Heme Oxygenase-1/Carbon Monoxide Induces Vascular Endothelial Growth Factor Expression via p38 Kinase-dependent Activation of Sp1*

Heng-Huei Lin, Shao-Chuan Lai, and Lee-Young Chau

From the Institute of Biomedical Sciences, Academia Sinica, Taipei 115, Taiwan

Heme oxygenase-1 (HO-1) is a stress-inducible enzyme catalyzing the oxidative degradation of heme to free iron, CO, and biliverdin. Previous studies demonstrated that HO-1 overexpression promoted VEGF expression and angiogenesis in the ischemic heart. However, the underlying mechanism remained elusive. Here we show that adenovirus-mediated HO-1 transduction of rat primary cardiomyocytes and H9C2 myocytes resulted in significant induction of VEGF expression, and a similar effect was seen in cells directly exposed to CO gas or a CO-releasing compound, tricarbonyldichlororuthenium (II) dimer. HO-1/CO-induced VEGF expression was significantly suppressed by pharmacological inhibition of p38 kinase, but not of AKT, activation. VEGF promoter-luciferase reporter assays, electrophoretic mobility shift assays, supershift assay, and chromatin immunoprecipitation showed that CO-induced VEGF promoter activation requires the binding of the Sp1 transcriptional factor to a cis-regulatory sequence located at the VEGF promoter. Western blot analysis and immunostaining experiments demonstrated that HO-1/CO induced p38-dependent phosphorylation of Sp1 at Thr-453 and Thr-739 both in vitro and in vivo. Overexpression of Sp1 protein with an alanine mutation at Thr-453 or Thr-739 suppressed CO-induced Sp1 binding to the VEGF promoter and its transcriptional activation. Collectively, these data suggest that p38-dependent phosphorylation of Sp1 at Thr-453/Thr-739 is crucial for HO-1/CO-induced VEGF expression in myocytes.

VEGF is a potent angiogenic factor to promote endothelial cell proliferation and new vessel formation. Its expression is essential not only during embryonic development but also in the adaptive response to postnatal hypoxia, inflammation, and wounding (1). Under hypoxia condition, VEGF expression is transcriptionally up-regulated by the binding of hypoxia-inducible factor-1 to a hypoxia response element in its gene promoter (2). Because hypoxia-inducible factor-1 can also be activated under certain normoxia stress conditions and by many growth factors and cytokines, it is regarded as a major transcriptional factor in the induction of VEGF expression (1, 2). However, several studies have demonstrated that VEGF expression can also be up-regulated by transcriptional activation mediated by other transcriptional factors, such as Sp1, STAT3, and nuclear receptors under various stimulatory conditions (1). Given the importance of VEGF in normal and pathophysiological neovascularization, a complete understanding of its regulation at the transcriptional level by specific stimuli and the cellular signaling pathways involved is important in vascular biology.

Heme oxygenase-1 (HO-1) is a stress-inducible enzyme catalyzing the oxidative degradation of cellular heme to free iron, CO, and biliverdin (3). Considerable evidence has supported the protective functions of HO-1 in the cardiovascular system. Biliverdin and its metabolite, bilirubin, are potent antioxidants, whereas CO is an important signaling molecule with vasodilatory, anti-inflammatory, and anti-apoptotic effects (3). Moreover, HO-1 promotes angiogenesis and mediates the angiogenic responses induced by VEGF and stromal cell-derived factor-1 (4). HO-1 overexpression in endothelial cells and vascular smooth muscle cells induces VEGF synthesis (5–8), as does exposure of vascular cells to CO gas or a CO-releasing compound (6, 7, 9). However, the molecular mechanism by which HO-1 or CO induces VEGF expression has not been explored. Recently, our group reported that myocardial HO-1 gene transduction mediated by adenovirus enhances neovascularization in the ischemic heart by inducing stromal cell-derived factor-1 and VEGF expression and the recruitment of circulating stem/progenitor cells (10). However, the detailed mechanism(s) underlying the regulation of VEGF and stromal cell-derived factor-1 gene expression by HO-1 remains elusive. We have therefore performed in vitro studies to investigate the molecular mechanism of HO-1-induced VEGF gene expression in primary neonatal cardiomyocytes and H9C2 myocytes and the role of CO in the HO-1-mediated regulation of VEGF. The signaling pathway and downstream nuclear factor responsible for the activation of VEGF gene transcription were characterized and confirmed in the heart tissues of animals receiving myocardial HO-1 gene transduction.

EXPERIMENTAL PROCEDURES

Materials—Tricarbonyldichlororuthenium (II) dimer (CORM-2), LY294002, SB203580, and poly(dI-dC) were from...
HO-1/CO-induced VEGF Expression

Sigma; the protease inhibitor mixture was from Calbiochem; the Lipofectamine 2000 was from Invitrogen; the dual luciferase reporter assay system was from Promega; the VEGF Quantikine ELISA kit was from R & D; the rabbit antibodies against p38, Akt, phospho-p38, or phospho-Akt were from Cell Signaling; the rabbit antibodies against GAPDH, Sp2 (sc-643X), Sp3 (sc-2027), and VEGF (sc-152) and the normal rabbit IgG were from Santa Cruz; the phosphoprotein antibody sampler pack containing rabbit antibodies against phosphoserine, phosphothreonine, and phosphotyrosine was from Zymed Laboratories Inc.; the rabbit antibody against Sp1(07-645) and the EZ ChIP™ kit were from Upstate; the anti-phospho-Sp1-T739 antibody (BS4755) was from Abcam; and the anti-phospho-Sp1-T739 antibody (BS4755) was from Bio-World Technology.

Adenovirus Preparation—A recombinant adenovirus carrying the human HO-1 (Adv-HO-1) gene driven by the CMV promoter was amplified in HEK293 cells and purified as described previously (11).

Cell Culture—H9C2 rat embryonic myoblast cells were cultured in DMEM containing 10% FBS. Neonatal cardiomyocytes were isolated from male Sprague-Dawley rats (1–2 days old) as described previously (12) and cultured in DMEM containing 10% FBS and 0.1 mM of bromodeoxyuridine for 3 days prior to the experiments. For virus infection, H9C2 cells or primary cardiomyocytes (4 × 10⁵) were subcultured in 3.5-cm dishes in complete medium for 24 h and then were changed to serum-free DMEM containing 100 multiplicity of infection of empty Adv or Adv-HO-1 for 2 h. An equal volume of complete medium was added to each culture, and incubation was continued for 22 h. The culture medium was then replaced by DMEM containing 1% FBS. After 24 h in culture, the cells were harvested for experiments. For CO exposure, the cells cultured in 3.5-cm dishes were changed to DMEM containing 1% FBS and placed for 24 h in a chamber containing the indicated concentration of CO, which was equilibrated by a flow of 0.5% CO mixed with compressed air. For CORM-2 treatment, the cells were incubated for various times in DMEM containing 1% FBS and the indicated concentration of CORM-2 or inactive CORM-2 (CORM-2 preincubated overnight at 37 °C in DMEM). To scavenge CO, hemoglobin was first dissolved in H₂O at 4 °C for 2 h before use.

Real Time Quantitative PCR—Total RNA was extracted using TRIzol reagent, and 2 μg of RNA was reverse transcribed to cDNA using Superscript II reverse transcriptase (Invitrogen). Real time PCR for rat VEGF and GAPDH was performed using LightCycler® FastStart DNA Master SYBR Green reagent I (Roche Applied Science). The primer sequences used for amplification of rat VEGF were: sense, 5’-TCCTACCTTT-3’; and antisense, 5’-CGCA-CACGCCATTAGG-3’. The primer sequences used for amplification of rat GAPDH were: sense, 5’-ACTCCCATTC-CTCCACCTTT-3’; and antisense, 5’-TTACTCTTGGGA-GGCCATGT-3’.

Western Blotting—The cells were rinsed twice with ice-cold PBS, lysed in reducing SDS sample buffer, and subjected to electrophoresis on 10% SDS-polyacrylamide gels, and then the proteins were transblotted onto nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in buffer containing 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20 (TBST) at room temperature for 1 h, followed by incubation with anti-phospho-AKT (1:1000), anti-phospho-p38 (1:1000), anti-HO-1 (1:1000), or anti-GAPDH (1:2000) antibodies diluted in TBST buffer containing 1% nonfat milk at room temperature for another 1 h. After three washes with TBST buffer, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:1000) diluted in TBST containing 1% nonfat milk at room temperature for 1 h, followed by three washes with TBST. Antigen was then detected using the enhanced chemiluminescence system. To reblock the membranes with anti-AKT (1:1000) or anti-p38 (1:500) antibodies, the membranes were washed twice with stripping buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 100 mM 2-mercaptoethanol at 55 °C for 15 min with shaking, followed by three washes with TBST. The membranes were then blocked and immunoblotted with the indicated antibodies as described above.

Plasmid Preparation—A PGL2-firefly luciferase reporter construct containing a 2.65-kb KpnI-BssHI fragment of the human VEGF gene 5’-flanking sequence encompassing nucleotides −2274 to +379 relative to the transcription initiation site was obtained from the ATCC. A series of deletion constructs containing 790, 268, or 131 bp of the 5’-flanking sequence was prepared by double restriction enzyme digestion using KpnI in combination with PstI, SacII, or ApaI, respectively, followed by religation. Reporter constructs containing a point mutation were prepared by site-directed mutagenesis using PCR. pCMV-HA-Sp1 and pCMV-HA-Sp1-T739A were kindly provided by Drs. J. J. Hung and W. C. Chang (National Cheng-Kung University, Taiwan). HA-Sp1 construct containing a T453A mutation was prepared by a PCR mutagenesis method. FLAG-tagged dominant-negative (DN) MKK3 (DN-MKK3) and DN-MKK6 constructs were prepared by subcloning DN-MKK3 and DN-MKK6 cDNAs (obtained from Dr. J. Han of the Scripps Research Institute) into the pFLAG-CMV-2 expression vector.

Promoter Assay—H9C2 cells were seeded in 12-well plates at a density of 8 × 10⁴ cells/well. Next day, the cells were transfected with 1 μg of the test promoter construct and 0.1 μg of the pRL-TK plasmid carrying the Renilla luciferase gene as an internal control using Lipofectamine 2000 according to the manufacturer’s instructions. After 22 h, the cells were changed to DMEM containing 1% FBS, treated with the indicated agent for 18 h, and washed twice with ice-cold PBS, and then cell lysates were prepared using the kit lysis buffer. Firefly and Renilla luciferase activities were measured using a dual luciferase reporter assay system.

Nuclear Extract Preparation—H9C2 cells cultured in 10-cm dishes were rinsed twice with ice-cold PBS and lysed on ice for 15 min in 500 μl of 10 mM HEPES, pH 8.0, 1.5 mM MgCl₂, 10 mM KCl, 1 mM DTT, 10 mM NaF, 1 mM Na₃VO₄, 1 mM Na₃P₂O₇, and protease inhibitor mixture. Nonidet P-40 was then added directly to the cell lysates to a final concentra-
tion of 0.5%, and the mixture was vigorously vortexed for 10 s and then centrifuged at 12,000 × g for 5 min at 4 °C. The nuclear pellet was resuspended in 20 mM HEPES, pH 8.0, 1.5 mM MgCl₂, 420 mM NaCl, 0.2 mM EDTA, 1 mM DTT, 10 mM NaF, 1 mM Na₃VO₄, 1 mM Na₄P₂O₇, and 25% glycerol, then nuclear proteins were extracted on ice for 30 min, and the nuclear extract was cleared by centrifugation at 15,000 × g for 15 min at 4 °C. The protein concentration was determined using the Bio-Rad protein assay.

EMSA—A double-stranded oligonucleotide (sense, 5′-AGAGTTCGCGGGCGATGGG-3′; and antisense, 5′-CCCATCCGCCCCGAAATCTC-3′) containing the potential Sp1 site (−238/−233) in the human VEGF promoter was end-labeled with [γ-³²P]ATP using T4 polynucleotide kinase. The radiolabeled DNA probe (50,000 cpm) was then incubated for 15 min at room temperature with 5 μg of nuclear extract in 20 μl of binding buffer containing 25 mM HEPES, pH 8.0, 50 mM KCl, 0.1 mM EDTA, 50 mM NaCl, 1 mM MgCl₂, and 1 μg of poly(dI-dC), and then the DNA-protein complex was analyzed on a 6% native polyacrylamide gel at 130 mA for 1 h. The protein-DNA complex was eluted from the protein G-agarose by two elution steps of incubation at room temperature for 15 min each with 100 μl of 1% SDS and 0.1 M NaHCO₃, followed by centrifugation at 600 × g for 1 min, then 8 μl of 5 M NaCl was added to the 200 μl of eluted complex, and the mixture was incubated at 65 °C overnight to reverse the cross-linking. The mixture was then treated with RNase A and proteinase K, and the DNA was purified using a Qiagen MinElute kit and used for PCR. The primers used for amplification of the rat VEGF promoter were: sense, 5′-GAAATTGCCCCAACCTCCTCTGGT-3′; and antisense, 5′-GGTTCATAGGCTTGAAGA-3′.

Immunofluorescence Staining—H9C2 cells grown on glass coverslips coated with 0.1% gelatin were incubated with or without 50 μM CORM-2 for 1 h and then were fixed with 4% paraformaldehyde for 10 min at room temperature. The cells were then permeabilized with cold acetone for 10 s, blocked with TBS containing 5% BSA for 30 min at room temperature, and incubated at room temperature for 1 h with rabbit IgG or rabbit anti-phospho-Sp1 T453 (1:50) or rabbit anti-phospho-Sp1 T739 (1:50) antibodies diluted in TBS. After two washes with TBS, the coverslips were incubated for 1 h in the dark with AlexaFluor568-conjugated goat anti-rabbit IgG. The nuclei were stained with DAPI. The coverslips were mounted on glass slides with 50% glycerol and examined by confocal microscopy.

Animal Operation and Immunohistochemistry—The animal experimental protocol was approved by the Institutional Animal Care and Utilization Committee of the Academia Sinica of Taiwan. Male C56BL/6J mice (8–12 weeks old) were anesthetized by intramuscular injection of a combination of ketamine (0.8 mg/kg of body weight), xylazine (0.2 mg/kg), and atropine (0.016 mg/kg), then the chest cavity was opened, and empty Adv or Adv-HO-1 (1 × 10⁹ plaque-forming units) was injected into the left ventricular myocardium as described previously (10). After 7 days, the animals were sacrificed, and the hearts were harvested, fixed with 4% paraformaldehyde, and paraffin-embedded. Tissue sections (7 μm) were pre-treated with target retrieval solution (DakoCytomation) at 95 °C for 30 min and then incubated with 3% hydrogen peroxide for 15 min at room temperature to block endogenous peroxidase. The sections were then incubated with 5% normal goat serum in TBS for 30 min at room temperature, followed by incubation for 1 h at 37 °C with rabbit anti-HO-1 (1:100), rabbit anti-VEGF (1:50), rabbit anti-phospho-Sp1 T453 (1:50), or rabbit anti-phospho-Sp1 T739 (1:50) antibodies diluted in TBS. After two washes with TBS, the sections were incubated for 1 h at 37 °C with horseradish peroxidase-conjugated anti-rabbit IgG antibodies (1:500) diluted in TBS. Diaminobenzidine chromogen was used for the detection of HO-1 and VEGF, and 3-amino-9-ethylcarbazole chromogen was used for the detection of phospho-Sp1. The sections were then counterstained with hematoxylin.

Statistical Analysis—The data are expressed as the means ± S.D. for at least three independent experiments and analyzed by one-way analysis of variance. A value of p < 0.05 was considered statistically significant.
**RESULTS**

**HO-1/CO Induces VEGF Expression in Cardiomyocytes**—We performed adenovirus-mediated HO-1 gene transduction in primary neonatal cardiomyocytes and the H9C2 myocyte cell line and found that VEGF gene expression determined by quantitative real time PCR was significantly higher in cells overexpressing HO-1 (Fig. 1A). To examine whether CO, a byproduct derived from heme degradation by HO-1, has a role in the HO-1-mediated effect, H9C2 cells were treated with 100 μM hemoglobin, a CO scavenger, for 48 h prior to cell harvest, and this resulted in a substantial reduction in HO-1-induced VEGF mRNA levels (Fig. 1B). VEGF mRNA levels in H9C2 cells receiving saline or the indicated adenovirus followed by treatment with or without 100 μM hemoglobin (Hb) for 48 h, *p < 0.05 versus Adv-HO-1-treated cells without hemoglobin treatment. C–E, H9C2 cells were exposed to the indicated concentration of CO gas (C) or CORM-2 (D) for 24 h or were treated with 50 μM CORM-2 for the indicated time (E). iCORM, inactivated CORM-2 (50 μM); DMSO, dimethyl sulfoxide. *, < 0.05 versus the untreated control.

**FIGURE 1.** CO mediates the effect of HO-1 on VEGF expression in myocytes. A, primary neonatal cardiomyocytes (left panel) or H9C2 myocytes (right panel) were infected with the indicated adenovirus (100 multiplicity of infection) for 48 h, and then total RNA was extracted, and the levels of VEGF and GAPDH (internal control) mRNAs were determined by real time PCR. *, p < 0.05 versus the control saline group. B, VEGF mRNA levels in H9C2 cells receiving saline or the indicated adenovirus followed by treatment with or without 100 μM hemoglobin (Hb) for 48 h. *, p < 0.05 versus Adv-HO-1-treated cells without hemoglobin treatment. C–E, H9C2 cells were exposed to the indicated concentration of CO gas (C) or CORM-2 (D) for 24 h or were treated with 50 μM CORM-2 for the indicated time (E). iCORM, inactivated CORM-2 (50 μM); DMSO, dimethyl sulfoxide. *, < 0.05 versus the untreated control.

**FIGURE 2.** p38 kinase mediates the induction of VEGF expression by CO. A–C, H9C2 cells were infected with the indicated adenovirus for 48 h (A and B) or were treated with 50 μM CORM-2 for the indicated time (C), and then levels of phosphorylated AKT (p-AKT) and phosphorylated p38 (p-p38) were examined by Western blot analysis. B, the levels of phosphorylated Akt and phosphorylated p38 shown in A were quantified by densitometry. *, p < 0.01 versus Adv-infected cells. D and E, H9C2 cells (D) or primary cardiomyocytes (E) were treated without (DMSO, with dimethyl sulfoxide) or with 50 μM CORM-2 in the absence or presence of 20 μM LY294002 (AKT inhibitor), ODQ (guanylate cyclase inhibitor), or SB203580 (p38 inhibitor) for 24 h, and then VEGF mRNA levels were assessed by real time PCR. *, p < 0.05 versus cells treated with CORM-2 alone.

**RESULTS**

**HO-1/CO Induces VEGF Expression in Cardiomyocytes**—We performed adenovirus-mediated HO-1 gene transduction in primary neonatal cardiomyocytes and the H9C2 myocyte cell line and found that VEGF gene expression determined by quantitative real time RT-PCR was significantly higher in cells overexpressing HO-1 (Fig. 1A). To examine whether CO, a byproduct derived from heme degradation by HO-1, has a role in the HO-1-mediated effect, H9C2 cells were treated with 100 μM hemoglobin, a CO scavenger, for 48 h prior to cell harvest, and this resulted in a substantial reduction in HO-1-induced VEGF gene expression (Fig. 1B). To confirm the effect of CO on VEGF expression, H9C2 myocytes were exposed to CO gas or treated with a CO-releasing compound, CORM-2, for 24 h, which resulted in induction of VEGF gene expression in a dose-dependent manner (Fig. 1, C and D), whereas CORM-2 inactivated by overnight incubation at 37 °C in DMEM had no effect (Fig. 1D). CORM-2-induced
VEGF expression was also significantly suppressed by co-treatment of H9C2 cells with hemoglobin (supplemental Fig. S1), supporting that the effect of CORM-2 is mediated by CO release. A time course experiment showed that induction of VEGF expression was evident after 12 h of CORM-2 treatment (Fig. 1E). Consistent with these mRNA expression results, levels of VEGF protein were also substantially increased in the culture medium of CORM-2-treated H9C2 cells (supplemental Fig. S2).

**p38 Mediates HO-1/CO-induced VEGF Expression**—To identify the signaling pathway involved in induction of VEGF expression by HO-1/CO, the phosphorylation states of the kinases p38 and AKT, which have been shown to be activated by HO-1/CO in cardiomyocytes (13), were examined in H9C2 myocytes transduced with Adv-HO-1 or treated with CORM-2. As shown in Fig. 2 (A and B), HO-1 overexpression induced an increase in levels of phosphorylated AKT and phosphorylated p38. Likewise, CORM-2 treatment increased levels of both phosphorylated AKT and phosphorylated p38, an effect detected as early as after 30 min of treatment and maintained for up to 8 h, the longest time point examined (Fig. 2C). Experiments were then performed to examine the effects of AKT and p38 activation on VEGF gene induction. As shown in Fig. 2D, CORM-2-induced VEGF gene expression in H9C2 myocytes was markedly suppressed by co-treatment of the cells with the p38 inhibitor, SB203580, but not by co-treatment with the AKT inhibitor, LY294002, at concentrations that were effective in blocking the activation of p38 or AKT, respectively (supplemental Fig. S3). Moreover, when soluble guanylate cyclase, which can be activated by CO (3), was inhibited with 1H-[1,2,4]oxadiazolo-(4,3-a)quinoxalin-1-one (ODQ), no effect on VEGF gene induction was seen (Fig. 2D). The effect of p38 blockade on CORM-2-induced VEGF expression was also observed in primary cardiomyocytes (Fig. 2E). To further determine the upstream signaling that mediates p38 activation in CORM-2-treated cells, we performed additional experiments to examine the phosphorylation states of MKK3 and MKK6, the main activators of p38 (14, 15). As shown in supplemental Fig. S4A, an increase in MKK3/MKK6 phosphorylation was evident at 5 min after CORM-2 treatment, which was much earlier than the increase in p38 phosphorylation. To confirm the role of MKK3/MKK6 in p38 phosphorylation, H9C2 cells were transient transfected with FLAG-tagged DN-MKK3 or FLAG-tagged DN-MKK6 cDNA construct for 30 h prior to CORM-2 treatment. As demonstrated in supplemental Fig. S4B, expression of DN-MKK3 or DN-MKK6 ablated p38 phosphorylation following CORM-2 treatment. These results support that MKK3 and MKK6 are
the upstream kinases responsible for CO-induced p38 activation in myocytes.

Localization of the CO Regulatory Element in the VEGF Gene Promoter—To confirm the role of p38 signaling in the transcriptional regulation of VEGF by CO, H9C2 myocytes were transiently transfected with a luciferase reporter plasmid bearing the human VEGF gene promoter region encompassing the region −2274 to +379 relative to the transcriptional initiation site. As shown in Fig. 3A, CORM-2 treatment increased luciferase activity by 3-fold, and this effect was abolished by co-treatment with SB203580 but not with LY2944002. To identify the cis-regulatory element responsible for this CORM-2 effect, the luciferase activity of cells transfected with a series of promoter 5′-deletion constructs was examined. The results showed that CORM-2-induced luciferase activity was still observed using a construct containing region −268 to +379, but not using a shorter construct containing region −131 to +379 (Fig. 3B), indicating that region −268 to −131 contains the CORM-2-responsive element. SB203580 treatment did not affect the basal luciferase activity of the (−268/+379) construct (supplemental Fig. S5). However, it abolished CORM-2-induced activity, supporting the involvement of p38 in the activation of the transcriptional factor binding to this CORM-2-responsive element. Computational analysis identified three potential binding sites for the transcriptional factor Sp-1 within this region. To investigate the importance of these potential Sp1-binding sites, we tested the effect of mithramycin, an inhibitor of Sp1 binding to DNA sequence, on promoter activity. As shown in Fig. 3C, the CORM-induced luciferase activity of the (−2274/+379) construct was abolished by mithramycin treatment. We then prepared promoter constructs containing mutations in each of these sites individually. As shown in Fig. 3D, mutation in the Sp1-binding site at −238/−233, but not those at −205/−200 and −177/−172, completely abolished the luciferase activity induced by CORM-2, supporting the idea that the Sp1-binding sequence at −238/−233 mediates the transcriptional activation of the VEGF gene by CORM-2.

Functional Role of Sp1 in CO-induced VEGF Gene Expression—To assess whether Sp1 bound to this cis-regulatory DNA sequence, an EMSA experiment was performed. As shown in Fig. 4A, CORM-2 treatment of H9C2 cells time-dependently increased the specific binding of nuclear proteins to the DNA sequence containing the −238/−233 GC-rich sequence. A supershift experiment was then performed using control IgG or specific antibodies against Sp1, Sp2, or Sp3 and the results clearly showed that only the anti-Sp1 antibody caused supershift of the DNA-protein complex (Fig. 4B), supporting the role of Sp1 in DNA-protein complex formation. Furthermore, DNA-protein complex formation was substantially reduced when the nuclear extracts prepared from cells co-treated with CORM-2 and SB203580 were used (Fig. 4C), indicating that the DNA binding was p38 kinase-dependent. It has been shown that human and rat VEGF promoters are highly homologous (16). A potential Sp1-binding sequence (GGGTGG, −191/−186), corresponding to the −238/−233 (GGGCGG) sequence of human VEGF promoter, was identified in rat VEGF promoter (supplemental Fig. S6). We then performed the ChIP assay to confirm the binding of Sp1 to the rat VEGF promoter in H9C2 cells. As shown in Fig. 4D,
CORM-2 induced a significant increase in Sp1 binding to the rat VEGF promoter region containing the −191/−186 sequence, which was again significantly attenuated by inhibition of p38 activation. Because there were studies showing that activated p38 can bind to gene promoters (17, 18), we then performed the ChIP assay using the specific antibody against phosphorylated p38. However, we failed to detect p38 binding to the VEGF promoter (supplemental Fig. S7), suggesting that p38 is not associated with the transcriptional complex required for CO-induced VEGF gene expression in the present experimental setting.

**HO-1/CO Induces p38-dependent Phosphorylation of Sp1 at Thr-453 and Thr-739**—The phosphorylation status of Sp1 following CORM-2 treatment of H9C2 cells was then examined. Western blot analysis of Sp1 immunoprecipitated from H9C2 cells revealed that CORM-2 treatment significantly increased the phosphorylation of Sp1 threonine residues, but not of tyrosine or serine residues (supplemental Fig. S8). Because a previous study showed that Sp1 phosphorylation at Thr-453 and Thr-739 was implicated in Erk-mediated VEGF gene transcription (19), we then examined whether p38-dependent Sp1 phosphorylation at both threonines occurred in H9C2 cells treated with HO-1/CO. We performed Western blot analysis using specific antibodies against Sp1 phosphorylated at Thr-453 and Thr-739, and increased phosphorylation of Sp1 Thr-453 and Thr-739 was observed in CORM-2-treated cells, and this effect was abolished by SB203580 (Fig. 5C). Immunofluorescence experiments showed significant nuclear staining for phosphorylated Sp1-Thr-453 and phosphorylated Sp1-Thr-739 in CORM-2-treated cells (Fig. 5D).
739 in cells after HO-1 transduction or CORM-2 treatment (Fig. 5, D and E).

Significance of Thr-453 and Thr-739 Phosphorylation—To assess the functional importance of Sp1 Thr-453 and Thr-739 phosphorylation in CO-induced VEGF gene transcription, H9C2 cells were transfected with the VEGF promoter reporter construct alone or with a plasmid coding for the HA-tagged WT Sp1 or the Sp1T453A or Sp1T739A mutant for 24 h and then were incubated without or with 50 μM CORM-2 for 18 h. A, luciferase activities. *, p < 0.05 versus the respective untreated control. B, expression of HA-tagged Sp1 proteins examined by Western blot analysis. C, cells transfected without or with plasmids bearing WT-Sp1, Sp1T453A and Sp1T739A were incubated with or without 50 μM CORM-2 for 1 h, and then chromatin immunoprecipitation (IP) was performed using control IgG or anti-Sp1 antibody, and the immunoprecipitated DNA was extracted and PCR was performed to amplify the rat VEGF promoter region containing the corresponding Sp1-binding site. D, the ChIP results were quantified by densitometry and expressed as fold increase relative to the level of 5% input. DMSO, dimethyl sulfoxide.

DISCUSSION

Our previous study demonstrated that HO-1 overexpression in myocardium induces VEGF expression (10). Here we showed that CO derived from heme degradation mediated HO-1-induced VEGF expression by inducing p38 kinase activation in primary cardiomyocytes and a myocyte cell line. The roles of MAP kinases in the regulation of VEGF gene expres-
Erk activation mediates VEGF gene transcription following Ras induction or growth factor stimulation (20, 21). Moreover, activation of JNK and p38 following cytokine or growth factor stimulation leads to increased VEGF expression by increasing the stability of its mRNA or gene transcription (22–27). The VEGF promoter contains several potential binding sites for transcriptional factors, including hypoxia-inducible factor-1, Sp1, AP1, and AP2 (1). Erk induces VEGF gene transcription by activating the cooperative binding of Sp1 and AP2 to a proximal GC-rich region between nucleotides −88 and −66 in the human VEGF promoter (20). JNK activation mediates tumor necrosis factor-induced VEGF gene transcription by inducing AP-1 expression (27). p38 is implicated in heregulin-β1-induced VEGF gene transcription involving a CA-rich sequence between nucleotides −2249 and −2242 in the VEGF promoter (23). Moreover, two GC-rich sequences located between nucleotides −73 and −62 in the rat VEGF promoter are required for the Sp1 binding and promoter activation induced by p38 and JNK overexpression in rat cardiomyocytes (26). In an attempt to obtain mechanistic insights into CO-induced VEGF gene transcription, we performed a reporter assay using a series of 5′-deletion constructs of the human VEGF promoter in H9C2 cells, and the results showed that a regulatory sequence located at −238/−233 (GGGCGG) was required for VEGF gene induction by CO. EMSA and supershift assays demonstrated that Sp1 bound to this sequence. The sequence alignment of human and rat VEGF promoter sequences revealed a similar Sp1-binding site (GGGTGG) located at the sequence −191/−186 of rat VEGF promoter (16). ChIP assay performed in H9C2 cells confirmed that CORM-2 treatment induced the binding of Sp1 protein to this region of rat VEGF promoter. It appears that different MAPK signaling pathways activated under different conditions can mediate VEGF gene transcription by regulating the binding of different transcriptional factors to various regulatory sequences located in distinct regions of the VEGF promoter.

Numerous studies have demonstrated that phosphorylation of Sp1 at different sites by various kinases affects its transcriptional function and protein stability (28). Erk phosphorylates Sp1 at Thr-453 and Thr-739, and mutation of either residue to alanine reduces Erk-dependent transcriptional activation of the VEGF promoter (19). Moreover, Erk-induced Sp1 phosphorylation of these two residues mediates the repression of PDGF receptor-α transcription induced by fibroblast growth factor-2 (29), supporting the functional importance of phosphorylation at Thr-453 and Thr-739 in Sp1-regulated gene transcription. In the present study, Western blotting and immunofluorescence staining experiments using specific antibodies against these two phosphorylated threonine residues in

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**FIGURE 7.** HO-1 induces Sp1 phosphorylation at Thr-453 and Thr-739 in vivo. Mice received a myocardial injection of saline, empty Adv, or Adv-HO-1, and then, after 7 days, the hearts were harvested and serial sections were subjected to immunostaining with antibodies against HO-1, VEGF, phospho-Sp1-T453, or phospho-Sp1-T739. Immunostaining for HO-1 or VEGF is shown in brown (×100 magnification), and immunostaining for phospho-Sp1-T453 or phospho-Sp1-T739 in the nuclei of the myocardium is in red (×1000 magnification). The red box outlines the corresponding area showing the immunostains for p-T453 or p-T739.
Sp1 demonstrated increased Sp1 phosphorylation at these residues in cells overexpressing HO-1 or treated with CORM-2. The physiological relevance of Sp1 phosphorylation in VEGF expression was again revealed in immunohistochemical studies on heart sections from mice with HO-1 gene transduction, in which immunostaining for Sp1 phosphorylated at Thr-453 or Thr-739 was detected in nuclei in myocytes. These observations clearly support the in vivo biological significance of Sp1 phosphorylation in the HO-1-mediated induction of VEGF expression. To examine the significance of Sp1 phosphorylation at Thr-453 and Thr-739 in VEGF gene transcription in the context of CO stimulation, we performed transient transfection experiments to assess the effects of WT Sp1 or Sp1 with a T453A or T739A mutation on VEGF gene promoter activation in H9C2 cells following CORM-2 treatment. The results showed that overexpression of the WT Sp1 significantly enhanced CORM-2-induced VEGF promoter activity. T453A or T739A mutation greatly reduced the effect of Sp1 overexpression on CORM-2-induced VEGF promoter activation. Likewise, the Chip assay demonstrated that overexpression of WT Sp1 resulted in a significant increase of CORM-2-induced Sp1 binding to the VEGF promoter, which was diminished by the mutation at Thr-453 or Thr-739. These observations support the importance of Thr-453 and Thr-739 phosphorylations in CO-induced Sp1 binding to VEGF promoter and promoter activation, which is consistent with the previous report showing that phosphorylation of both Thr-453 and Thr-739 is required for maximal VEGF promoter activation induced by Erk (19). It is interesting to note that Sp1 mediates Erk- and p38-dependent transcriptional activation of VEGF via binding to the regulatory sequences located at different region of the promoter. Whether other nuclear factors that are involved in the formation of the stable transcriptional complex with Sp1 at the specific region of the VEGF promoter are subjected to the differential regulation by Erk and p38 remains to be elucidated.

In summary, the present study supports the importance of the p38 kinase-Sp1 axis in HO-1/C0-induced VEGF gene transcription in myocytes. Although both Erk and p38 activate Sp1 through phosphorylation at the same residues, the involvement of different Sp1-binding sites in VEGF promoter for the transcriptional activation highlights the complexity of the transcriptional networks involved in VEGF gene regulation in various cellular contexts. Because HO-1 induction is commonly observed in many pathophysiological states associated with cellular stress and because VEGF is a potent angiogenic factor implicated in neovascularization in various disease states, including ischemic heart disease, the demonstration that HO-1 or CO acts as a biological inducer of VEGF and the mechanistic insights obtained extend our understanding of the pro-angiogenic function of HO-1/CO in vascular biology.