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ARTICLES

Human Heme Oxygenase: Cell Cycle-Dependent Expression and DNA Microarray Identification of Multiple Gene Responses After Transduction of Endothelial Cells

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Abstract The purpose of the present study was to examine the role of human heme oxygenase (human HO-1) in cell cycle progression following exposure to heme or human HO-1 gene transfer and to identify target genes associated with human HO-1-meditated increases in cell cycle progression using cDNA microarray technology. Heme-induced robust human HO-1 expression in quiescent human microvessel endothelial cells cultured in 1% FBS and the levels of human HO-1 expression progressively declined without a change in the cell cyclin. To identify genes regulated by human HO-1 in the cell cycle, human endothelial cells were transduced with a retroviral vector encoded with human HO-1 gene or an empty vector. Transgene expression and functionality of the recombinant protein were assessed by Western blotting, enzyme activity, carbon monoxide, cGMP production, and cell cycle analysis. Human cDNA gene array and quantitative real-time RT-PCR were used to identify both known and novel differentially expressed genes in cells overexpressing human HO-1. Major findings were upregulation of several genes associated with cell cycle progression, including cyclin E and D; downregulation of cyclin-dependent kinase inhibitors p21 and p27, cyclin-dependent kinases 2, 5, and 6, and monocyte chemoattractant protein-1; and upregulation of growth factors, including vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor I (VEGFRI), endothelial growth factor (EGF) and hepatic-derived growth factor (HDGF). These findings identify an array of gene responses to overexpression of human HO-1 and elucidate new aspects of human HO-1 signaling involved in cell growth. J. Cell. Biochem. 90: 1098–1111, 2003. © 2003 Wiley-Liss, Inc.

Key words: bilirubin; oxidative stress; cyclin; gene profiling; cell proliferation; quantitative real-time RT-PCR

Heme oxygenase-1 (HO-1) has been identified in many cell types [Nath et al., 1992; Abraham et al., 1995, 1996; da Silva et al., 2001]. In the vasculature, the products of HO-1 activity, biliverdin-bilirubin and carbon monoxide (CO), have been shown to protect endothelial cells from oxidative stress generated by such agents as heme, H_2O_2, and tumor necrosis factor (TNF) [Morita et al., 1997; Yet et al., 1999; Kushida et al., 2002a.b]. CO is considered to be both a regulator of vascular tone, acting exclusively as a vasodilator, and an enhancer of cell proliferation [Kushida et al., 2002b]. CO also has a proliferative effect in endothelial cells, but not in vascular smooth muscle cells [Li Volti et al., 2002; Liu et al., 2002]. Inhibition of HO-1 enhances cell death [Otterbein and Choi, 2000; Soares et al., 2002], which can be prevented by the antioxidant bilirubin. HO inhibitors decrease bilirubin formation [Kappas and Drummond, 1986; Kappas, 2002] and subsequently increase oxidative stress, as measured

Abbreviations: (HO-1)/(HO-2), heme oxygenase-1 and -2; CO, carbon monoxide; RT/PCR, reverse transcription-polymerase chain reaction; VEGF, vascular endothelial growth factor; VEGFR-I, vascular endothelial growth factor receptor-1; HSP, heat shock protein; CDK, cycline-dependent kinase; ID_2, helix-loop-helix protein Id_2; sGC, soluble guanylate cyclase; PGE_2, prostaglandin E_2; HDGF, hepatic-derived growth factor; EGF, endothelial growth factor

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by increased expression of endothelial adhesion molecules [Wagener et al., 1999] and formation of vasoconstrictors [Sessa et al., 1989; Haider et al., 2001].

Evidence of the cell survival effect of enhanced HO-1 in vivo is reflected in the attenuation of inflammatory responses associated with a variety of experimental and clinical circumstances [Willis et al., 1996; Laniado-Schwartzman et al., 1997; Hancock et al., 1998; Amersi et al., 1999; Minamino et al., 2001]. It has also been shown that CO, generated by HO-1, prevents endothelial cell apoptosis [Brouard et al., 2000], an effect reversed by inhibitors of the enzyme [Brouard et al., 2000; Otterbein et al., 2000]. The anti-apoptotic effect of HO-1-mediated CO generation in endothelial cells has been shown to occur via the activation of p38 mitogen-activated protein kinase [Brouard et al., 2000; Clark et al., 2000]. The effect of HO-1 activity appears to be independent of nitric oxide (NO) generation [Duckers et al., 2001].

We have shown previously that upregulation of HO-1 in endothelial cells enhances cell proliferation and angiogenesis [Deramaudt et al., 1999]. More recently, we demonstrated that transduction of human HO-1 into spontaneously hypertensive rats promotes growth [Abraham et al., 2002] and may play a significant role in cell cycle progression [Morita et al., 1997; Yet et al., 1999; Kushida et al., 2002a,b]. The aims of the present study were to determine whether human HO-1 expression is increased in the quiescent state in response to known inducers of the enzyme, and to define the mechanism of enhanced cell cycle progression or determine whether expression of HO-1 may target genes involved in cell growth. To achieve these aims, we determined the effects of the HO inducer, heme, on HO-1 and HO-2 proteins in quiescent endothelial cells. Further, we examined the effect of human HO-1 gene transduction on possible target genes involved in cell cycle progression, utilizing DNA microarray analysis [Brown and Botstein, 1999; Schubert, 2003; Stears et al., 2003]. Transduction with the human HO-1 gene to enhance overall HO-1 activity provides a specificity, which chemical inducers of the enzyme do not provide [Quan et al., 2001].

Our results demonstrate that, in quiescent cells, human HO-1 is expressed at significant levels in a time-dependent fashion in response to heme. The data also demonstrate that overexpression of the human HO-1 gene is associated with global gene profiling changes. In addition to detecting changes in the levels of the multiple genes affected by human HO-1 over expression, we have identified several previously unreported human HO-1-responsive genes known to be critical for cell cycle progression.

MATERIALS AND METHODS

Cell Culture Conditions and Retroviral Constructs and Production

Human dermal microvessel endothelial cells were a kind gift of Dr. Michael Dillon (National Center for Infectious Diseases, Atlanta, GA) and grown in MCDB131 medium (GIBCO-BRL, Grand Island, NY) supplemented with 10% FBS, 10 ng/ml endothelial growth factor (EGF) (Sigma, St. Louis, MO) and 1 μg/ml hydrocortisone (Sigma). 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 as described previously [Yang et al., 1999]. PA317 retroviral packaging cells were transfected with the retroviral vectors LSN-HHO-1 or LXSN using Lipofectamine reagent (Life Technologies, Grand Island, NY). Individual G418-resistant clones were selected as described previously [Yang et al., 1999]. For each isolated clone, the viral titer was determined by infection of NIH/3T3 fibroblasts. The clones of packaging cell lines (PA317/LSN-HHO-1 and PA317/LXSN) with viral titers of $0.14-1.5 \times 10^7$ cfu/ml were employed in the experiments described.

Cell Preparation and DNA Distribution

To investigate the effect of heme on DNA fragmentation of endothelial cells, cells were treated with 10 μM heme for different time and stained with 4,6-diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, OR). They were then analyzed by EPICS flow cytometry (Beckman Coulter, Miami, FL). Cells were analyzed by flow cytometry following staining with DAPI as previously described [Kushida et al., 2002b].

Western Blot Analysis and Measurement of HO-1 Activity, CO Production, cGMP, Prostaglandin E_2 (PGE_2), and VEGF Levels

Cells were harvested using cell lysis buffer as previously described [Li Volti et al., 2002]. The

lysate was collected for Western blot analysis and protein levels were visualized by immunoblotting with antibodies against human HO-1 or HO-2 (Stressgen Biotechnologies, Victoria, BC, Canada). The antibodies used for HO-1 do not crossreact with HO-2. Similarly, the antibodies for HO-2 do not crossreact with HO-1 protein. Western blot analysis of p16, p21, p27, and p53 and CO production was performed as previously described [Kushida et al., 2002a]. SDS–PAGE immunoblots were also compared to the corresponding molecular weight band on the membranes. HO activity was assayed in cell lysate as previously described [Abraham et al., 1987]. The amount of bilirubin generated was determined by scanning spectrophotometer (Lambda 17 UV/VIS; Perkin-Elmer Cetus Instruments, Norwalk, CT) and was defined as the difference between 460 and 530 NM (extinction coefficient, 40 $mM^{-1} cm^{-1}$ for bilirubin). Results were expressed as nmol of bilirubin per mg of protein per hour.

For measurement of cGMP levels, endothelial cells were plated in 60-mm culture dishes and grown to approximately 80% confluence. Cellular cGMP content was determined using a commercial ELISA kit (Cayman Chemicals, Ann Arbor, MI) following the instructions provided by the manufacturer. PGE_2 levels were determined in the media of endothelial cell cultures using an enzyme-linked immunoassay (EIA). Endothelial cells were counted and seeded in 24-well plates (1.2×10^4 cells/wells). Control cells and cells overexpressing human HO-1 were cultured as described above and the media were removed and stored at $-80°C$. Solid-phase enzyme immunoassay was performed using an ELISA kit following instructions provided by the manufacturer.

Vascular endothelial growth factor (VEGF) protein levels in the culture medium were measured by enzyme-linked immunoabsorbent assay using a VEGF Quantikine immunoassay Kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Cell Proliferation and In Vitro Angiogenesis

Cells were seeded in 24-well culture plates (4×10^4) and grown for 24 h. Cell proliferation was carried out using a 5-bromo-2′deoxyuridine colorimetric kit (Roche Molecular Biochemical Indianapolis, IN) according to the manufacturer's instructions. A growth factor-induced basement membrane Matrigel matrix (BD Bioscience, Bedford, MA) was used for assessment of in vitro capillary formation.

cDNA Microarray, Hybridization, and Imaging

Three independent sets of experiments were performed. These experiments comprised cells transduced with human HO-1 or control cells in the exponentially grown state (50–60% confluence). In each experiment, total RNA from control and transduced cell cultures was extracted using Trizol (Sigma) and treated with RNase-free DNAse I to remove any residual genomic DNA. The Atlas Human 1, 2 Array I and the Atlas Human 1, 2 Array II (Clontech), containing 1,176 genes each, were used. Array membranes were individually hybridized with [α-^{32}P] dATP labeled cDNA according to the manufacturer's protocol. cDNA was purified using a NucleoSpin extraction spin column and the radioactivity of the probes was counted in a Beckman LS 1801 (Beckman, Inc., Fullerton, CA). Each array membrane was prehybridized in ExpressHyb buffer, along with the addition of 1.5 mg of denatured sheared salmon testes DNA for 30 min at 68°C. The prepared cDNA probes were then added to ExpressHyb buffer and allowed to hybridize with the cDNA on the Array membranes at 68°C overnight. After hybridization, the membranes were washed four times with wash solution 1 ($2 \times$ sodium chloride sodium citrate [SSC], 1% sodium dodecyl sulfate [SDS]) for 30 min at 68°C, followed by a wash with wash solution 2 ($0.1 \times$ SSC, 0.5% SDS) for 30 min and a final wash with $2 \times$ SSC for 5 min. The radioactive signals were visualized by both autoradiography and phosphorimaging, and the intensities of each signal were compared between the control and the transfected cells (Atlas Image 2.0, Clontech). Values were corrected for differences in hybridization efficiency between the two membranes by dividing the average expression of all genes in the respective arrays (global normalization). Adjusted values, that is, those with relative differences in gene expression of more than 2.0 up or down, were selected.

Quantitative Real-Time RT-PCR

Gene expression changes revealed by the Array analysis were validated for 10 genes by real-time RT-PCR, using the RNA samples utilized in the Array experiment. Forward and reverse primers (FP and RP), used to amplify the selected genes to be confirmed, are listed in

TABLE I. Sequence of Oligonucleotide Primers for 10 Genes to Validate by Quantitative Real-Time RT-PCR

Gene name	Gene bank	Forward primer sequence	Reverse primer sequence	Fragment length
HO-1	X06985	CCAGCGGGCCAGCAACAAAGTGC	AAGCCTTCAGTGCCCACGGTAAGG	265
HO-2	D21243	GTGGCCCAGCGAGCACTGAAACTC	AGGGAACCCATCCTCCAAGGTCTC	255
HSP27	X54079	ACGAGGAGCGGCAGGACGAGCATG	CGGGCTAAGGCTTTACTTGGCGGCA	260
HSP70	M11717	AAGAGCACCGGCAAGGCCAACAAGAT	CACGAGATGACCTCTTGACACTTGTCCA	263
VEGF	M32977	CAGCGCAGCTACTGCCATCCAATCGAGA	GCTTGTCACATCTGCAAGTACGTTCGTTTA	424
Id_2	M97796	GAAAGCCTTCAGTCCCGTGAGGTCCGTT	CTGGTGATGCAGGCTGACAATAGTGGGATG	271
sGC	AF038499	CAGGACTCCAAACCCAGAAT	CTTCGGAGAGCACAGGTACA	230
Cyclin A1	U66838	TCAGGACTGAGAACCTGGCTAAGTACG	CCTAATTGCTTGCTGAGGTCGATGGG	251
Cyclin D3	D13639	TCAGGACTTTGTGAGTTAGCATGACCCT	TACAGACTGTAAATAGAGTCGGGTAGGC	352
CDK_6	X66365	GCACCTGGAGACCTTCGAGCACC	GTGACGACCACTGAGGTTAGAGCC	348

Table I. To control the integrity of RNA and for differences attributable to errors in experimental manipulation from tube to tube, primers for human β-actin, a housekeeping gene, were used in separate PCR reactions. Aliquots of cDNA (0.1 and 0.2 μg) and known amounts of external standard (purified PCR product, 10^3 to 10^9 copies) were amplified in parallel reactions using the FP and RP as indicated in Table II. Each PCR reaction (final volume 20 μl) contained 0.5 μM of primers, 2.5 mM Mg^{2+}, and $1 \times$ Light cycler DNA master SYBR Green (Roche Diagnostics, Indianapolis, IN). PCR amplifications were performed with a LightCycler (Roche Molecular Biochemicals, Indianapolis, IN) using the following four cycle programs: (i) denaturation of cDNA (1 cycle: 95°C for 10 min); (ii) amplification (40 cycles: 95°C for 0 s, 60°C for 5 s, 72°C for 10 s); (iii) melting curve analysis (1 cycle: 95°C for 0 s, 70°C for 10 s, 95°C for 0 s); (iv) cooling (1 cycle: 40°C for 3 min). The temperature transition rate was 20°C/s except for the third segment of the melting curve analysis, which was 0.2°C/s. Fluorimeter gain value was 6. Real-time detection of fluorometric intensity of SYBR Green I, indicating the amount of the PCR product formed, was measured at the end of each elongation phase. Quantification was performed by comparing the fluorescence of PCR products of unknown concentration with the fluorescence of the external standards. For this analysis, fluorescence values were measured in the log-linear phase of amplification and the second derivative maximum method of the Light Cycler Data Analysis software (Roche Molecular Biochemicals). Specificity of the PCR products was determined by melting curve analysis, followed by gel electrophoresis, visualized by ethidium bromide staining and DNA sequencing.

TABLE II. Cell Cycle Progression and DNA Distribution in Serum Deprived Endothelial Cells (1% FBS) for 24 h and Treated With Heme (10 μM for 4, 16, and 24 h)

	DNA distribution (%)		
Cells	G_0/G_1	S	G_2/M
Control	78.7 ± 2.8	16.1 ± 3.5	5.1 ± 4.7
Heme 4 h	85.7 ± 3.8	10.5 ± 4.7	4.5 ± 3.2
Heme 16 h	78.7 ± 4.8	13.4 ± 2.7	7.9 ± 1.9
Heme 24 h	80.9 ± 3.5	12.1 ± 3.5	7.0 ± 2.2

Exponentially grown cells were stained with DAPI and analyzed by flow cytometry. Results are the mean = SD; n = 3.

Statistical and Data Analyses

Spot intensities from scanned membranes were analyzed using the Atlas Image software (Clontech). Gene expression data were normalized using the sum methods included in the Atlas Image software. The data are presented as mean ± SE for the number of experiments. Statistical significance ($P < 0.05$) between the experimental groups was determined by the Fisher's 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 an unpaired *t*-test for two groups.

RESULTS

Differential Effects of Heme on HO-1 and HO-2 Protein Levels in G_0/G_1 During Cell Cycle Growth

We compared the effects of heme on HO-1 and HO-2 proteins in FBS serum-starved cells, that

is, cells in G_0/G_1 phase of the cell cycle. Cell cycle analysis revealed that more than 78–85% of the cells were in G_0/G_1 phase when the cells were cultured in 1% FBS media for 24 h. The addition of heme (10 μM) to endothelial cells G_0/G_1 resulted in an increase of HO-1 protein in a time-dependent manner (Fig. 1A) with a maximum increase between 12–16 h. Heme failed to increase HO-2 protein at any time point. Cells treated with heme increased HO-1 to a lesser extent when they were cultured in 10% FBS (data not shown). Induction of HO-1 by heme was accompanied by a similar time-dependent increase in HO activity and bilirubin synthesis (Fig. 1B). Bilirubin levels increased gradually with time. The basal levels of HO activity were 0.86 nmol formed bilirubin/5×10^6 cells/30 min and increased to 1.90 nmol formed bilirubin/5×10^6 cells/30 min at 16 h. Treatment of endothelial cells with stannous mesoporphyrin (SnMP) (10 μM) 1 h prior to the addition of heme reduced bilirubin formation by 85%, indicating that bilirubin and CO, produced by the addition of heme, occurred via the catalytically active HO-1 pathway independent of HO-2, which is not cell cycle-dependent. By flow cytometry analysis, we demonstrated that the addition of heme to serum-starved cells did not drive

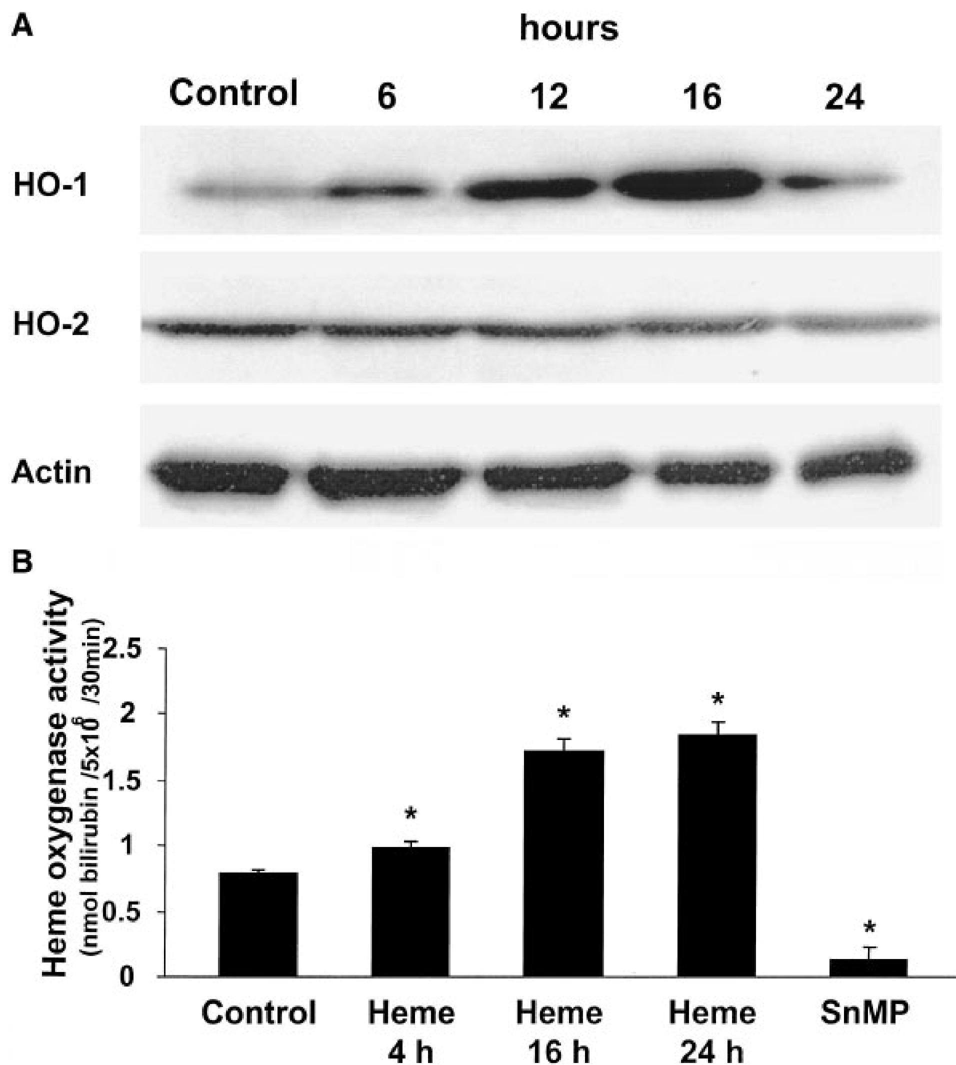

Fig. 1. Time course of HO-1 and HO-2 protein expression (**A**) and HO-1 activity (**B**) after addition of heme (10 μM) to serum-starved cells. A: HO-1, HO-2, and α actin (as internal control) protein levels in heme-treated cells as measured by Western blot analysis. Each lane was loaded with 30 μg of cell lysate proteins; (B) HO-1 activity by spectrophotometric measurement of bilirubin levels in heme-treated cells. These panels are representative of four different experiments with similar results.

the cells into cell cycle. In fact, the percentage of cells in G_0/G_1 remained between 79% and 86% and did not significantly change following the addition of heme at 4, 16, and 24 h (Table I). As seen in Table I, the addition of heme to endothelial cells did not change the ratio of cells in S and G_2/M phase. In control cells, the S phase and G_2/M phase were 16.1 ± 3.5 and 5.1 ± 4.7, respectively. After the addition of heme for 24 h to starved cells, the S and G_2/M was 12.1 ± 3.5 and 7.0 ± 2.2, which was not significantly from the control cells. The increase in HO-1 protein expression and activity brought about by the addition of heme (Fig. 1A,B) was not accompanied by changes in the cell cycle.

Assessment of Human HO-1 Functional Expression in Endothelial Cells Using a Retroviral Vector

To investigate the direct effect of HO-1 on cell cycle progression and DNA distribution, we transduced endothelial cells with human HO-1, using a retroviral vector. The use of the chemical inducer, heme, to manipulate HO-1 activity may not specifically target HO-1. This agent, for example, can affect processes unrelated to HO-1 expression and activity, such as NO synthase and guanylate cyclase which, by themselves, might contribute to the effects observed in cell cycle progression. The availability of retroviral vectors expressing HO-1 thus offers a highly specific means to assess the effect of HO-1 on cell cycle-dependent molecules.

We transduced endothelial cells via retrovirus-mediated delivery of the human HO-1 gene. Transgene expression was examined by Western blot analysis, and functional expression was determined by measurement of known functions of HO-1 gene expression. Among these functions are an increase in CO production, an increase in cGMP, an increase of angiogenesis [Deramaudt et al., 1998; Dulak et al., 2002; Jozkowicz et al., 2003], and a decrease in cellular heme and PGE_2. Western blot analysis, demonstrating recombinant human HO-1 expression, resulted in an increase in human HO-1 protein with a non-significant decrease in HO-2 protein and soluble guanylate cyclase (sGC) (Fig. 2A,B). As seen in Figure 2C,D, HO-1 gene transfer increased the levels of CO formation and cGMP levels. Since overexpression of human HO-1 decreases cellular heme content [Quan et al., 2001], the basal levels of cyclooxygenase (COX) activity-derived PGE_2 were determined and were shown to be significantly decreased compared to control (Fig. 2E). In contrast, upregulation of HO-1 significantly increased the basal levels of VEGF from 62 ± 14 pg/ml in control cells to 91 ± 18 in cells overexpressing HO-1 ($P < 0.05$) (Fig. 2F).

Effect of Human HO-1 on Cell Proliferation and Angiogenesis

In the next set of experiments, we measured other known consequences of HO-1 gene transfer in the endothelial cell, especially cell proliferation and angiogenesis. As expected, overexpression of HO-1 was associated with an increase in CO and VEGF. At 24 h after FCS stimulation, cell proliferation increased by 33% (Fig. 3A). Similarly, the rate of tubular length formation (data not shown), that is, angiogenesis in cells transduced with human HO-1 (Fig. 3C), was significantly increased by 46% compared to control cells ($P < 0.05$) (Fig. 3B). This confirmed that endothelial cells transduced with human HO-1 gene responded as expected by increasing molecules, such as CO and VEGF, involved in cell proliferation.

Downregulation of p21 and p27 Expression Following Human HO-1 Gene Transfer

Following the above results, we assessed the effect of human HO-1 gene transfer on the cyclin kinase inhibitors, p16, p21, p27, and p53, which play an important role in the cell cycle. As shown in Figure 4, overexpression of the human HO-1 gene in substantially decreased p21 and p27 compared to cells transduced with empty vector. In contrast, p16 and p53 expression did not change significantly in control cells or cells overexpressing HO-1.

Effect of Human HO-1 Gene Expression on Endothelial Cell Gene Changes Using cDNA Microarray and RT-PCR Analysis

We extended the study through the use of cDNA microarray to facilitate identification of other genes involved in cell cycle progression, which might be modulated by human HO-1 gene transfer. To determine gene changes resulting from human HO-1 overexpression in the cells, mRNA from endothelial cells exponentially grown to 50–60% confluent was used for cDNA microarrays. Exponentially grown cells were chosen since it has been shown that the HO-1 gene is expressed maximally at 16 h and provides cytoprotection against oxidative damage

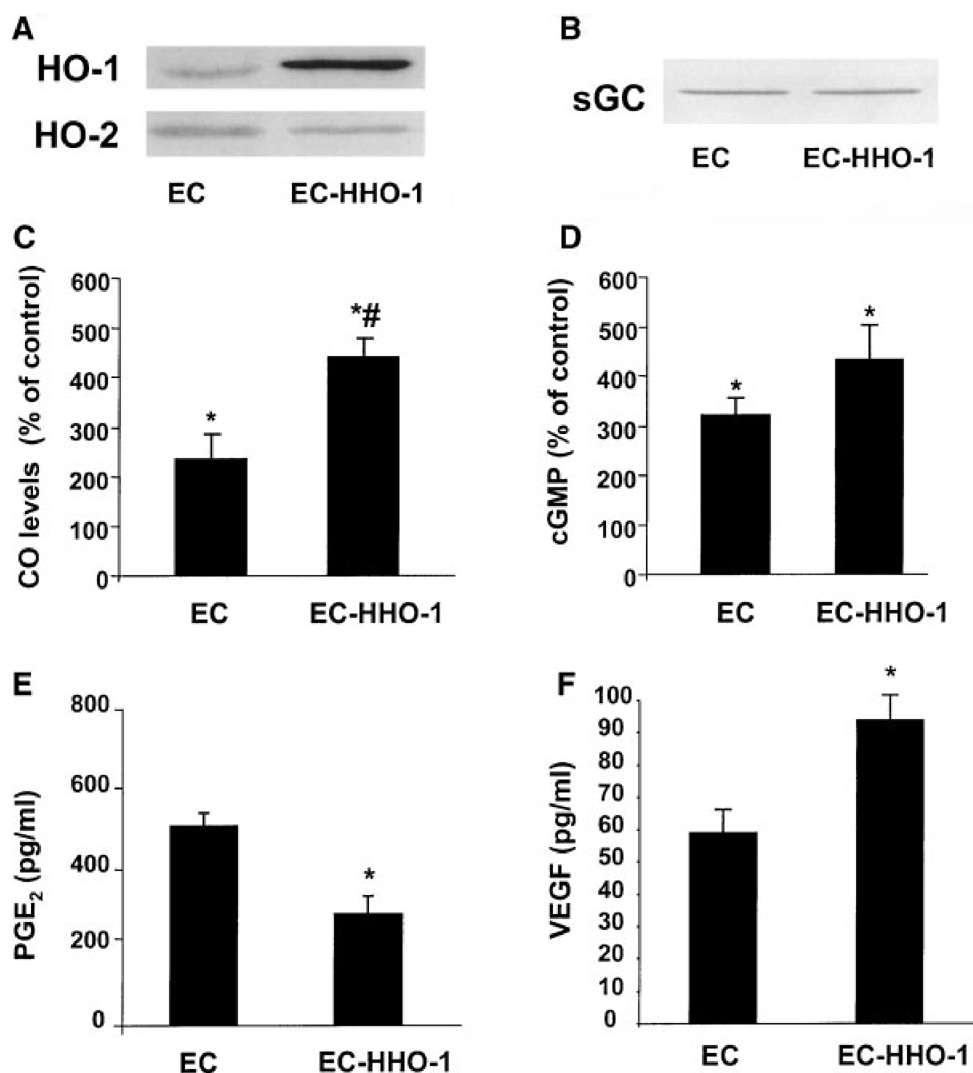

Fig. 2. **A**: Western blot analysis of HO-1 and HO-2. Cell lysates from control cells and cells transduced with human HO-1 gene were visualized by immunoblotting with antibodies against the respective antibody proteins; (**B**) Western blot analysis of sGC protein levels; (**C**) CO production; (**D**) cGMP levels in control endothelial cells and cells transduced with HHO-1 gene. **E**: Levels of PGE_2; (**F**) levels of VEGF in culture media expressed as the mean ± SE (n = 3; $*P < 0.05$ compared with the corresponding control).

during DNA synthesis [Kushida et al., 2002b; Malaguarnera et al., 2002]. For this purpose, mRNA pools from control endothelial cells and cells overexpressing human HO-1 were isolated and prepared for cDNA microarray hybridization. Analysis of the array data identified a great number of genes whose steady-state RNA levels were increased or decreased as a result of human HO-1 overexpression. Genes with a ratio of 2.0 or above were considered positively regulated by the human HO-1 gene; those that had a ratio of 0.5 or below were considered negatively regulated. These data include genes that satisfied the threshold value in three independent experiments. The initial gene expression changes obtained from cDNA hybridization in the microarray system were further verified in 10 genes by quantitative real-time and semi-quantitative RT-PCR. As shown in Figure 5, quantitative real-time RT-PCR confirmed that human HO-1 induced the expression of Id2, VEGF, sGC, cyclin A and D_3 and decreased the expression of HO-2, heat shock protein 27 (HSP27), heat shock protein 70 (HSP70), and cyclin-dependent kinase (CDK6).

Human HO-1 and Cell Cycle Regulator Genes

Among the genes found to be up- or down-regulated, we concentrated our attention on

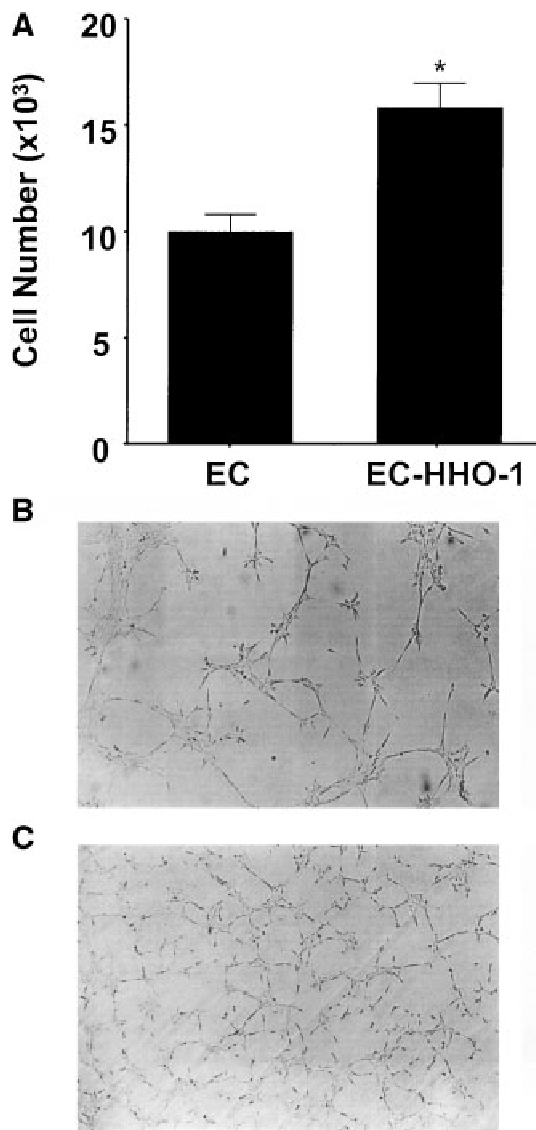

Fig. 3. **A**: Cell proliferation and (**B**,**C**) angiogenesis. A: Proliferation of endothelial cells in control cells and cells overexpressing human HO-1 as described in Materials and Methods. B: Upper panel control (at magnification of 10×); (C) lower panel cells transduced with HO-1 (at magnification 5×). Quantitative analysis was performed as described in Materials and Methods. This panel is a representative of six different experiments with similar results. Statistical analysis was performed by *t*-test; $^*P < 0.05$, control vs. human HO-1 sense.

those groups of genes related to cell cycle regulation and cell proliferation. Figure 6 shows autoradiographic images from the Atlas TM Human 1, 2 Array I, hybridized with 32P-labeled probes for a number of crucial genes involved in cell cycle progression such as cyclin A1, cyclin G_2, cyclin K, and VEGF. Among the genes downregulated are DAD 1, BAK, CDK 4, and CDK 6. In addition, the heat shock proteins,

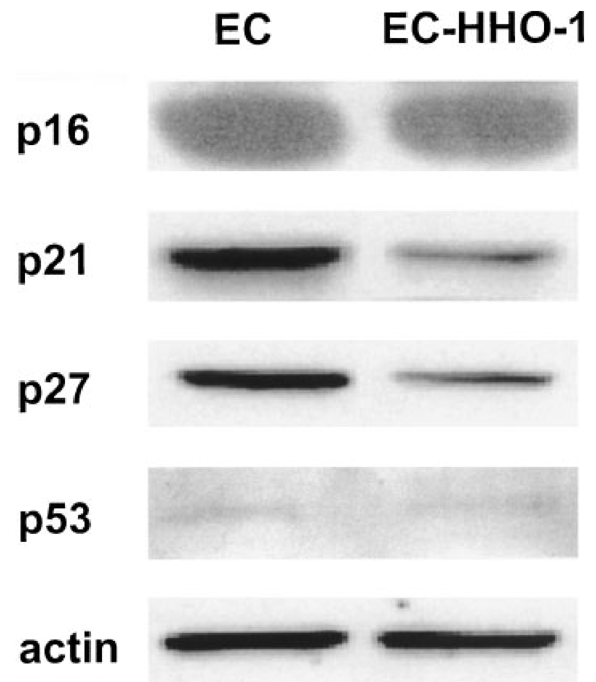

Fig. 4. Western blot analysis of proteins upregulated or downregulated by human HO-1 overexpression. p16, p21, p27, and p53 protein levels in cell lysates from control cells and cells transduced with human HO-1 gene were visualized by immunoblotting with antibodies against the respective proteins. Representative blots are shown (n = 3).

HSP27 and HSP70, as well as HO-2 (Figs. 6 and 7), were among the genes downregulated following overexpression of the HO-1 gene.

The finding that many of the genes induced by human HO-1 encode proteins known to function during DNA replication and mitosis, including cyclin E, CDK_2, and cyclin A1, was not surprising given the fact that human HO-1 activity was increased early in the cell cycle, possibly to enable synthesis of growth factors needed for cell proliferation. VEGF and vascular endothelial growth factor receptor I (VEGFR-1) were increased by about ten- and ninefold, respectively (Fig. 7D) ($P < 0.05$) and sGC and HO-1 were also among the upregulated genes. As seen in Figure 7A, significant ($P < 0.05$) increases (seven to eightfold) in gene expression were observed for the cell cycle regulator, cyclin D_3, as well as for cyclin A1 (fourfold), cyclin E, (fivefold), and cyclin K (twofold) (Fig. 7A). The known effect on cell cycle progression [Kushida et al., 2002b], overexpression of HO-1 was associated with an increase in cell cycle progression in regulatory molecules, such as the antiapoptotic bcl-x (Fig. 7C). In contrast, a

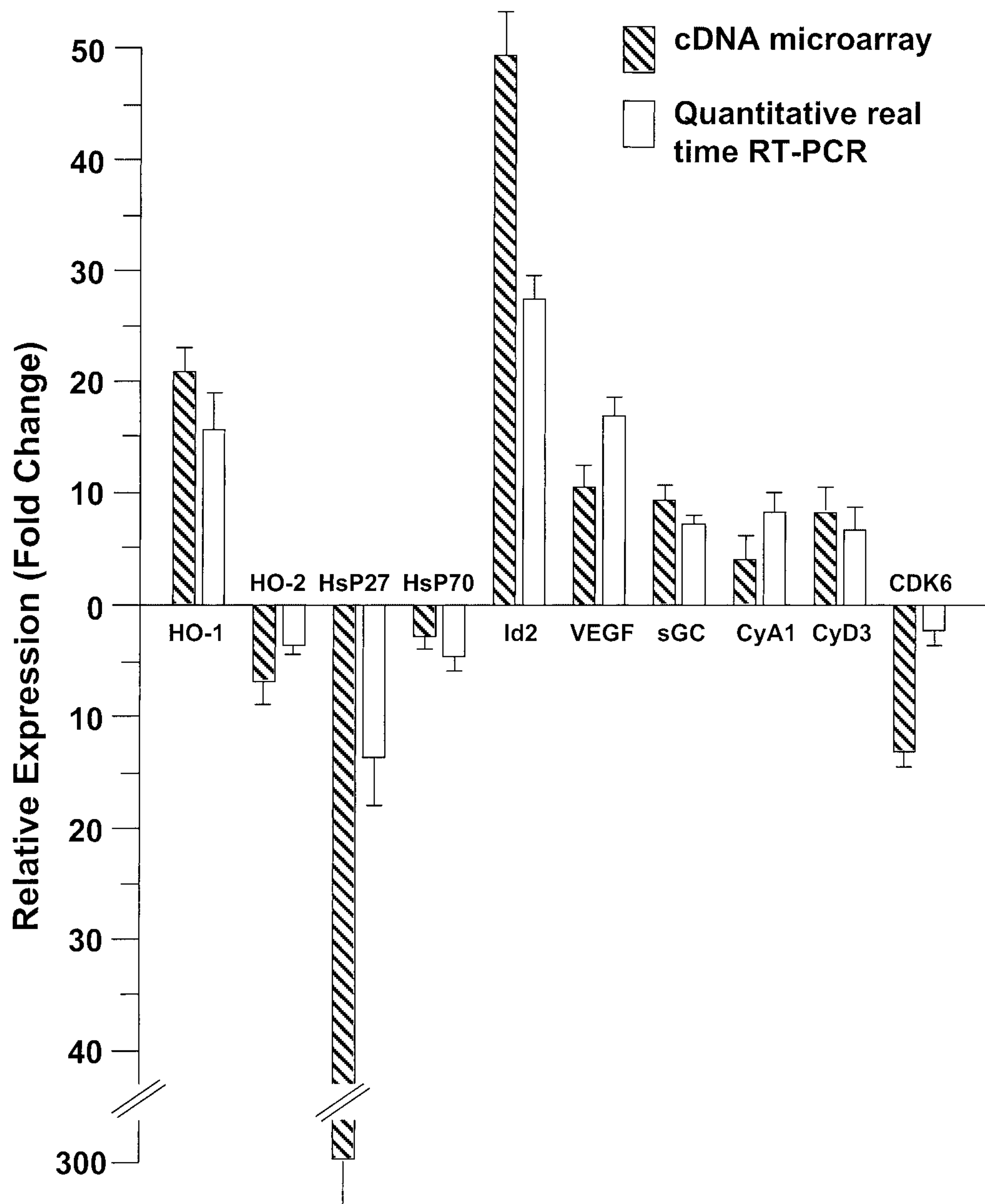

Fig. 5. Validation of differential expression of 10 genes in stable-transfected human endothelial cells expressing human HO-1 sense. The same total RNA sample used for microarray analysis was reverse transcribed and subjected to quantitative RT-PCR analysis. The specific primers for the 10 genes selected are listed in Table I. The fold change ± SEM was determined by averaging the three results obtained for each gene. For microarray, the three hybridizations were averaged, and the average fold change of transfected cells over control± is shown.

decrease of the mRNA levels, as a result of human HO-1 overexpression, was revealed for the antiproliferative and pro-apoptotic genes, DAD1, BAK, Caspases 2 and 6, (Fig. 7C), and CDK_4 and CDK_6 (Fig. 7B). Since HO-1 overexpression is known to increase cell proliferation and cell cycle progression, presumably via an increase in VEGF [Dulak et al., 2002; Jozkowicz et al., 2003], we searched for other growth factors that possibly might be influenced by HO-1 overexpression. As seen in Figure 7D, several growth factors were increased, such as EGF, hepatic-derived growth factor (HDGF), and hematopoietic growth factor. Among the growth factor genes having the greatest response to human HO-1 overexpression were T-cell growth factor p40 (22-fold), followed by TGF-β-3 (14-fold) and HDGF (9-fold).

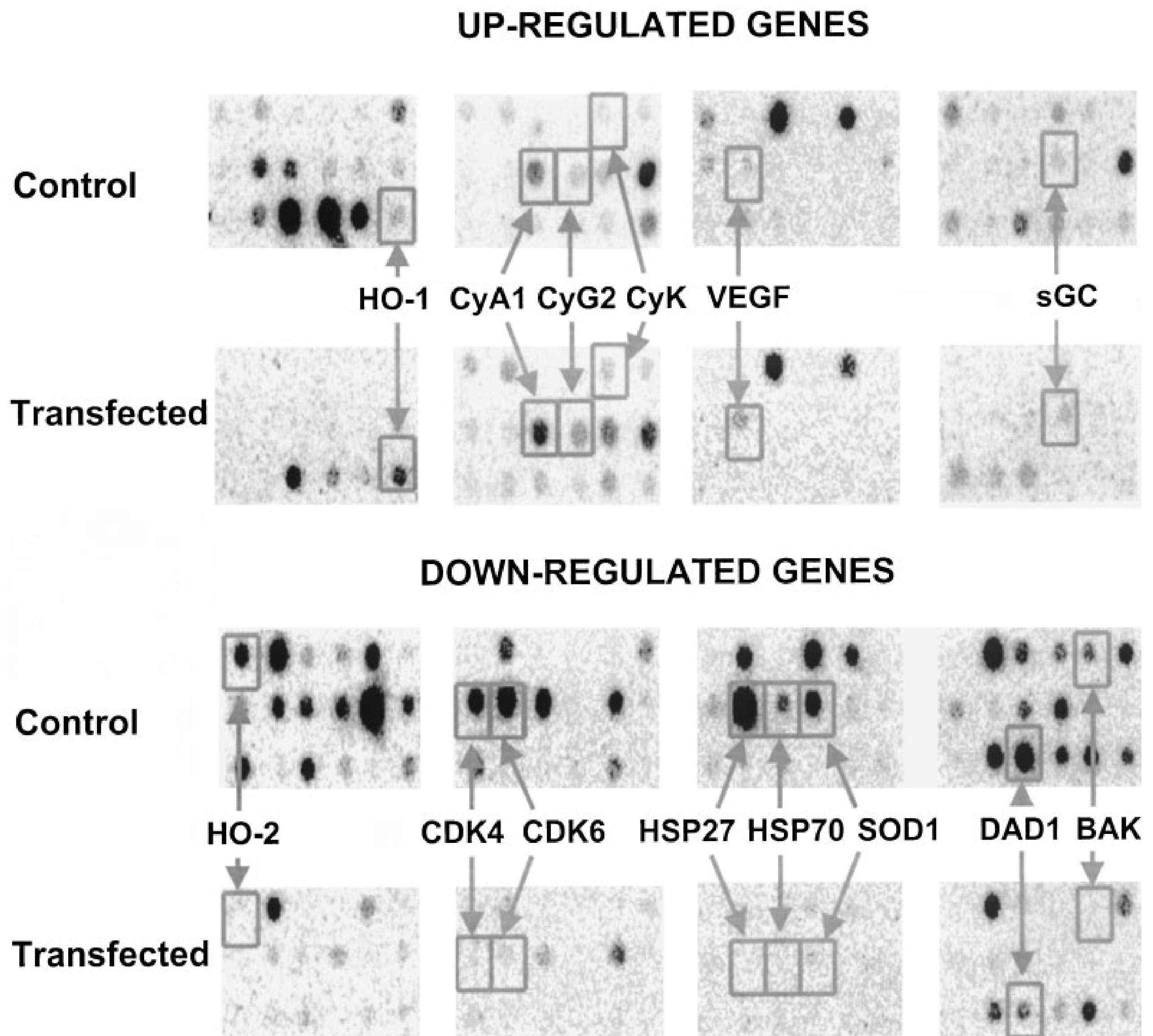

Fig. 6. Autoradiographic images from the Atlas TM Human 1, 2 Array I. Filtered hybridized with 32P-labeled probes prepared from total RNA isolated from control endothelial cells and cells transduced with HO-1. The squares on the figure indicate probable key genes whose expression varies by at least a factor of 2.5-fold in the transduced cells.

DISCUSSION

This study demonstrates that HO-1 in endothelial cells participates in the regulation of cell cycle progression. Three findings substantiate this conclusion. Firstly, the finding that heme, which does not drive endothelial cells into the cell cycle (Table I), induced HO-1 protein in a time-dependent fashion (Fig. 1). We have recently obtained similar results using angiotensin II, an inducer of HO-1 [Colombrita et al., 2003], which has been shown to be associated with an increase of superoxide anion [Griendling et al., 2000] and in isoprostanes levels [Abraham et al., 2003], did not change cell cycle. Secondly, overexpression of the human HO-1 gene was associated with cell cycle progression and enhanced angiogenesis (Fig. 2). This effect appears to be independent of HO-2. Others [Malaguarnera et al., 2002] have also shown that HO-1, but not HO-2, increases angiogenesis in endothelial cells and enhances cell proliferation. Finally, human HO-1 overexpression was associated with suppression of

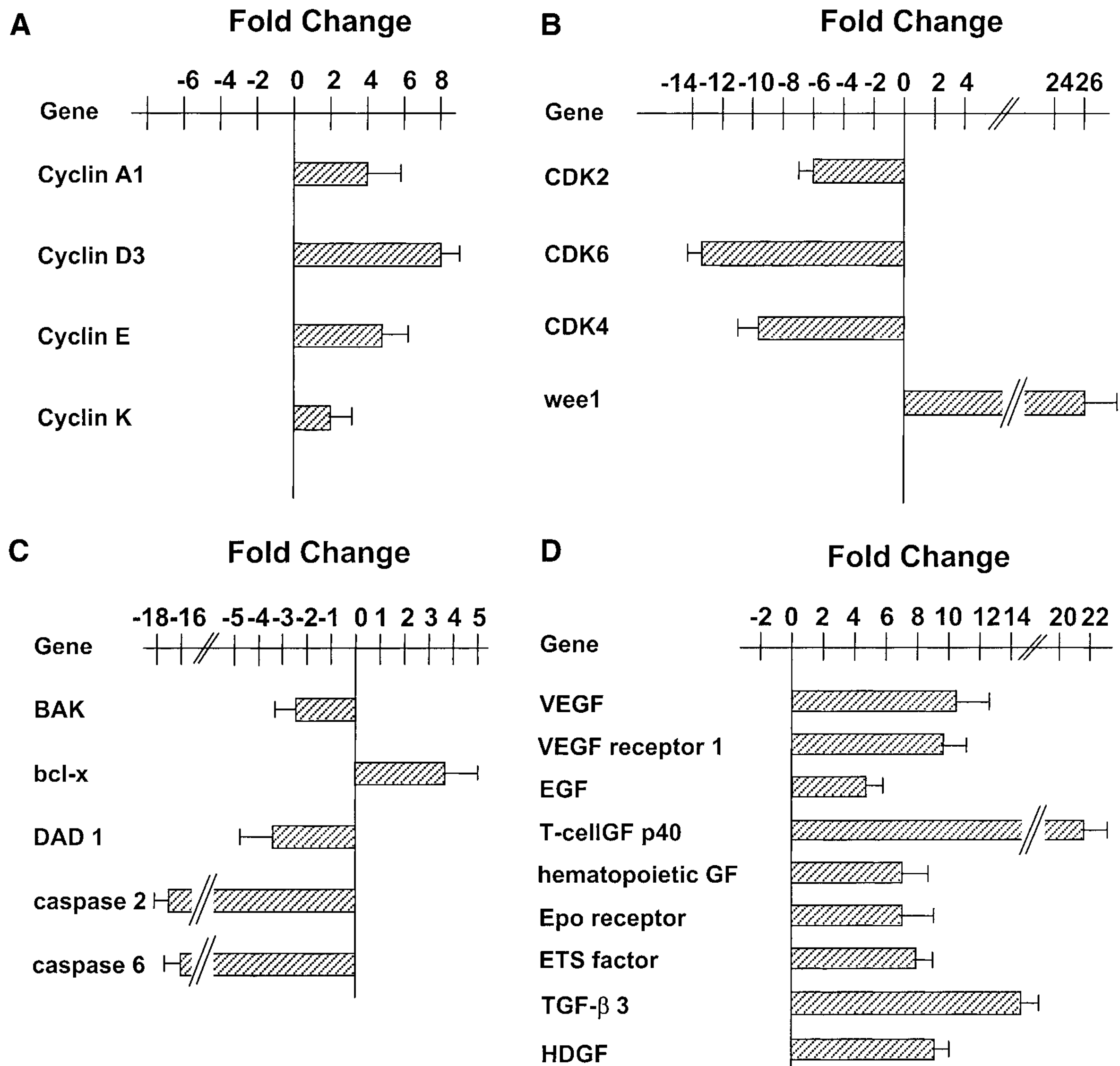

Fig. 7. Up- and downregulated genes after human HO-1 overexpression. Bar graph depicting the magnitude of change in expression of genes, belonging to several functional categories, in stable-transduced human endothelial cells overexpressing the human HO-1 gene and identified using the Atlas TM Human 1,2 Array I and Human 1,2 Array II. Expression levels were quantified by phosphoimaging. NC represents no change in expression. **A**: Cyclin genes known to be involved in the cell cycle progression; (**B**) genes related to cellular proliferation; (**C**) genes related to apoptosis and growth arrest and; (**D**) growth factor genes and oncogenes.

p21 and p27 genes without affecting p53 and p16 genes (Fig. 4), which contribute to enhancement of cell cycle progression. Taken together, this evidence suggests that HO-1, by increasing bilirubin levels, might act as a first line defensive mechanism against stress and DNA damage in the early cell cycle phase whereas DNA synthesis is sensitive to degradation.

DNA microarray analysis provides a powerful means for identifying global changes in gene expression associated with changes in a single gene [Brown and Botstein, 1999; Fan et al., 2002; Pollack et al., 2002]. We were able to utilize this technology in the present study to demonstrate that overexpression of the human HO-1 gene is associated with concurrent upregulation of 19 genes and downregulation of 11 genes. We confirmed the DNA microarray findings by quantitative real-time RT-PCR analysis for 10 of these genes (Fig. 5). Among the genes whose expression was affected by changes in human HO-1 were those involved in cell proliferation. These include, among others, cyclin A1, D3 and E, and VEGF, which were

upregulated, and CDK6, CDK4, BAK, DAD 1, and Caspases 2 and 6 which were downregulated. A number of other genes listed in Figures 5–7, whose biological functions are known, have a relationship to the vascular system, signaling pathways and angiogenesis. In fact, in agreement with these results [Jozkowicz et al., 2003] have shown that HO-1 overexpression increases VEGF and cell growth.

The striking downregulation of Caspase family gene expression, as shown by the microarray and which we have confirmed by determination of Caspase activity (unpublished data), in response to human HO-1 overexpression, is consistent with the hypothesis that human HO-1 activity and, thus, bilirubin and CO production, decrease apoptosis and protect cells from oxidative stress injury [Stocker et al., 1987; Dore et al., 1999]. The decrease in superoxide dismutase (SOD) (Fig. 6) is a very good indication of increased functional activity of HO-1, which is in agreement with the antioxidant effects of HO-1 overexpression and, consequently, elevation of the antioxidant, bilirubin. The upregulation of HO-1, and subsequent bilirubin elevation, may change the cellular redox state and cause the downregulation of the heat shock proteins, including HsP27 and Hsp70, as well as HO-2 (Figs. 5–7).

An additional pathway through which human HO-1 might regulate cell cycle progression is by controlling the levels of p21 and p27 (Fig. 4) and increasing the helix-loop-helix protein Id_2 (ID_2) (Fig. 7). The observed increase in VEGF and VEGFR-1 in endothelial cells transduced with human HO-1 may also contribute to the increase in cell cycle progression and cell proliferation. The expression of growth factors in the gene array may be critical for endothelial cell growth in response to inflammation.

The findings of the differential gene profile identified as a consequence of human HO-1 direct attention to the critical genes that may play a role in the signaling mechanism of cell cycle progression in endothelial cells. Recognition of human HO-1 early-responder genes is important to an understanding of the role of HO in homeostasis and in pathological conditions that are influenced by the enzyme, as well as for understanding the mechanism by which human HO-1 deficiency suppresses endothelial cell proliferation, growth, and function.

In summary, the global gene expression generated by transduction of the human HO-1 gene in endothelial cells greatly enlarges the relationship of this enzyme to cellular metabolic processes and permits the generation and experimental evaluation of new hypotheses concerning the manner in which diverse genes are regulated by the heme-heme oxygenase system.

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REFERENCES

Abraham NG, Lin JH, Dunn MW, Schwartzman ML. 1987. Presence of heme oxygenase and NADPH cytochrome P-450 (c) reductase in human corneal epithelium. Invest Ophthalmol Vis Sci 28:1464–1472.

Abraham NG, Lavrovsky Y, Schwartzman ML, Stoltz RA, Levere RD, Gerritsen ME, Shibahara S, Kappas A. 1995. Transfection of the human heme oxygenase gene into rabbit coronary microvessel endothelial cells: Protective effect against heme and hemoglobin toxicity. Proc Natl Acad Sci USA 92:6798–6802.

Abraham NG, Drummond GS, Lutton JD, Kappas A. 1996. The biological significance and physiological role of heme oxygenase. Cell Physiol Biochem 6:129–168.

Abraham N, Quan S, Shenouda S, Kappas A. 2002. Selective increase in human heme oxygenase-1 gene expression attenuates development of hypertension and increases body growth in spontaneously hypertensive rats. In: Wang R, editor. Carbon monoxide and cardiovascular functions. Boca Raton: CRC Press. pp 233–245.

Abraham NG, Kushida T, McClung J, Quan S, Kafaro R, Darzynkiewicz Z, Wolin MS. 2003. Heme oxygenase-1 attentuates glucose mediated cell growth arrest and apoptosis in human microvessel endothelial cells. Circ Res 93:131–139.

Amersi F, Buelow R, Kato H, Ke B, Coito AJ, Shen XD, Zhao D, Zaky J, Melinek J, Lassman CR, Kolls JK, Alam J, Ritter T, Volk HD, Farmer DG, Ghobrial RM, Busuttil RW, Kupiec-Weglinski JW. 1999. Upregulation of heme oxygenase-1 protects genetically fat Zucker rat livers from ischemia/reperfusion injury. J Clin Invest 104: 1631–1639.

Brouard S, Otterbein LE, Anrather J, Tobiasch E, Bach FH, Choi AM, Soares MP. 2000. Carbon monoxide generated by heme oxygenase 1 suppresses endothelial cell apoptosis. J Exp Med 192:1015–1026.

Brown PO, Botstein D. 1999. Exploring the new world of the genome with DNA microarrays. Nat Genet 21:33–37.

Clark JE, Foresti R, Sarathchandra P, Kaur H, Green CJ, Motterlini R. 2000. Heme oxygenase-1-derived bilirubin ameliorates postischemic myocardial dysfunction. Am J Physiol Heart Circ Physiol 278:H643–H651.

Colombrita C, Lombardo G, Scapagnini G, Abraham NG. 2003. Heme oxygenase-1 expression level is cell cycle dependent. Biochem Biophys Res Commun 308:1001–1008.

da Silva JL, Zand BA, Yang LM, Sabaawy HE, Lianos E, Abraham NG. 2001. Heme oxygenase isoform-specific expression and distribution in the rat kidney. Kidney Int 59:1448–1457.

Deramaudt BM, Braunstein S, Remy P, Abraham NG. 1998. Gene transfer of human heme oxygenase into coronary endothelial cells potentially promotes angiogenesis. J Cell Biochem 68:121–127.

Deramaudt BJM, Remy P, Abraham NG. 1999. Upregulation of human heme oxygenase gene expression by Ets-family proteins. J Cell Biochem 72:311–321.

Dore S, Takahashi M, Ferris CD, Zakhary R, Hester LD, Guastella D, Snyder SH. 1999. Bilirubin, formed by activation of heme oxygenase-2, protects neurons against oxidative stress injury. Proc Natl Acad Sci USA 96:2445–2450.

Duckers HJ, Boehm M, True AL, Yet SF, San H, Park JL, Clinton WR, Lee ME, Nabel GJ, Nabel EG. 2001. Heme oxygenase-1 protects against vascular constriction and proliferation. Nat Med 7:693–698.

Dulak J, Motterlini R, Huk I, Pachinger O, Weidinger F, Jozkowicz A. 2002. Carbon monoxide and iron, by-products of heme oxygenase, modulate vascular endothelial growth factor synthesis in vascular smooth muscle cells. In: Abraham NG, Alam J, Nath KA, editors. Heme oxygenase in biology and medicine. New York: Kluwer academic/Plenum publishers.

Fan J, Yang X, Wang W, Wood WH III, Becker KG, Gorospe M. 2002. Global analysis of stress-regulated mRNA turnover by using cDNA arrays. Proc Natl Acad Sci USA 99:10611–10616.

Griendling KK, Sorescu D, Lassegue B, Ushio-Fukai M. 2000. Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology. Arterioscler Thromb Vasc Biol 20:2175–2183.

Haider A, Olszanecki R, Gryglewski R, Schwartzman ML, Lianos E, Nasjletti A, Kappas A, Abraham NG. 2001. Regulation of cyclooxygenase by the heme-heme oxygenase system in microvessel endothelial cells. J Pharm Exp Ther 300:188–194.

Hancock WW, Buelow R, Sayegh MH, Turka LA. 1998. Antibody-induced transplant arteriosclerosis is prevented by graft expression of anti-oxidant and anti-apoptotic genes. Nat Med 4:1392–1396.

Jozkowicz A, Huk I, Nigisch A, Weigel G, Dietrich W, Motterlini R, Dulak J. 2003. Heme oxygenase and angiogenic activity of endothelial cells: Stimulation by carbon monoxide and inhibition by tin protoporphyrin-IX. Antioxid Redox Signal 5:155–162.

Kappas A. 2002. Development of heme oxygenase inhibitors for the prevention of sever jaundice in infants: Studies from laboratory bench to newborn nursery. In: Abraham NG, Alam J, Nath KA, editors. Heme oxygenase in biology and medicine. New York: Kluwer Academic/Plenum Publishers. pp 3–17.

Kappas A, Drummond G. 1986. Control of heme metabolism with synthetic metalloporphyrins. J Clin Invest 77: 335–339.

Kushida T, LiVolti G, Goodman AI, Abraham NG. 2002a. TNF-alpha-mediated cell death is attenuated by retrovirus delivery of human heme oxygenase-1 gene into human microvessel endothelial cells. Transplant Proc 34:2973–2978.

Kushida T, Quan S, Yang L, Ikehara S, Kappas A, Abraham NG. 2002b. A significant role for the heme oxygenase-1 gene in endothelial cell cycle progression. Biochem Biophys Res Commun 291:68–75.

Laniado-Schwartzman M, Abraham NG, Conners M, Dunn MW, Levere RD, Kappas A. 1997. Heme oxygenaseinduction with attenuation of experimentally induced corneal inflammation. Biochem Pharmacol 53: 1069–1075.

Li Volti G, Wang J, Traganos F, Kappas A, Abraham NG. 2002. Differential effect of heme oxygenase-1 in endothelial and vascular smooth muscle cell cycle progression. Biochem Biophys Res Commun 296:1077–1082.

Liu XM, Chapman GB, Wang H, Durante W. 2002. Adenovirus-mediated heme oxygenase-1 gene expression stimulates apoptosis in vascular smooth muscle cells. Circulation 105:79–84.

Malaguarnera L, Pilastro MR, Quan S, Ghattas MH, Yang L, Mezentsev AV, Kushida T, Abraham NG, Kappas A. 2002. Significance of heme oxygenase in prolactin-mediated cell proliferation and angiogenesis in human endothelial cells. Int J Mol Med 10:433–440.

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.

Morita T, Mitsialis SA, Hoike H, Liu Y, Kourembanas S. 1997. Carbon monoxide controls the proliferation of hypoxic smooth muscle cells. J Biol Chem 272:32804–32809.

Nath KA, Balla J, Jacob HS, Vercellotti GM, Levitt M, Rosenberg ME. 1992. Induction of heme oxygenase is a rapid protective response in rhabdomyolysis in the rat. J Clin Invest 90:267–270.

Otterbein LE, Choi AM. 2000. Heme oxygenase: Colors of defense against cellular stress. Am J Physiol Lung Cell Mol Physiol 279:L1029–L1037.

Otterbein LE, Bach FH, Alam J, Soares M, Tao LH, Wysk M, Davis RJ, Flavell RA, Choi AM. 2000. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. Nat Med 6: 422–428.

Pollack JR, Sorlie T, Perou CM, Rees CA, Jeffrey SS, Lonning PE, Tibshirani R, Botstein D, Borresen-Dale AL, Brown PO. 2002. Microarray analysis reveals a major direct role of DNA copy number alteration in the transcriptional program of human breast tumors. Proc Natl Acad Sci USA 99:12963–12968.

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

Schubert CM. 2003. Microarray to be used as routine clinical screen. Nat Med 9:9.

Sessa WC, Abraham NG, Escalante B, Schwartzman ML. 1989. Manipulation of cytochrome P-450 dependent renal thromboxane synthase activity in spontaneously hypertensive rats. J Hypertens 7:37–42.

Soares MP, Usheva A, Brouard S, Berberat PO, Gunther L, Tobiasch E, Bach FH. 2002. Modulation of endothelial cell apoptosis by heme oxygenase-1-derived carbon monoxide. Antioxid Redox Signal 4:321–329.

Stears RL, Martinsky T, Schena M. 2003. Trends in microarray analysis. Nat Med 9:140–145.

Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN. 1987. Bilirubin is an antioxidant of possible physiological importance. Science (Washington DC) 235: 1043–1047.

Wagener FADTG, da Silva J-L, Farley T, de Witte T, Kappas A, Abraham NG. 1999. Differential effects of heme oxygenase isoforms on heme mediation of endothelial intracellular adhesion molecule 1 expression. J Pharmacol Exp Ther 291:416–423.

Willis D, Moore AR, Frederick R, Willoughby DA. 1996. Heme oxygenase: A novel target for the modulation of the inflammatory response. Nat Med 2.2:87–90.

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.

Yet SF, Perrella MA, Layne MD, Hsieh CM, Maemura K, Kobzik L, Wiesel P, Christou H, Kourembanas S, Lee ME. 1999. Hypoxia induces severe right ventricular dilatation and infarction in heme oxygenase-1 null mice. J Clin Invest 103:R23–R29.