of Viral Particles and Protocols of Animal Experimentation

Viral particles from supernatants of retroviral packing cells (PT67/HOP-HHO-1) were sedimented by centrifugation at 9,000*g* for 12–14 h at 4°C in a 45 ml sterile centrifuge tube in a RC3B centrifuge. After centrifugation, the supernatant was removed by aspiration and the pellet was resuspended in 1 ml cell culture media or Hank's balanced salt solution. Retroviral particles sedimented as described above were further concentrated by centrifugation at 10,000*g*, 4°C for 12 h in a 1.5 ml tube. The supernatant was removed and the pellet was resuspended in 20–50 μl of physiological saline solution [Quan et al., 1999].

The concentrated retroviruses ($3–5 \times 10^9$ cfu/ml) were injected into newborn Sprague-Dawley (SD) rats (Charles River Lab.,Wilmington, MA) twice intraventricularly at Day 5 (20 μl) and Day 12 (40 μl). Following the injections, rats were allowed to recover and returned to their cages with the appropriate mothers for continued weaning. The animals were weaned, until they were 21 days old before they were housed in their own respective cages. At the end of 4 weeks, some rats were taken to measure the expression of HHO-1 mRNA in various tissues [Lu et al., 1995; Iyer et al., 1996].

Statistical Analysis

The data are presented as mean ± standard deviation (SD) for the number of experiments. Statistical significance ($P < 0.05$) between the experimental groups was determined by the Fisher method of analysis of multiple comparisons. For comparison between treatment groups, the Null hypothesis was tested by a single factor analysis of variance (ANOVA) for multiple groups or unpaired *t*-test for two groups.

RESULTS

Integration and Functional Expression of HHO-1 in RLMV Endothelial Cells

The supernatants of PA317/HHO-1 and PT67/HOP-HHO-1 retroviral packaging cells were used to infect RLMV endothelial cells. After selection with G418, integration and expression of the *HHO-1* gene were determined in G418-resistant cell clones RLMV/HHO-1 (RLMV transduced with viral promoter-driven HHO-1) and RLMV/HOP-HHO-1 (RLMV transduced with the HOP promoter-driven HHO-1). As shown in Figure 1, PCR analysis from genomic DNA showed that the HHO-1 cDNA fragments (555 bp) were detected in both RLMV/HHO-1 and RLMV/HOP-HHO-1 cells with primer sets of P2 (HHO-1 sense) and P4 (HHO-1 antisense). When primer sets of P1 (LXSN vector sense) and P4 were used, a 760-bp fragment (containing partial vector and partial HHO-1 sequence) and a 2,260-bp fragment (containing partial vector, HOP and partial HHO-1 sequence) were amplified in RLMV/HHO-1 and RLMV/HOP-HHO-1 cells, respectively. Neo^r gene fragments were found in all of the three RLMV endothelial cells (RLMV/HHO-1, RLMV/HOP-HHO-1, and control RLMV/LXSN).

To confirm the expression of HHO-1 mRNA transcripts, we conducted RT–PCR analysis for the RNA extracted from the three RLMV cell clones. Total RNA was first digested with RNase-free DNase to exclude the possibility of

contamination with genomic DNA. Then, the DNase was removed by phenol:chloroform:isoamyl alcohol extraction prior to cDNA synthesis. As shown in Figure 2A, HHO-1 mRNA was detectable in both RLMV/HHO-1 and RLMV/HOP-HHO-1 cells. However, no HHO-1 signals were found in untransduced RLMV and control vector LXSN-transduced RLMV cells (RLMV/LXSN).

These results indicate that the *HHO-1* and the *neo*^{r} genes have been integrated into the genomic DNA of the RLMV endothelial cells transduced with either viral promoter-driven HHO-1 or HOP-driven HHO-1, and that the HHO-1 mRNA transcripts are expressed in these two kinds of RLMV cells.

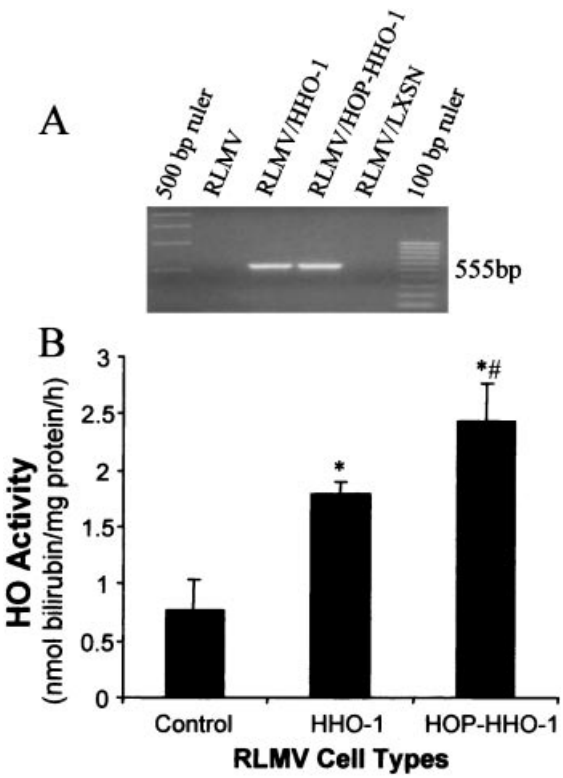

Fig. 2. **A**: Detection of human HO-1 transcripts by RT–PCR analysis in RLMV endothelial cells untransduced or transduced with retroviral vectors (LSN-HOP-HHO-1, LSN-HHO-1, and LXSN). The primer set of P2 and P4 (see Table I) amplified a 555-bp DNA fragment in both LSN-HOP-HHO-1 and LSN-HHO-1 transduced RLMV cells (RLMV/HOP-HHO-1 and RLMV/HHO-1, respectively). **B**: Total HO activity in RLMV/HOP-HHO-1, RLMV/HHO-1 and control RLMV cells. HO activity (nmol bilirubin/mg protein/h) is expressed as the mean ± SD of three experiments. *$P < 0.05$ vs. control cells, #$P < 0.05$ vs. cells transduced with *HHO-1* gene.

Enhancement of HO Activity and Heme-Induced HHO-1 Expression in RLMV Cells Transduced With HOP-Driven HHO-1

The efficacy of *HHO-1* gene transfer in RLMV cells was evaluated by comparing the levels of total HO activity in uninfected and retrovirus-infected endothelial cells. This cell line was chosen because of its relevance to vascular gene therapy. As seen in Figure 2B, total HO activity in RLMV/HHO-1 and RLMV/HOP-HHO-1 endothelial cells increased 2.3- and 3.0-fold, respectively, relative to RLMV cells ($P < 0.05$). In control LXSN-infected endothelial cells, HO activity was similar to that in uninfected cells (data not shown).

HHO-1 protein expression in HHO-1-transduced RLMV endothelial cells was determined by Western blot analysis. As shown in Figure 3, the basal level of HHO-1 protein in RLMV/HOP-HHO-1 cells was significantly higher (4.75-fold) than that in RLMV/HHO cells. The addition of heme (10 μM, 24 h) further increased HHO-1 protein expression (1.8-fold higher) in RLMV/HOP-HHO-1 cells, but not in RLMV/HHO-1 cells. HHO-1 protein expression was not found in either RLMV or control vector LXSN-transduced RLMV cells. Moreover, there were no differences in HO-2 protein levels, further indicating that the overexpression of HO-1 by gene transfer did not modulate HO-2 expression. These findings reveal that the HHO-1 expression driven by the 1.5 kb HHO-1 promoter is much stronger than that driven by the viral LTR promoter and can be induced by heme or other stimulators (such as stannous chloride) in HHO-1-transduced RLMV endothelial cells (Fig. 3C).

Effect of *HHO-1* Gene Transduction on Endogenous CO and cGMP Production

CO production in RLMV endothelial cells transduced with the HHO-1 promoter-driven *HHO-1* gene and in cells transduced with the viral promoter-driven *HHO-1* gene was examined by GC-MS. The results of three representative experiments are shown in Figure 4. CO production was significantly higher in RLMV/HOP-HHO-1 cells than in RLMV/HHO-1 cells (150 ± 12 vs. 80 ± 15 nmol/mg protein/4 h, $P < 0.05$). The addition of heme (10 μM, 24 h) further increased the levels of CO production in both RLMV/HOP-HHO-1 cells (530 ± 75 nmol/mg protein/4 h) and RLMV/HHO-1 cells

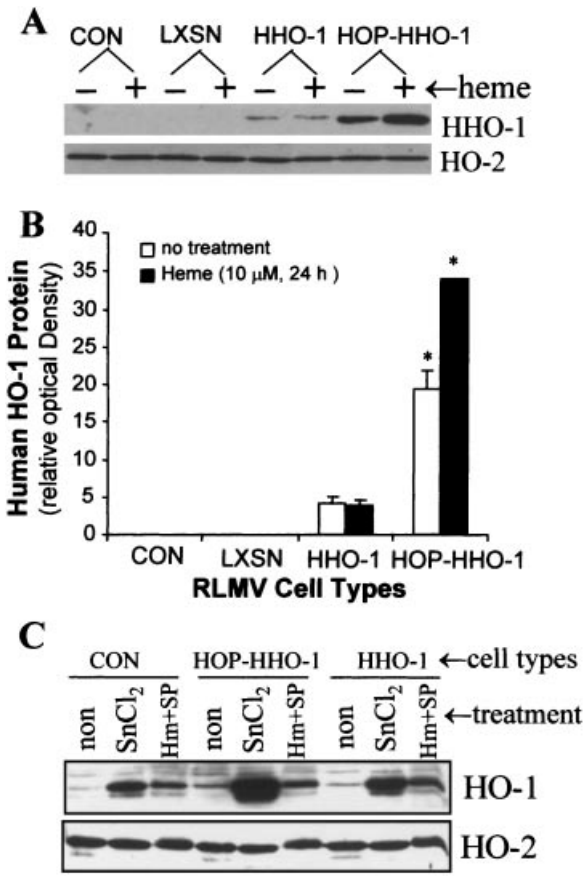

Fig. 3. Western blot analysis of RLMV endothelial cells untransduced or transduced with retroviral vectors (LSN-HOP-HHO-1, LSN-HHO-1, and LXSN). **A**: Cells were treated without or with heme (10 μM, 24 h) and HHO-1 protein was detected using anti-human HO-1 antibody. **B**: Relative optical density of HHO-1 protein expression, normalized for the darkest band on each gel, is shown to facilitate comparison. Values are expressed as the mean ± SD (n = 3). $*P < 0.05$ compared with RLMV/HHO-1 cells. **C**: RLMV cells untransduced or transduced with HHO-1 or HOP-HHO-1 were treated with $SnCl_2$ (50 μM, 24 h) or heme (10 μM, 24 h) after preincubation with SnMP (10 μM, 30 min). non, nontreatment; Hm, heme; SP, SnMP. Total (rat and human) HO-1 protein was detected using anti HO-1 antibody.

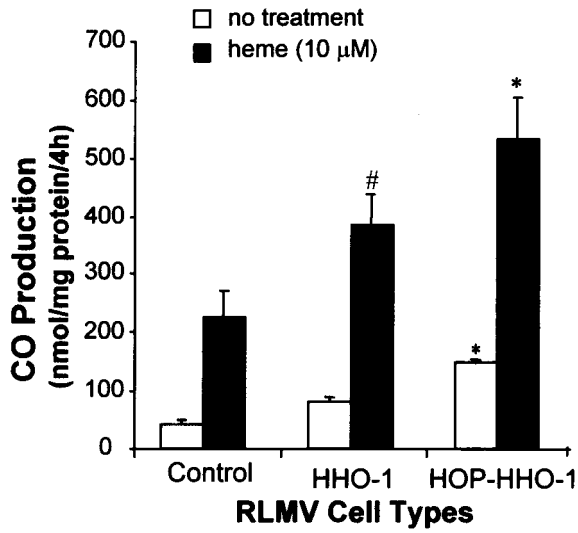

Fig. 4. CO production in RLMV endothelial cells untransduced and transduced with retroviral vectors (LSN-HHO-1 and LSN-HOP-HHO-1). Measurement of CO production is described in METHODS. Values are expressed as the mean ± SD of four experiments. $*P < 0.05$ vs. RLMV/HHO-1 cells; $\#P < 0.05$ vs control RLMV cells.

(385 ± 50 nmol/mg protein/4 h); the former was significantly higher than the latter. These results indicated that CO production in cells transduced with the HHO-1 promoter-driven *HHO-1* gene is higher than that in cells transduced with the viral promoter-driven *HHO-1* gene, and the challenge with heme significantly increased CO production.

To determine whether overexpression of the *HHO-1* gene affects the endogenous cellular cGMP level, we measured cGMP content in nontransduced RLMV cells and cells transduced with HOP-driven *HHO-1* gene (RLMV/HOP-HHO-1) (Fig. 5). There was no significant difference in basal cGMP levels between untreated RLMV and RLMV/HOP-HHO-1 cells, which were cultured in media containing 0.5% fetal bovine serum for 24 h. The addition of heme (10 μM, 24 h) increased the levels of cGMP in both RLMV/HOP-HHO-1 cells (61.5 ± 5 pmol/mg protein) and control RLMV cells (44 ± 6.1 pmol/mg protein)($P < 0.05$). To demonstrate that the elevated cGMP level is mediated by the overexpression of HO, we preincubated cells with Sn-mesoporhyrin (SnMP) (10 μM) for 30 min prior to the administration of heme (10 μM) for 24 h. The results showed that the addition of SnMP reduced the cellular cGMP level by 43.8 and 61.9% in RLMV and RLMV-HOP-HHO-1 cells respectively ($P < 0.05$), indicating that HO activity contributed to the elevated cGMP content.

To ascertain the metabolic characteristics of the cells expressing HHO-1, we assessed the levels of nonmetabolized heme following exogenous heme addition in RLMV cells untransduced and transduced with HHO-1. Cells were cultured for 24 h, heme content and HO activity were determined. As shown in Table II, heme content was significantly lower in RLMV/HHO-1 cells than that in RLMV cells ($P < 0.05$), reflecting the increase in HO activity following *HHO-1* gene expression.

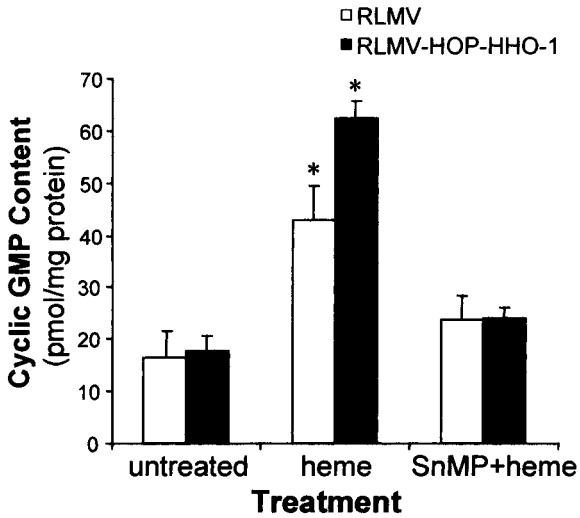

Fig. 5. Cellular cGMP content in RLMV endothelial cells untransduced or transduced with retroviral vectors (LSN-HOP-HHO-1). In untreated RLMV and RLMV-HOP-HHO-1 cells, 0.5% fetal bovine serum was used for 12 h cell culture prior to cellular cGMP assay. Then, some cells were treated with heme (10 μM) for 24 h, or preincubated with SnMP (10 μM) for 30 min followed by the addition of heme (10 μM, 24 h). Cellular cGMP concentrations are expressed as the mean ± SD (n = 6), $^*P < 0.05$ vs. untreated.

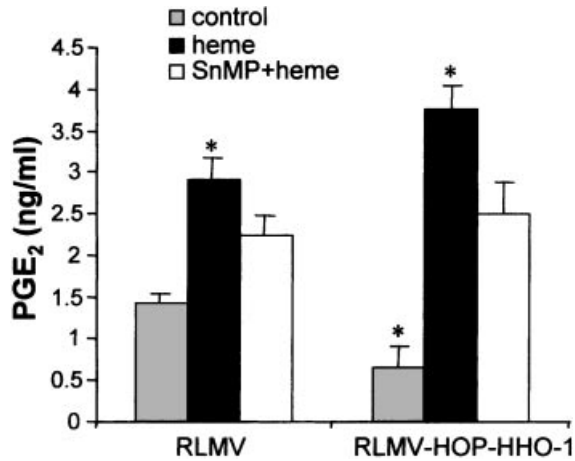

Fig. 6. The effect of heme on PGE_2 production in RLMV cells untransduced or transduced with HOP-driven *HO-1* gene (RLMV-HOP-HHO-1). The levels of PGE_2 in the culture media of RLMV and RLMV-HOP-HHO-1 cells were assayed by ELISA as described in METHODS. The results are expressed as mean ± SD (ng/ml, n = 4, $^*P < 0.05$ compared with the corresponding controls).

Effect of Heme on Prostaglandin Levels in RLMV Cells Transduced With HOP-Driven *HHO-1* Gene

To determine the impact of *HO-1* gene transfer on prostaglandin levels, we assessed PGE_2 production in RLMV cells untransduced or transduced with the HOP directed *HHO-1* gene (RLMV-HOP-HO-1). Figure 6 shows that the basal levels of PGE_2 in control RLMV cells were 2.41-fold higher than those in RLMV cells transduced with HOP-HHO-1 (1.45 ± 0.14 vs. 0.59 ± 0.26 ng/ml, $P < 0.05$). The addition of heme (10 μM, 24 h) increased PGE_2 production by 2.0- and 6.3-fold, respectively, in RLMV cells and RLMV cells transduced with HO-1 ($P < 0.05$). The preincubation with SnMP (10 μM, 30 min) prior to heme treatment partially attenuated the production of PGE_2 to a similar level ($P > 0.05$) in both untransduced RLMV cells and RLMV cells transduced with HOP-HHO-1.

TABLE II. Effect of Retroviral Mediated *HHO-1* Gene Transfer on Heme Content in RLMV Endothelial Cells

Cell types	Heme content (pmol/mg microsomal protein)
RLMV	280 ± 38
RLMV/HHO-1	142 ± 52*
RLMV/LXSN	263 ± 23

RLMV cells untransduced or transduced with HHO-1 (RLMV/HHO-1) were treated with heme (10 μM) for 24 h. Heme content was measured as described in Materials and Methods.
$^*P < 0.05$ vs. untransduced RLMV cells. Values expressed as mean ± SD of three experiments.

HHO-1 Expression in Newborn SD Rats Administered Concentrated Retroviruses Containing *HHO-1* Gene

To determine whether the human HO-1 promoter can be effective in driving *HO-1* gene in vivo, we infected newborn SD rats with concentrated retroviruses via a cardiac route. Newborn rats were chosen, because their cells have stronger ability to proliferate and differentiate than those of adult rats, and retroviruses only infect actively dividing cells. LSN-HOP-HHO-1 and LXSN (control) retroviruses were concentrated to $3–5 \times 10^9$ cfu/ml as described in METHODS. The rats were injected with 20 and 40 μl of retroviruses at day 5 and day 12, respectively. Following injection, the rats were returned to cages with their mothers for continued weaning, until day 21. The rats were housed in a room at 25°C with a 12-h light/12-h dark cycle. There was a 94.7% (90/95) survival rate 48 h after viral administration. At different time points, rats were taken for measurement of HHO-1 expression by RT–PCR. The results showed that 100% (6/6) of the rats injected with LSN-HOP-HHO-1 at

week 4 expressed both HHO-1 and neo^r transcripts in the liver, kidney, lung, heart, and spleen (Fig. 7). Only neo^r transcripts were detected in all (4) of the LXSN-infected rats. However, in 3 control rats, neither HHO-1 nor neo^r transcripts were found. These data indicate that the *HHO-1* gene can be efficiently delivered and expressed in newborn rats via retroviruses, and the human HO-1 promoter (HOP) can execute its function to drive *HHO-1* gene expression in vivo, as it does in vitro.

DISCUSSION

The present study describes the construction of a functional retroviral delivery system for transducing rat lung microvessel endothelial cells with the human *HO-1* gene driven by either a viral LTR promoter or a human HO-1 promoter. Our results showed that the HO-1 promoter can drive a stronger expression of the *HO-1* gene compared to the retroviral LTR promoter in cell culture and in vivo. Cells transduced with retroviral HHO-1 constructs displayed enhanced heme oxidation activity, resulting in a significant decrease in the amount of unmetabolized heme in cell cultures to which this natural substrate of HO was added. Nucleotide sequence analysis of the HO-1 promoter shows that the $+19$ to $-1{,}500$ bp region encompassed NF-KB, AP-1, and AP-2 consensus binding sites, whose activation by heme was associated with *HO-1* gene expression [Lavrovsky et al., 1993; Lavrovsky et al., 1994; Lavrovsky et al., 1996]. Other studies have indeed revealed a direct interaction between these transcriptional factors and *HO-1* gene expression [Kurata et al., 1996; Tacchini et al., 1999]. By employing a microassay procedure for quantitating CO, it was determined that production of CO was significantly enhanced, consistent with an increased rate of heme catabolism in HOP-HHO-1-transduced cells. This effect was demonstrated in 20 sequential passage cells and can be presumed to be "permanent."

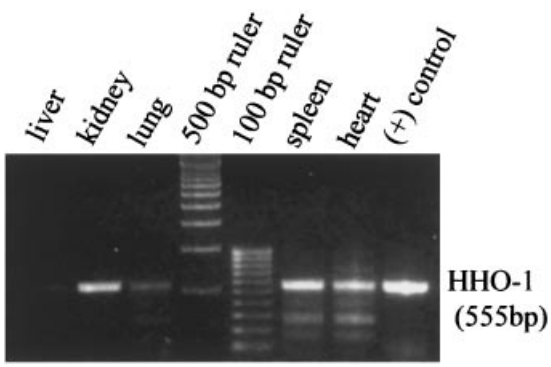

Fig. 7. Detection of human HO-1 transcripts by RT–PCR analysis in 4-week-old Sprague-Dawley rats. Newborn rats were injected twice (days 5 and 12) with concentrated retroviruses (LSN-HOP-HHO-1). Rats were taken at differing times and their tissues were prepared for RT–PCR analysis. Human HO-1 transcripts were detected at week 4 in the liver, kidney, lung, spleen, and heart of rats injected with retrovirus LSN-HOP-HHO-1.

This study demonstrates that the heme-HO system of endothelial cells participates in the regulation of prostaglandin production, presumably by influencing the availability of heme for the catalytically active COX and soluable guanylate cyclase activities. Two important observations substantiate this conclusion. The first is that human *HO-1* gene transfer into endothelial cells was associated with modulation of cGMP levels. Since the media containing 10% FBS increased cGMP production (data not shown), the basal cGMP levels were measured in cells cultured in media containing 0.5% FBS, which limited the availability of heme in FBS. There was no significant difference in basal cellular cGMP levels between untreated RLMV and RLMV-HOP-HHO-1 cells. However, the addition of heme significantly increased cellular cGMP content, which could be partially reversed by preincubation with SnMP. These results suggest that overexpression of HO-1 contributed to the increased CO production and, at least in part, to the elevated cellular cGMP level. The second important observation is that the basal levels of the COX product, PGE_2, were greatly diminished in endothelial cells transduced with the HOP-HHO-1. These effects have been correlated with the levels of cellular heme and one of the end products of heme catabolism, CO. Supplementation of heme to endothelial cells resulted in the potential enhancement of cGMP and PGE_2 in endothelial cells transduced with human HO-1. This activation of COX products can be partially reversed by the addition of SnMP, a known inhibitor of HO activity. There is no evidence, however, that cellular heme influences either the rate of arachidonic acid acylation or reacylation, the balance of which determines the amount of arachidonic acid available for prostaglandin synthesis [Farooqui et al., 2000]. On the other hand, it is known that

heme bound to histidine residues of the peroxidase binding site of COX isoforms is required for catalytic activity [Smith and Marnett, 1991]. Therefore, it is likely that the alteration in prostaglandin production observed in endothelial cells subjected to interventions that increase or decrease cellular heme are the result of directional alterations in the levels of catalytically active COX. The design of the current study does not allow for evaluation of the mechanisms by which alterations in the status of the heme-HO-1 system of endothelial cells impact prostaglandin or cGMP production.

The availability of a *HHO-1* gene delivery system carrying sense or antisense nucleotide sequences under the control of human HO-1 promoter offers many possibilities for experimental examination of the relationship of human heme oxygenase activity to various physiological processes. For example, the administration of HO-1 inducers is known to lower blood pressure and enhance tissue growth in spontaneously hypertensive rats [Sabaawy et al., 2001], whereas the administration of HO-1 inhibitors produces systemic vasoconstriction [Martasek et al., 1991; Christodoulides et al., 1995; Maines, 1997]. These effects have been correlated with the levels of cellular heme and one of the end products of heme catabolism, CO. An important role for heme oxygenase in the control of vascular tone and blood pressure and in the regulation of growth is indicated by these findings. However, the use of metal inducers, such as heme and metalloporphyrins, to manipulate heme oxygenase activity is far from specifically targeting heme oxygenase. These agents can affect processes unrelated to heme oxygenase expression and activity, such as guanylate cyclase and nitric oxide production, which by themselves can contribute to the effects seen in response to these agents. To this end, we, as well as others, have embarked on developing the means to specifically target the expression and activity of heme oxygenase in an isoform and site-specific manner [Imai et al., 2001; Minamino et al., 2001]. We have constructed an adenoviral-*HO-1* gene vector carrying human HO-1 and have demonstrated its efficiency in increasing *HO-1* gene expression and heme oxygenase activity in vitro and in vivo [Abraham et al., 1995; Abraham et al., 2000]. The effect, however, is short lasting (2–4 weeks). In contrast, retroviral gene transfer offers long lasting expression of the gene carried [Chertkov et al., 1993; Quan et al., 1999; Sabaawy et al., 2001]. The availability of retroviral vectors expressing the *HHO-1* gene thus offers a means to assess the long-term consequences on the physiological processes of sustained alterations in heme oxygenase activity. Importantly, newborn rats injected with concentrated viral particles (LSN-HHO-1 or LSN-HOP) resulted in the expression of human HO-1 mRMA in tissues (kidney, liver, lung, heart, and spleen) after 4–6 weeks.

In summary, the availability of an effective retroviral *HOP-HHO-1* gene construct opens the potential for a longer term study to understand the regulatory action of the heme-HO system in endothelial cells on cGMP and COX activity. Upregulation of HO-1 leading to reduction in cellular heme and elevation of CO brings about a differential impact on several proteins, such as those involved in the synthesis of prostaglandin and cGMP, which can potentially have a long-term influence on heme homeostasis.

ACKNOWLEDGMENT

We thank Ms. Jennifer Brown for her secretarial assistance.

REFERENCES

Abraham NG, da Silva JL, Lavrovsky Y, Stoltz RA, Kappas A, Dunn MW, Schwartzman ML. 1995. Adenovirus-mediated heme oxygenase-1 gene transfer into rabbit ocular tissues. Invest Ophthalmol Vis Sci 36:2202–2210.

Abraham NG, Jiang S, Yang L, Zand BA, Laniado-Schwartzman M, Marji J, Drummond GS, Kappas A. 2000. Adenoviral vector-mediated transfer of human heme oxygenase in rats decreases renal heme-dependent arachidonic acid epoxygenase activity. J Pharmacol Exp Ther 293:494–500.

Cao L, Blute TA, Eldred WD. 2000. Localization of heme oxygenase-2 and modulation of cGMP levels by carbon monoxide and/or nitric oxide in the retina. Vis Neurosci 17:319–329.

Chertkov JL, Jiang S, Lutton JD, Harrison J, Levere RD, Tiefenthaler M, Abraham NG. 1993. The hematopoietic stromal microenvironment promotes retrovirus-mediated gene transfer into hematopoietic stem cells. Stem Cells 11:218–227.

Christodoulides N, Durante W, Kroll MH, Schafer AI. 1995. Vascular smooth muscle cell heme oxygenases generate guanylyl cyclase-stimulatory carbon monoxide. Circulation 91:2306–2309.

Christou H, Morita T, Hsieh CM, Koike H, Arkonac B, Perrella MA, Kourembanas S. 2000. Prevention of hypoxia-induced pulmonary hypertension by enhancement of endogenous heme oxygenase-1 in the rat. Circ Res 86:1224–1229.

Coceani F. 1993. Carbon monoxide and dilation of blood vessels. Science 260:739.

Coceani F, Kelsey L, Seidlitz E. 1996. Carbon monoxide-induced relaxation of the ductus arteriosus in the lamb: evidence against the prime role of guanylyl cyclase. Br J Pharmacol 118:1689–1696.

Deramaudt TB, da Silva JL, Remy P, Kappas A, Abraham NG. 1999. Negative regulation of human heme oxygenase in microvessel endothelial cells by dexamethasone. Proc Soc Exp Biol Med 222:185–193.

Elbirt KK, Bonkovsky HL. 1999. Heme oxygenase: recent advances in understanding its regulation and role. Proc Assoc Am Physicians 111:438–447.

Farooqui AA, Horrocks LA, Farooqui T. 2000. Deacylation and reacylation of neural membrane glycerophospholipids. J Mol Neurosci 14:123–135.

Fuhrop JH, Smith KM. 1975. Hemes: determination as pyridine hemochromes, in Porphyrins and metalloporphyrins. New York: Elsevier Scientific Publishing Co. pp 804–807.

Furchgott RF, Jothianandan D. 1991. Endothelium-dependent and -independent vasodilation involving cyclic GMP: relaxation induced bynitric oxide, carbon monoxide and light. Blood Vessels 28:52–61.

Graser T, Vedernikov YP, Li DS. 1990. Study on the mechanism of carbon monoxide induced endothelium-independent relaxation in porcine coronary artery and vein. Biomed Biochim Acta 49:293–296.

Hartsfield CL, Alam J, Choi AM. 1998. Transcriptional regulation of the heme oxygenase 1 gene by pyrrolidine dithiocarbamate. FASEB J 12:1675–1682.

Hartsfield CL, Alam J, Choi AM. 1999. Differential signaling pathways of *HO-1* gene expression in pulmonary and systemic vascular cells. Am J Physiol 277: L1133–L1141.

Haugen EN, Croatt AJ, Nath KA. 2000. Angiotensin II induces renal oxidant stress in vivo and heme oxygenase-1 in vivo and in vitro. Kidney Int 58:144–152.

Imai T, Morita T, Shindo T, Nagai R, Yazaki Y, Kurihara H, Suematsu M, Katayama S. 2001. Vascular smooth muscle cell-directed overexpression of heme oxygenase-1 elevates blood pressure through attenuation of nitric oxide-induced vasodilation in mice. Circ Res 89:55–62.

Iyer SN, Lu D, Katovich MJ, Raizada MK. 1996. Chronic control of high blood pressure in the spontaneously hypertensive rat by delivery of angiotensin type 1 receptor antisense. Proc Natl Acad Sci USA 93:9960–9965.

Johnson RA, Lavesa M, Askari B, Abraham NG, Nasjletti A. 1995. A heme oxygenase product, presumably carbon monoxide, mediates a vasodepressor function in rats. Hypertension 25:166–169.

Kanse SM, Takahashi K, Warren JB, Ghatei M, Bloom SR. 1991. Glucocorticoids induce endothelin release from vascular smooth muscle cells but not endothelial cells. Eur J Pharmacol 199:99–101.

Kozma F, Johnson RA, Zhang F, Yu C, Tong X, Nasjletti A. 1999. Contribution of endogenous carbon monoxide to regulation of diameter in resistance vessels. Am J Physiol 276:R1087–R1094.

Kurata S, Matsumoto M, Nakajima H. 1996. Transcriptional control of the heme oxygenase gene in mouse M1 cells during their TPA-induced differentiation into macrophages. J Cell Biochem 62:314–324.

Lavrovsky Y, Schwartzman ML, Abraham NG. 1993. Novel regulatory sites of the human heme oxygenase-1 promoter region. Biochem Biophys Res Commun 196: 336–341.

Lavrovsky Y, Schwartzman ML, Levere RD, Kappas A, Abraham NG. 1994. Identification of binding sites for transcription factors NF-kappa B and AP-2 in the promoter region of the human heme oxygenase 1 gene. Proc Natl Acad Sci USA 91:5987–5991.

Lavrovsky Y, Drummond GS, Abraham NG. 1996. Down-regulation of the human heme oxygenase gene by glucocorticoids and identification of 56b regulatory elements. Biochem Biophys Res Commun 218:759–765.

Lee PJ, Alam J, Sylvester SL, Inamdar N, Otterbein L, Choi AM. 1996. Regulation of heme oxygenase-1 expression in vivo and in vitro in hyperoxic lung injury. Am J Respir Cell Mol Biol 14:556–568.

Lu D, Yu K, Raizada MK. 1995. Retrovirus-mediated transfer of an angiotensin type I receptor (AT1-R) antisense sequence decreases AT1-Rs and angiotensin II action in astroglial and neuronal cells in primary cultures from the brain. Proc Natl Acad Sci USA 92: 1162–1166.

Maines MD. 1997. The heme oxygenase system: a regulator of second messenger gases. Annu Rev Pharmacol Toxicol 37:517–554.

Martasek P, Schwartzman ML, Goodman AI, Solangi KB, Levere RD, Abraham NG. 1991. Hemin and L-arginine regulation of blood pressure in spontaneous hypertensive rats. J Am Soc Nephrol 2:1078–1084.

Minamino T, Christou H, Hsieh CM, Liu Y, Dhawan V, Abraham NG, Perrella MA, Mitsialis SA, Kourembanas S. 2001. Targeted expression of heme oxygenase-1 prevents the pulmonary inflammatory and vascular responses to hypoxia. Proc Natl Acad Sci USA 98:8798–8803.

Motterlini R, Gonzales A, Foresti R, Clark JE, Green CJ, Winslow RM. 1998. Heme oxygenase-1-derived carbon monoxide contributes to the suppression of acute hypertensive responses in vivo. Circ Res 83:568–577.

Muraosa Y, Shibahara S. 1993. Identification of a cis-regulatory element and putative trans-acting factors responsible for 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-mediated induction of heme oxygenase expression in myelomonocytic cell lines. Mol Cell Biol 13:7881–7891.

Ohta K, Yachie A, Fujimoto K, Kaneda H, Wada T, Toma T, Seno A, Kasahara Y, Yokoyama H, Seki H, Koizumi S. 2000. Tubular injury as a cardinal pathologic feature in human heme oxygenase-1 deficiency. Am J Kidney Dis 35:863–870.

Polte T, Abate A, Dennery PA, Schroder H. 2000. Heme oxygenase-1 is a cGMP-inducible endothelial protein and mediates the cytoprotective action of nitric oxide. Arterioscler Thromb Vasc Biol 20:1209–1215.

Prabhakar NR. 1999. NO and CO as second messengers in oxygen sensing in the carotid body. Respir Physiol 115:161–168.

Quan S, Seiter K, Feldman E, Yang L, Argani I, Farley TJ, Abraham NG, Ahmed T. 1999. Human CD34 + hematopoietic cells transduced by retrovirus-mediated interferon alpha gene maintains regeneration capacity and engraftment in NOD/SCID mice. Exp Hematol 27:1511–1518.

Quan S, Yang L, Abraham NG, Kappas A. 2001. Regulation of human heme oxygenase at the gene level using sense and antisense retroviral constructs. Proc Natl Acad Sci USA 98:12203–12208.

Reeve VE, Tyrrell RM. 1999. Heme oxygenase induction mediates the photoimmunoprotective activity of UVA radiation in the mouse. Proc Natl Acad Sci USA 96:9317–9321.

Rizzardini M, Terao M, Falciani F, Cantoni L. 1993. Cytokine induction of haem oxygenase mRNA in mouse liver. Interleukin 1 transcriptionally activates the haem oxygenase gene. Biochem J 290(Pt 2):343–347.

Sabaawy HE, Zhang F, Nguyen X, Elhosseiny A, Nasjletti A, Schwartzman ML, Dennery PA, Kappas A. 2001. Human heme oxygenase-1 gene transfer lowers blood pressure and promotes growth in spontaneously hypertensive rats. Hypertension 38:210–215.

Shi W, Haberland ME, Jien ML, Shih DM, Lusis AJ. 2000. Endothelial responses to oxidized lipoproteins determine genetic susceptibility to atherosclerosis in mice. Circulation 102:75–81.

Shibahara S, Muller RM, Taguchi H. 1987. Transcriptional control of rat heme oxygenase by heat shock. J Biol Chem 262:12889–12892.

Shibahara S, Sato M, Muller RM, Yoshida T. 1989. Structural organization of the human heme oxygenase gene and the function of its promoter. Eur J Biochem 179:557–563.

Smith WL, Marnett LJ. 1991. Prostaglandin endoperoxide synthase: structure and catalysis. Biochim Biophys Acta 1083:1–17.

Tacchini L, Radice L, Bernelli-Zazzera A. 1999. Differential activation of some transcription factors during rat liver ischemia, reperfusion, and heat shock. J Cell Physiol 180:255–262.

Taddei S, Virdis A, Ghiadoni L, Salvetti A. 2000. Vascular effects of endothelin-1 in essential hypertension: relationship with cyclooxygenase-derived endothelium-dependent contracting factors and nitric oxide. J Cardiovasc Pharmacol 35:S37–S40.

Takeda A, Onodera H, Sugimoto A, Itoyama Y, Kogure K, Shibahara S. 1994. Increased expression of heme oxygenase mRNA in rat brain following transient forebrain ischemia. Brain Res 666:120–124.

Takeda A, Smith MA, Avila J, Nunomura A, Siedlak SL, Zhu X, Perry G, Sayre LM. 2000. In Alzheimer's disease, heme oxygenase is coincident with Alz50, an epitope of tau induced by 4-hydroxy-2-nonenal modification. J Neurochem 75:1234–1241.

Taketani S, Kohno H, Yoshinaga T, Tokunaga R. 1988. Induction of heme oxygenase in rat hepatoma cells by exposure to heavy metals and hyperthermia. Biochem Int 17:665–672.

Terry CM, Clikeman JA, Hoidal JR, Callahan KS. 1998. Effect of tumor necrosis factor-alpha and interleukin-1 alpha on heme oxygenase-1 expression in human endothelial cells. Am J Physiol 274:H883–H891.

Togane Y, Morita T, Suematsu M, Ishimura Y, Yamazaki JI, Katayama S. 2000. Protective roles of endogenous carbon monoxide in neointimal development elicited by arterial injury. Am J Physiol Heart Circ Physiol 278: H623–H632.

Vedernikov YP, Graser T, Vanin AF. 1989. Similar endothelium-independent arterial relaxation by carbon monoxide and nitric oxide. Biomed Biochim Acta 48:601–603.

Wang R, Wu L, Wang Z. 1997. The direct effect of carbon monoxide on KCa channels in vascular smooth muscle cells. Pflugers Arch 434:285–291.

Yachie A, Niida Y, Wada T, Igarashi N, Kaneda H, Toma T, Ohta K, Kasahara Y, Koizumi S. 1999. Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 deficiency. J Clin Invest 103:129–135.

Yang L, Quan S, Abraham NG. 1999. Retrovirus-mediated *HO* gene transfer into endothelial cells protects against oxidant-induced injury. Am J Physiol 277:L127–L133.

Zanetti M, Katusic ZS, O'Brien T. 2000. Expression and function of recombinant endothelial nitric oxide synthase in human endothelial cells. J Vasc Res 37:449–456.

Zhang F, Kaide JI, Wei Y, Jiang H, Yu C, Balazy M, Abraham NG, Wang W, Nasjletti A. 2001. Carbon monoxide produced by isolated arterioles attenuates pressure-induced vasoconstriction. Am J Physiol Heart Circ Physiol 281:H350–H358.