Activator Protein-2α Mediates Carbon Monoxide–Induced Stromal Cell–Derived Factor-1α Expression and Vascularization in Ischemic Heart

Heng-Huei Lin, Yen-Hui Chen, Ming-Tsai Chiang, Pei-Ling Huang, Lee-Young Chau

Objective—Increased cardiac stromal cell-derived factor-1α (SDF-1α) expression promotes neovascularization and myocardial repair after ischemic injury through recruiting stem cells and reducing cardiomyocyte death. Previous studies have shown that heme oxygenase-1 and its reaction byproduct, carbon monoxide (CO), induce SDF-1α expression in ischemic heart. However, the mechanism underlying heme oxygenase-1/CO-induced cardiac SDF-1α expression remains elusive. This study aims to investigate the signaling pathway and the transcriptional factor that mediate CO-induced SDF-1α gene expression and cardioprotection.

Approach and Results—CO gas and a CO-releasing compound, tricarbonyldichlororuthenium (II) dimer, dose-dependently induced SDF-1α expression in primary neonatal cardiomyocytes and H9C2 cardiomyoblasts. Promoter luciferase-reporter assay, electrophoretic mobility shift assay, and chromatin immunoprecipitation demonstrated that the activator protein 2α (AP-2α) mediated tricarbonyldichlororuthenium (II) dimer-induced SDF-1α gene transcription. Tricarbonyldichlororuthenium (II) dimer induced AP-2α expression via protein kinase B (AKT)-dependent signaling. AKT inhibition or AP-2α knockdown reduced tricarbonyldichlororuthenium (II) dimer-induced SDF-1α expression. Coronary ligation induced transient increases of cardiac AP-2α and SDF-1α expression, which were declined at 1 week postinfarction in mice. Periodic exposure of coronary-ligated mice to CO (250 ppm for 1 hour/day, 6 days) resumed the induction of AP-2α and SDF-1α gene expression in infarcted hearts. Immunohistochemistry and echocardiography performed at 4 weeks after coronary ligation revealed that CO treatment enhanced neovascularization in the myocardium of peri-infarct region and improved cardiac function. CO-mediated SDF-1α expression and cardioprotection was ablated by intramyocardial injection of lentivirus bearing specific short hairpin RNA targeting AP-2α.

Conclusions—Our data demonstrate that AKT-dependent upregulation of AP-2α is essential for CO-induced SDF-1α expression and myocardial repair after ischemic injury. (Arterioscler Thromb Vasc Biol. 2013;33:785-794.)

Key Words: activator protein 2α ■ carbon monoxide ■ heme oxygenase-1 ■ ischemic heart ■ stromal cell–derived factor-1α ■ vascularization

The recruitment of circulating bone marrow–derived stem and progenitor cells to ischemic tissues, and the participation of these cells in neovascularization contribute significantly to the repair process. Stromal cell–derived factor-1α (SDF-1α) is one of the key factors implicated in orchestrating stem/progenitor cell homing to injured tissues through interacting with chemokine (C-X-C motif) receptor-4 (CXCR4) receptor.1,2 It was previously shown that SDF-1α is transiently induced in ischemic heart.3,4 Blockade of SDF-1α/CXCR4 interactions markedly suppresses bone marrow–derived cell recruitment to the ischemic heart.4 Conversely, administration or forced overexpression of SDF-1α in ischemic myocardium promotes stem/progenitor cell recruitment, and subsequent neovascularization and tissue regeneration.4,7 Furthermore, SDF-1α promotes cardiomyocyte survival against ischemic insults through paracrine or autocrine SDF-1α/CXCR4 axis.6,7 These findings support the therapeutic potential of SDF-1α in cardiac repair.

Heme oxygenase-1 (HO-1) is a stress–response enzyme catalyzing the oxidative degradation of heme to liberate free iron, carbon monoxide (CO), and biliverdin.5 Earlier studies have shown that HO-1 exerts potent cardioprotective functions by reducing cardiomyocyte death and inflammatory reaction after myocardial ischemia/reperfusion via the antioxidant and anti-inflammatory effects of bilirubin and CO, respectively.5-13 Recently, studies from our group and others also demonstrated that HO-1 promotes neovascularization in ischemic heart.14-16 HO-1 and CO can induce the expressions of vascular endothelial growth factor and SDF-1α, which then promote the recruitment of circulating stem/progenitor cells for neovascularization.14,15 Nonetheless, the molecular mechanism

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underlying the regulation of SDF-1α gene expression by HO-1/CO remains elusive. To this end, here we performed the in vitro experiments to investigate the signaling pathway and the downstream transcriptional factor responsible for CO-induced SDF-1α gene expression in primary cardiomyocytes and H9C2 cardiomyoblasts. We provide evidence indicating that the transcriptional factor, activator protein 2α (AP-2α), is upregulated after CO treatment through protein kinase B (AKT)-dependent signaling pathway and mediates SDF-1α gene expression. The involvement of AP-2α in CO-mediated cardioprotection in vivo was also confirmed by AP-2α gene knockout experiments performed in mice with myocardial infarction (MI).

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
HO-1/CO Induces SDF-1α Expression
To confirm our previous finding that HO-1 overexpression promotes SDF-1α gene induction in myocardium in vivo,14 we performed HO-1 gene transduction experiment in primary neonatal cardiomyocytes and H9C2 cardiomyoblasts. As shown in Figure 1A and 1B, when cells were infected with adenovirus bearing HO-1 gene for 48 hours, SDF-1α gene expression examined by quantitative real-time PCR was significantly higher, as compared with that of control cells without virus infection or cells infected with empty adenovirus. Cotreatment of H9C2 cells with a CO scavenger, hemoglobin (Hb; 100 µmol/L), resulted in a significant reduction of HO-1–induced SDF-1α gene expression (Figure 1B). Moreover, when H9C2 cells were exposed to CO gas or treated with a CO-releasing compound, tricarbonyldichlororuthenium (II) dimer (CORM-2), for 24 hours, SDF-1α gene expression was induced by CO (100 and 250 ppm) or CORM-2 (10–50 µmol/L) dose-dependently (Figure 1C and 1D). Likewise, SDF-1α protein level was significantly increased in the culture media of CORM-2–treated cells (Figure 1A in the online-only Data Supplement).

CO-Induced SDF-1α Expression Requires a New Protein Synthesis
Time course experiment revealed that SDF-1α mRNA and protein were significantly increased at 18 hours, reached a peak at 24 hours, and sustained up to 36 hours after CORM-2 treatment (Figure 1E and Figure 1B in the online-only Data Supplement). Treatment of cells with the protein synthesis inhibitor, cycloheximide (10 µmol/L),17 resulted in a marked reduction of CORM-2–induced SDF-1α expression (Figure IIA in the online-only Data Supplement), suggesting that a new protein synthesis is required for CO-induced SDF-1α gene expression. Because CO can induce HO-1 expression in cardiomyocytes,18 we then examined whether HO-1 is implicated in CORM-2–mediated effect. As shown in Figure IIB in the online-only Data Supplement, CORM-2 induced HO-1 expression in H9C2 cells. Treatment of cells with HO inhibitor, tin protoporphyrin IX, completely abolished HO activity (Figure IIC in the online-only Data Supplement) without significantly affecting the increased SDF-1α expression induced by CORM-2 (Figure IID in the online-only Data Supplement), indicating that HO-1 is not implicated in CO-induced SDF-1α gene expression.

Figure 1. Effect of heme oxygenase-1 (HO-1)/carbon monoxide (CO) on stromal cell-derived factor-1α (SDF-1α) gene expression. A, SDF-1α mRNA levels determined in primary neonatal cardiomyocytes receiving saline or indicated adenovirus vectors for 48 hours. *P<0.05 vs saline-treated group. B, SDF-1α mRNA levels determined in H9C2 cells receiving saline or indicated adenovirus vectors in the absence or presence of 100 µmol/L hemoglobin (Hb) for 48 hours. *P<0.05 vs saline-treated group; and †P<0.01 vs adenovirus bearing HO-1 gene (Adv-HO-1)–treated cells without Hb treatment. H9C2 cells were treated with indicated doses of CO gas (C) or tricarbonyldichlororuthenium (II) dimer (CORM-2) and inactive CORM-2 (50 µmol/L) (D) for 24 hours. *P<0.05 vs untreated control. E, H9C2 cells were treated with CORM-2 (50 µmol/L) for indicated times. *P<0.01 vs zero time point.
AKT Mediates CO-Induced SDF-1α Gene Expression

HO-1/CO induce p38 and AKT phosphorylation in cardiomyocytes. As shown in Figure 2A, treatment of primary cardiomyocytes or H9C2 cells with AKT inhibitor, LY294002, but not by p38 inhibitor, SB203580, markedly suppressed CORM-2–induced SDF-1α gene expression. It has been shown that CO can promote reactive oxygen species (ROS) production via targeting mitochondrial complex IV or NADPH oxidase. To examine whether ROS mediates the activation of AKT induced by CO or CORM-2 in myoblasts, the intracellular ROS levels in H9C2 cells treated with CO gas or CORM-2 were assessed by a redox-sensitive fluorescent dye, CM-H2DCFDA. As demonstrated in Figure IIIA in the online-only Data Supplement, CORM-2 and CO gas induced increase of ROS in H9C2 cells. Moreover, AKT phosphorylation induced by CORM-2 and CO gas was completely blocked by cotreatment with antioxidant (N-acetylcysteine; Figure IIIB in the online-only Data Supplement), indicating that ROS mediated the effect of CO on AKT activation. To further confirm the role of AKT in SDF-1α gene expression, H9C2 cells were transfected with cDNA construct of hemagglutinin-tagged wild-type (WT)-AKT, constitutive active AKT (CA-AKT), or dominant negative AKT (DN-AKT) for 24 hours before treatment with or without CORM-2. As shown in Figure 2B, SDF-1α mRNA was substantially increased in cells overexpressing CA-AKT, but not WT-AKT or DN-AKT. CORM-2–induced SDF-1α expression was prominent in cells overexpressing WT-AKT and CA-AKT, but was suppressed in cells overexpressing DN-AKT. To explore the role of AKT signaling in the transcriptional regulation of SDF-1α gene in CORM-2–treated cells, H9C2 cells were transiently transfected with a luciferase reporter plasmid bearing the rat SDF-1α gene promoter region –547 to +30 relative to the transcriptional initiation site before CORM-2 treatment. As shown in Figure 2C, CORM-2 treatment increased luciferase activity by 3-fold,
which was attenuated by cotreatment with LY2944002, but not SB203580. The basal SDF-1α promoter activity was significantly increased in cells transfected with CA-AKT construct, but not WT-AKT or DN-AKT construct (Figure 2D), whereas CORM-2–induced SDF-1α promoter activity was markedly reduced by cotransfection with DN-AKT construct, but not WT-AKT or CA-AKT (Figure 2D).

**Localization of CO-Regulatory Element in SDF-1α Gene Promoter**

To identify the cis-regulatory element responsible for CO effect, cells were transfected with a series of promoter deletion constructs. It was shown that CORM-2–induced luciferase activity was still observed in construct with region –245 to +30, but no longer in shorter construct with region –86 to +30, indicating that the region –245 to –86 contains the CO-responsive element (Figure 3A). The computational analysis revealed 3 potential binding sites for AP-2α, including ggCCTCggC (–192/–184), gCCggggAC (–173/–165), and gCCgAgggC(–164/–156), within this region. To confirm the importance of these AP-2α–binding sites, we prepared the promoter constructs with mutations in each of these sites for the promoter assay. As shown in Figure 3B, CORM-2–induced luciferase activity of (–245/+30) construct was not significantly affected by the mutations at –192/–184, but partially reduced by the mutations at either –173/–165 or –164/–156 site. When these 2 adjacent AP-2α–binding sites were mutated, CORM-2–induced luciferase activity was completely abolished, indicating that these 2 sites are required to mediate CORM-2–induced SDF-1 gene transcription.

**CO Induces AP-2α Expression Through AKT Activation**

We then performed experiment to investigate whether CORM-2 induces AP-2α expression. As shown in Figure 3C, CORM-2 induced a significant increase of AP-2α mRNA,

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**Figure 3.** Activator protein 2α (AP-2α)-binding sites mediates carbon monoxide (CO)-induced stromal cell-derived factor-1α (SDF-1α) gene promoter activation. H9C2 cells transfected with different 5′-deletional SDF-1α promoter reporter constructs (A) or with the SDF-1α promoter reporter construct with the wild-type (WT) sequence or the indicated nucleotide mutations (B) were treated with dimethyl sulfoxide (DMSO) or tricarbonyldichlororuthenium (II) dimer (CORM-2; 50 μmol/L) for 18 hours before luciferase activity assay. *P<0.05 vs DMSO-treated control. H9C2 cells were treated with CORM-2 (50 μmol/L) for indicated times. The levels of AP-2α mRNAs (C) and proteins (D) were examined by quantitative real-time PCR and Western blot analysis, respectively. *P<0.01 vs zero time point. H9C2 cells were transfected with WT-AKT, constitutive active AKT (CA-AKT), or dominant negative AKT (DN-AKT) construct as indicated for 24 hours, followed by treatment with DMSO or CORM-2 (50 μmol/L) for 12 hours. The levels of AP-2α mRNAs (E) and proteins (F) were examined by quantitative real-time PCR and Western blot analysis, respectively. *P<0.01 vs DMSO–treated cells transfected with WT-AKT; and †P<0.01 vs WT-AKT–transfected cells treated with CORM-2.
which was prominent at 6 hours, reached a peak at 12 hours, and then returned to basal level at 24 hours in H9C2 cells. Western blot analysis revealed a similar time course of AP-2α protein expression in CORM-2–treated cells (Figure 3D and Figure IV in the online-only Data Supplement). Additional experiment demonstrated that CORM-2 failed to induce the expression of hypoxia-inducible factor-1α (HIF-1α; Figure V in the online-only Data Supplement), which has been shown to induce SDF-1α gene expression.21 Experiment was then performed to assess whether AP-2α expression is mediated by AKT activation. Overexpression of hemagglutinin-tagged CA-AKT, but not WT-AKT or DN-AKT, significantly increased AP-2α mRNA and protein expression levels in H9C2 cells (Figure 3E and 3F and Figure IVB in the online-only Data Supplement), whereas CORM-2–induced AP-2α expression was substantially reduced by overexpression of DN-AKT, but not WT-AKT or CA-AKT, as shown in the same figures.

Role of AP-2α in CO-Induced SDF-1α Gene Expression

To examine whether CO induces an increased AP-2α binding to SDF-1 promoter in vivo, we performed the chromatin immunoprecipitation assay in H9C2 cells. In parallel with an increased AP-2α protein expression, CORM-2 induced AP-2α binding to SDF-1α promoter containing the –173/–156 sequence (Figure 4A). To confirm the importance of AP-2α in SDF-1α promoter activation and gene expression, H9C2 cells were transfected with specific small interfering RNA targeting AP-2α. As shown in Figure 4B and 4C, CORM-induced AP-2α expression was markedly reduced. Concomitantly, CORM-2–induced SDF-1α promoter activation and gene expression was substantially suppressed (Figure 4D and 4E). In contrast, transfection with control small interfering RNA had no effect on increased SDF-1α promoter activity and SDF-1 gene expression in CORM-2–treated cells.

Figure 4. Activator protein 2α (AP-2α) regulates stromal cell-derived factor-1α (SDF-1α) gene expression. A, H9C2 cells were incubated without (N) or with tricarbonyldichlororuthenium (II) dimer (CORM-2; 50 μmol/L) for 6 hours, and chromatin immunoprecipitation (ChIP) was performed using control IgG or anti–AP-2α antibody. One tenth of the immunoprecipitate was examined by Western blot analysis with anti–AP-2α antibody, and the rest subjected to DNA extraction and PCR to amplify the region encompassing the corresponding AP-2α-binding sites in the rat SDF-1α promoter. B through E, H9C2 cells were transfected with SDF-1α promoter reporter construct (–547 to +30) together without (N) or with indicated small interfering RNA (siRNA) for 22 hours, followed by treatment with dimethyl sulfoxide (DMSO) or CORM-2 (50 μmol/L) for 18 hours before Western blot analysis to examine AP-2α protein expression (B and C), luciferase activity assay (D), and quantitative real-time PCR to determine SDF-1α mRNA level (E). *P<0.05 vs DMSO-treated cells; and †P<0.01 vs CORM-2–treated cells transfected with control siRNA.
CO Promotes AP-2α/SDF-1α Expressions in Ischemic Heart

To investigate whether AP-2α has a role in SDF-1α expression in ischemic heart, we examined the expressions of AP-2α and SDF-1α in hearts of C57BL/6J mice subjected to coronary ligation. As shown in Figure 5A, significant increases of AP-2α and SDF-1α expressions were detected at day 2, peaked at day 4, and then declined at day 7, after the induction of MI. To explore whether CO can resume AP-2α and SDF-1α gene inductions after their declines in the ischemic heart, C57BL/6J mice with coronary ligation for a week were exposed to 250 ppm CO gas for 1 hour per day for 6 consecutive days. As shown in Figure VIA and VIB in the online-only Data Supplement, coronary ligation resulted in increased AKT phosphorylation in hearts, which was further enhanced by CO treatment. Likewise, both mRNA and protein levels of cardiac AP-2α and SDF-1α determined at the end of the last CO exposure were significantly higher as compared with those of untreated counterparts (Figure 5B and Figures VIC, VID, and VIIIA in the online-only Data Supplement). When we performed the immunostaining on heart sections with antibody against c-kit, the marker for progenitor cells, the results showed that coronary ligation resulted in significant recruitment of c-kit+cells to the infarcted heart (Figure VIIA and VIB in the online-only Data Supplement). CO treatment further promoted the recruitment of these cells.

Figure 5. Carbon monoxide (CO) inhalation promotes activator protein 2α (AP-2α)/stromal cell-derived factor-1α (SDF-1α) expression and myocardial repair. A, B6 mice were subjected to sham operation (0 day) or coronary ligation for indicated days. The hearts were collected and AP-2α and SDF-1α mRNA levels were determined (n=4/group). *P<0.02 vs sham-operated group. B, B6 mice were subjected to sham operation (n=10) or coronary ligation (n=20) for a week. Ten of the infarcted mice were exposed to 250 ppm CO gas for 1 hour per day for 6 days. Five mice from each treated group were euthanized, and the mRNA levels of AP-2α and SDF-1α in heart tissues were examined. *P<0.01 vs sham-operated group; and †P<0.01 vs ligation alone. C, The cardiac functions of the remaining mice were assessed by echocardiography at 4 weeks. The quantitative results on left ventricular internal dimension (LVID) and percentage of fraction shortening (FS) determined (n=5/group). *P<0.01 vs sham-operated group; and †P<0.05 vs ligation alone. D, The Masson trichrome-stained heart sections. Bar indicates 1 mm. The quantitative results of infarct size (E) and collagen content (F) in various treated groups (n=5/group) were determined. *P<0.01 vs sham-operated group; and †P<0.01 vs ligation alone. G, The CD31 immunostaining in the peri-infarct regions. Bar indicates 100 μm. H, The quantitative results on vascular density determined (n=5/group). *P<0.01 vs sham-operated group; and †P<0.01 vs ligation alone.
Immunostaining performed with antibodies against Gr-1 and CD11b, the markers for neutrophils and macrophages, respectively, revealed that CO treatment significantly reduced the infiltration of these inflammatory cells in the infarcted hearts (Figure VII–VIIIF in the online-only Data Supplement). When the cardiac functions of the infarcted mice were assessed at 4 weeks post-MI, the results showed that MI-induced ventricular dilatation and functional impairment were significantly attenuated by the short-term periodic CO treatment (Figure 5C and Table I in the online-only Data Supplement). Consistently, histological examination of the heart sections revealed that the infarct size and fibrotic area were much smaller in CO-treated infarcted mice as compared with untreated counterparts (Figure 5D–5F). Immunostaining experiment performed with a specific antibody against CD31, an endothelial marker, demonstrated higher vascular density in the peri-infarcts of CO-treated mice (Figure 5G and 5H). The cardioprotective effect was also observed in mice receiving 6 days of intravenous administration of CORM-2 (4 mg/kg of body weight) starting at the second week after the coronary ligation (Figure IX in the online-only Data Supplement). Moreover, CO-mediated cardiac protection was also observed in coronary-ligated HO-1 knockout mice (Figure X in the online-only Data Supplement), indicating that the effect of CO is not dependent on HO-1 expression.

**AP-2α Knockdown Ablates CO-Induced Cardioprotection**

To investigate the role of AP-2α in CO-induced SDF-1α expression and cardiac protection post-MI, we performed the in vivo gene knockdown experiment using lentivirus bearing specific short hairpin RNA (shRNA) targeting AP-2α (AP-2α shRNA). We first examined the lentivirus-mediated gene knockdown efficiency in vivo using lentivirus bearing specific shRNA targeting lamin A/C. When animals were subjected to intramyocardial injection of the negative control (–shRNA) or lamin A/C-shRNA lentivirus (1×10^9 virus particles/mouse) for 7 days, the lamin A/C expression in the ventricular tissues of mice treated with lamin A/C-shRNA lentivirus was significantly reduced as compared with control counterparts (Figure XI in the online-only Data Supplement). We also examined the infection efficiency of lentivirus in infarcted heart using the lentivirus bearing green fluorescent protein construct. When the animal was subjected to coronary ligation, followed by the injection of the green fluorescent protein-lentivirus (1×10^9 virus particles) at 5 different sites around the peri-infarct region for 7 days, the green fluorescent protein expression was evident in the myocardium of the peri-infarct region (Figure XII in the online-only Data Supplement). We then performed the experiments by injecting the (–)shRNA or AP-2α shRNA lentivirus around the peri-infarct region of coronary ligated mice. After 1 week, each treated group of mice was randomly divided into 2 subgroups with 1 subgroup remaining in normal air condition and another subgroup of mice being exposed to periodic CO gas treatment as described above. As shown in Figure 6A and 6B, CO-induced AP-2α protein expression in the ventricular tissues of the infarcted mice was significantly reduced in mice treated with AP-2α shRNA, but not with (–)shRNA. In parallel, CO-induced SDF-1α mRNA (Figure 6C) and protein (Figure VIIIIB in the online-only Data Supplement) expressions were also abolished in mice treated with AP-2α shRNA. Echocardiography examination of these mice at 4 weeks after MI revealed that the beneficial effect of CO on the ventricular dilatation and the cardiac performance was diminished in the group receiving AP-2α shRNA treatment (Figure 6D and Table II in the online-only Data Supplement). Likewise, the reduced infarct size and fibrosis observed in CO-treated infarcted mice were attenuated by AP-2α shRNA treatment (Figure 6E and 6F). AP-2α knockdown also abolished the enhanced neovascularization in CO-treated mice (Figure 6G). However, the suppressive effect of CO on the inflammatory cell infiltration in infarcted heart was not significantly affected by AP-2α knockdown (Figure XIII in the online-only Data Supplement).

**Discussion**

SDF-1α plays a crucial role in tissue repair after ischemic injury. However, the transient expression of SDF-1α observed after the induction of MI is not sufficient for myocardial repair. Increasing our understanding of the mechanism underlying the regulation of SDF-1α expression in cardiomyocytes will lay the ground for translational research that results in better treatment of ischemic heart disease. Hypoxia has been shown to induce SDF-1α expression through the activation of HIF-1α, which is believed to participate in the upregulation of SDF-1α in the ischemic heart. However, the potential involvement of other signaling pathways and transcriptional factors in the regulation of cardiac SDF-1α expression has not been fully explored. We previously reported that HO-1 overexpression in ischemic heart promotes SDF-1α gene expression, which then promotes neovascularization in infarcted mice. The involvement of HO-1 in the regulation of SDF-1α expression was further substantiated by the observation that the level of transient cardiac SDF-1α gene expression post-MI is much lower in HO-1−/− mice than in WT mice. These findings support that HO-1 can induce SDF-1α expression under normoxia condition. Here, we showed that CO mediates HO-induced SDF-1α gene expression in cardiomyocytes and H9C2 cardiomyoblasts in vitro. Notably, CO-induced SDF-1α expression required a new protein synthesis. This observation may explain the late induction of SDF-1α expression, which was not evident until 18 hours after CO treatment. Although CO has been shown to exert beneficial effect on several disease settings by inducing HO-1 expression, our data showed that HO-1 is not required for CO-induced SDF-1α expression and cardioprotection. Nevertheless, considering that HO-1 can exert cardioprotective effects via multiple pathways, the possibility for CO-induced HO-1 to act together with CO to confer further protection in the infarcted heart cannot be completely ruled out. Moreover, HIF-1α has been shown to regulate SDF-1α expression after vascular injury under normoxia condition. Studies by others have also shown that CO can increase the stabilization and translational stimulation of HIF-1α in macrophages and astrocytes. However, we did not detect significant increase in HIF-1α expression.
level in CO-treated H9C2 cells, indicating that the effect of CO on HIF-1α expression is cell type–specific and unlikely responsible for SDF-1α gene induction in the present context. By performing SDF-1α gene promoter assay, we identified 2 adjacent AP-2α–binding sites in the proximal promoter region of SDF-1α gene as the cis-regulatory element responsible for CO-induced SDF-1α promoter activation. Chromatin immunoprecipitation assay also revealed the increase of AP-2α protein binding to the SDF-1α gene promoter after CO treatment. Moreover, AP-2α expression was significantly induced by CO via AKT-dependent pathway. Additional experiment demonstrated that CORM-2 and CO gas induced an increase of intracellular ROS, which mediated AKT activation in myoblasts. This observation was consistent with previous findings by others. The functional importance of AKT–AP-2α axis in SDF-1α gene expression again was evidenced by the experiment showing that AKT inhibition or small interfering RNA–mediated AP-2α gene knockdown significantly reduced CO-induced SDF-1α promoter activity and SDF-1α gene expression, whereas overexpression of CA-AKT or AP-2α was sufficient to induce SDF-1α gene transcription in H9C2 cells. Collectively, these results support the role of ROS/AKT signaling in sequential expression of AP-2α and SDF-1α in CORM-2/CO-treated myoblasts.

AP-2α is a member of the helix-span-helix transcription factors implicated in proliferation and differentiation of various cell types during development. AP-2α is a member of the helix-span-helix transcription factors implicated in proliferation and differentiation of various cell types during development. 

Figure 6. Lentivirus-mediated activator protein 2α (AP-2α) knockdown abrogates carbon monoxide (CO)-induced stromal cell–derived factor-1α (SDF-1α) gene expression and cardiac repair. B6 mice underwent coronary ligation and received intramyocardial injection of saline or lentivirus bearing negative short hairpin RNA (−) shRNA or specific shRNA targeting AP-2α (AP-2α shRNA) in the infarct borders (n=20/group). A, After 1 week, each treated group of mice was randomly divided into 2 subgroups with 1 subgroup periodically exposed to CO gas (250 ppm, 1 hour/day) for 6 days. After the last CO treatment, 5 mice from each subgroup were euthanized. AP-2α protein expression level was determined by Western blot analysis. Data shown are the representative results of 3 mice in each group. B, AP-2α protein levels were quantified by densitometry. *P<0.02 vs sham group; and †P<0.05 vs CO-treated control group. C, SDF-1α mRNA levels in ventricular tissues determined. *P<0.01 vs normal air-treated group infected with same lentivirus; and †P<0.01 vs CO-treated control group. D, At 4 weeks, the cardiac functions of the remaining mice were assessed by echocardiography. The quantitative results on left ventricular internal dimension (LVID) and fraction shortening (FS) were determined. *P<0.01 vs normal air-treated group infected with same lentivirus; and †P<0.01 vs CO-treated control group. After animals were euthanized, the infarct sizes (E), collagen content (F), and vascular densities (G) in various treated groups were assessed. *P<0.05 vs normal air-treated group infected with same lentivirus; and †P<0.05 vs CO-treated control group.
is predominant in the developing embryo, and its deficiency results in the defects of cranial closure and craniofacial development. Studies have shown that AP-2α is highly induced in various cancers and has a role in tumorigenesis. However, little is known about its role in cardiovascular diseases. Similar to the time course of SDF-1α expression, AP-2α was highly induced within the first few days and then returned to basal level at 7 days after the induction of MI. When we exposed mice that were subjected to coronary ligation for a week to a periodic CO exposure for 6 days, the expression levels of both AP-2α and SDF-1α in the ischemic hearts were significantly reduced. Moreover, the echocardiography performed at 4 weeks after the induction of MI revealed that the cardiac performance was much improved in CO-treated mice, which was associated with increased neovascularization in the infarcted hearts of the same group of mice. Consistent with the present finding, an early study also showed that treatment with CO donor alleviates cardiac remodeling in infarcted mice, To further confirm that the AP-2α-mediated SDF-1α gene induction is crucial to CO-induced cardioprotection, we performed the in vivo gene knockdown experiment in coronary-ligated mice. The results showed that CO-induced AP-2α, as well as SDF-1α expressions were significantly reduced in mice receiving the myocardial administration of lentivirus bearing AP-2α-shRNA, but not lentivirus bearing control shRNA. Likewise, functional and pathohistological assessments revealed that CO-mediated cardiac repair was substantially attenuated by AP-2α knockdown. These results provide the convincing evidence in support of the proangiogenic and anti-inflammatory effects. Notably, AP-2α knockdown attenuated enhanced neovascularization but had no effect on the reduction of inflammatory cells in CO-treated infarcted heart, indicating that the proangiogenic and anti-inflammatory effects of CO are mediated by distinct pathways. Our data showed that attenuation of neovascularization significantly hampered CO-mediated cardioprotection even in the condition that the inflammatory reaction remained suppressed. These observations highlight the importance of enhanced neovascularization in cooperation with reduced inflammation in cardiac repair post-MI.

The importance of SDF-1α/CXCR4 axis in myocardial repair is well documented. Nevertheless, a recent study using CXCR4 heterozygous mice has revealed the double-edged role of SDF-1α/CXCR4 axis in the recruitment of both inflammatory and progenitor cells post-MI. Although the infarct size was reduced in CXCR4−/− mice compared with WT mice as a result of the attenuation of acute recruitment of inflammatory cells, the ventricular function was not improved in these mice because of the decreased neovascularization and coronary flow recovery after MI. Apparently, a therapeutic strategy that can modulate a temporal activation SDF-1α/CXCR4 axis after the acute inflammatory phase would provide better outcomes. Our findings suggest the possibility of using CO for the timely induction of SDF-1α to treat ischemic heart disease.

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**Disclosures**

None.

**References**


Significance

A timely cardiac stromal cell–derived factor-1α (SDF-1α) expression is crucial for cardiac repair postmyocardial infarction (MI). The present study demonstrates that carbon monoxide (CO), a byproduct derived from heme degradation catalyzed by heme oxygenase-1 (HO-1), is capable of inducing SDF-1α gene expression through AKT–activator protein 2α (AP-2α) axis in cardiomyocytes, both in vitro and in vivo. Moreover, a periodic short-term treatment of infarcted mice with CO gas or CO-releasing compound promotes neovascularization and cardiac repair. These findings support the possibility of using CO as an effective and timely therapeutic strategy for treating ischemic heart disease.
Activation Protein-2α Mediates Carbon Monoxide–Induced Stromal Cell–Derived Factor-1 α Expression and Vascularization in Ischemic Heart
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Supplemental Figure I. CORM-2 induces SDF-1α protein expression. (A) H9C2 cells were treated with DMSO or indicated concentrations of CORM-2 for 24 h. (B) Cells were treated with 50 μmol/L of CORM-2 for indicated times. The levels of SDF-1α protein accumulated in the cell culture media were determined by ELISA. *P< 0.01 vs untreated control cells.
Supplemental Figure II. HO-1 is not involved in CORM-2-induced SDF-1α expression.  (A) H9C2 cells were treated with DMSO or CORM-2 (50 μmol/L) in the absence or presence of 10 μmol/L cycloheximide (CHX) for 24 h.  The SDF-1α mRNA levels were then determined by quantitative real-time RT-PCR.  *P< 0.01 vs DMSO-treated cells; †P< 0.01 vs cells treated with CORM-2 alone.  Cells were treated with DMSO or CORM-2 in the absence or presence of tin-protoporphyrin IX (SnPP) (10 μmol/L) for 24 h.  The HO-1 protein level (B) and HO activity (C) were determined.  *P< 0.01 vs DMSO-treated cells; †P< 0.01 vs cells treated with CORM-2 alone.  (D) Cells were treated with DMSO or CORM-2 in the absence or presence of SnPP as described above.  The SDF-1α mRNA levels were then determined by quantitative real-time RT-PCR.  *P< 0.01 vs DMSO-treated cells.
Supplemental Figure III. ROS mediates AKT activation induced by CORM-2 and CO gas. (A) H9C2 cells were preloaded with CM-H2DCFDA, and then treated without (Cont) or with CORM-2 (50 μmol/L) or CO gas (250 ppm) as indicated in the absence or presence of 5 mmol/L N-acetylcysteine (NAC) for 30 min. The fluorescence was then examined by fluorescent microscope. Bar, 20 μm. (B) Cells were treated without (N) or with CORM-2 or CO gas as indicated in the absence or presence of NAC for 30 min. The level of AKT phosphorylation was examined by Western blot analysis.
Supplemental Figure IV. AKT mediates CORM-2-induced AP-2α protein expression. (A) H9C2 cells were treated with CORM-2 ((50 μmol/L) for indicated times. The protein expression levels of AP-2α were examined by Western blot analysis and quantified by densitometry. Data shown are mean ± SD of three independent experiments. *P<0.05 vs zero time point. (B) Cells were transfected with WT-AKT, CA-AKT or DN-AKT construct as indicated for 24 h, followed by treatment with DMSO or CORM-2 (50 μmol/L) for 12 h. AP-2α protein expression levels were examined by Western blot analysis and quantified by densitometry. Data shown are mean ± SD of three independent experiments. *P<0.01 vs DMSO-treated cells transfected with WT-AKT; †P<0.01 vs WT-AKT-transfected cells treated with CORM-2.

Supplemental Figure V. CORM-2 fails to induce HIF-1α expression. H9C2 cells were treated with CORM (50 μmol/L) or CoCl₂ (100 μmol/L) for indicated times. The expression levels of AP-2α and HIF-1α were then examined by Western blot analysis.
Supplemental Figure VI. CO inhalation induces AKT phosphorylation and AP-2α expression in infarcted heart. B6 mice were subjected to sham operation (n=3) or coronary ligation (n=6) for a week. Half of the infarcted mice were exposed to 250 ppm CO gas for 1 h per day for 6 days. The ventricular tissue lysates were then prepared and the phosphorylation state of AKT (A) and AP-2α expression level (C) were examined by Western blot analysis and quantified by densitometry (B & D), respectively. *P<0.05 vs sham control; †P< 0.05 vs ligation alone.
Supplemental Figure VII. CO inhalation promotes progenitor cell recruitment but suppresses inflammatory cells in infarcted heart. B6 mice were subjected to sham operation or coronary ligation for a week. Half of the infarcted mice were exposed to 250 ppm CO gas for 1 h per day for 6 days. Heart sections were prepared and immunostaining experiments were performed with specific antibodies against c-kit (A & B), CD11b (C & D) and Gr-1 (E & F), respectively. A, C, & E are the representative images taken from the peri-infarcted regions. Bar, 100 μm. B, D & F are the corresponding quantitative results (n=5/group). *P<0.01 vs sham control; †P<0.05 vs ligation alone.
Supplemental Figure VIII. AP-2α mediates CO-induced SDF-1 expression. (A) B6 mice were subjected to sham operation or coronary ligation for a week. Half of the infarcted mice were exposed to 250 ppm CO gas for 1 h per day for 6 days. Ventricular tissue lysates were prepared and the amounts of SDF-1 protein levels were determined by ELISA (n=5/group). *P<0.05 vs sham control; †P< 0.05 vs ligation alone. (B) B6 mice underwent coronary ligation and received intramyocardial injection of saline or lentivirus bearing negative shRNA ((-) shRNA) or specific shRNA targeting AP2α (AP-2α shRNA) in the infarct borders (n=10/group). After one week, each treated group of mice were randomly divided into two subgroups with one subgroup periodically exposed to CO gas (250 ppm, 1 h/day) for 6 days. After the last CO treatment, five mice from each subgroup were sacrificed. SDF-1 protein expression levels in ventricular tissues were determined by ELISA (n=5/group). *P< 0.05 vs normal air-treated group infected with same lentivirus; †P< 0.05 vs CO-treated control group.
Supplemental Figure IX. CORM-2 treatment promotes cardioprotection. B6 mice were subjected to sham operation or coronary ligation for 7 days. The infarcted mice then received i.v injection of saline, CORM-2 or iCORM-2 (4mg / kg body weight) as indicated per day for a period of 6 days (n = 4/group). At 4 weeks after coronary ligation, all mice were subjected to echocardiography and then sacrificed. (A) The quantitative results on percent of fraction shortening (FS) and left ventricular internal dimension (LVID) determined. *P< 0.05 vs saline-treated coronary-ligated group. (B) The representative images of Masson trichrome-stained heart sections of various treated groups. Bar, 1 mm. The quantitative results of infarct sizes (C) and collagen contents (D) in various treated groups were determined . *P< 0.05 vs saline-treated coronary-ligated group.
Supplemental Figure X. CO inhalation promotes cardioprotection in HO-1−/− mice. WT and HO-1−/− mice were subjected to sham operation or coronary ligation for 7 days. Half of the coronary ligated mice were exposed to 250 ppm CO gas for 1 h per day for 6 days. At 4 weeks after coronary ligation, all mice were subjected to echocardiography and then sacrificed. (A) The quantitative results of percent of fraction shortening (FS) and left ventricular internal dimension (LVID) determined (n=3/group). *P< 0.05 vs coronary-ligated WT group; †P<0.05 vs ligation alone group of same genotype. (B) The representative images of Masson trichrome-stained heart sections of various groups. Bar, 1 mm. The quantitative results of infarct sizes (C) and collagen contents (D) in various groups were determined . *P< 0.05 vs coronary-ligated WT group; †P<0.05 vs ligation alone group of same genotype.
Supplemental Figure XI. Lentivirus-mediated specific gene knockdown in heart.
Lentiviral vector bearing control shRNA or Lamin A/C shRNA \((1 \times 10^9\) virus particles/mouse) plus 10 ng of substance P was injected at five different sites of left ventricular myocardium of B6 mice \((n = 3/group)\). After 7 days, the left ventricular tissues were harvested and Lamin A/C protein expression levels were determined by Western blot analysis.

Supplemental Figure XII. Lentivirus-mediated GFP expression in infarcted heart.
C57BL/6J mice underwent coronary ligation, followed by injection of saline or lentivirus bearing GFP construct \((1 \times 10^9\) virus particles/mouse) at 5 different sites around the infarct borders. After 7 days, animals were sacrificed and the heart cryosections were examined for GFP expression. Magnification: 10X (A); 100X (B); Bar: 1 mm (A); 100 μm (B).
Supplemental Figure XIII. Lentivirus-mediated AP2α knockdown does not affect decreased inflammatory cell infiltration in CO-treated infarcted heart. B6 mice underwent coronary ligation and received intramyocardial injection of saline or lentivirus bearing negative shRNA ((-) shRNA) or specific shRNA targeting AP2α (AP-2α shRNA) in the infarct borders (n=20/group) (A) After one week, each treated group of mice were randomly divided into two subgroups with one subgroup periodically exposed to CO gas (250 ppm, 1 h/day) for 6 days. After the last CO treatment, 5 mice from each subgroup were sacrificed. Heart sections were prepared and immunostaining experiments were performed with specific antibodies against CD11b (A) and Gr-1 (B), respectively. Data shown are the quantified results (n=5/group). *P< 0.05 vs normal air-treated group infected with same lentivirus.
Supplemental Table I. Echocardiographic measurements of infarcted mice with or without CO treatment.

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
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<th>Ligation+CO</th>
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<tbody>
<tr>
<td>Heart rate</td>
<td>451.1±8.5</td>
<td>447.6±8.7</td>
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<td>LV mass/BW (mg/g)</td>
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<td>LVIDd (mm)</td>
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<td>LVIDs (mm)</td>
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<td>EF%</td>
<td>72.3±2.5</td>
<td>30.7±1.9*</td>
<td>47.2±2.4†</td>
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<tr>
<td>FS%</td>
<td>42.3±1.5</td>
<td>16.5±1.3*</td>
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<tr>
<td>Stroke volume (µl)</td>
<td>67.3±2.2</td>
<td>39.5±3.9*</td>
<td>52.2±3.6†</td>
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<td>Cardiac output (ml)</td>
<td>30.3±0.9</td>
<td>17.6±1.6*</td>
<td>23.3±1.9†</td>
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</table>

B6 mice were subjected to sham operation or coronary ligation for a week. Half of the infarcted mice were exposed to 250 ppm CO gas for 1 h per day for 6 days. The cardiac functions were then assessed by echocardiography at 4 weeks after coronary ligation. The number of mice in each group was five. LVIDd, left ventricular internal dimension at the end of diastole; LVIDs, left ventricular internal dimension at the end of systole; EF%, percent of ejection fraction; FS%, percent of fraction shortening. *P<0.05 vs sham-operated group; †P<0.05 vs ligation alone.
Supplemental Table II. Echocardiographic measurements of infarcted mice with or without AP-2α knockdown

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Control</th>
<th>(−)shRNA</th>
<th>AP-2α shRNA</th>
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<td></td>
<td>Ligation</td>
<td>Ligation/CO</td>
<td>Ligation</td>
<td>Ligation/CO</td>
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<tr>
<td>Heart rate</td>
<td></td>
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<td></td>
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<tr>
<td>B6 mice</td>
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<td>LV mass/BW (mg/g)</td>
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<tr>
<td>LVIDs(cm)</td>
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<td>EF%</td>
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<tr>
<td>FS%</td>
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<td>Stroke volume (μl)</td>
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<td>45.8 ± 2.8</td>
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<tr>
<td>Cardiac output (ml)</td>
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<td>20.2 ± 1.1</td>
<td>27.8 ± 1.3</td>
<td>19.3 ± 3.8</td>
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</table>

B6 mice underwent coronary ligation and received intramyocardial injection of saline (control) or lentivirus bearing negative shRNA (−) shRNA) or specific shRNA targeting AP2α (AP-2α shRNA) in the infarct border. After one week, half of the infarcted mice in each treated group were exposed to 250 ppm CO gas for 1 h per day for 6 days. The cardiac functions were then assessed by echocardiography at 4 weeks after coronary ligation. The number of mice in each group was five. LVIDd, left ventricular internal dimension at the end of diastole; LVIDs, left ventricular dimension at the end of systole; EF%, percent of ejection fraction; FS%, percent of fraction shortening. *P < 0.05 vs normal air-treated control group #P < 0.05 vs normal air-treated group infected with same lentivirus.
MATERIALS AND METHODS

DNA constructs
Adenovirus (Adv) bearing human HO-1 cDNA was prepared as described previously. A fragment of rat SDF-1 5′-flanking sequence encompassing -547 to +30 (-547/+30) relative to the transcription initiation site was obtained by polymerase chain reaction (PCR) using rat genomic DNA isolated from H9C2 cells as the template and subcloned into the KpnI/XhoI restriction site of the pGL2-basic vector. A series of deletion constructs containing -245/+30, -86/+30, -51/+30 bp of 5′-flanking sequence were also prepared by PCR using the longest fragment (-547/+30) as the template. The constructs containing the mutations were generated by PCR-based mutagenesis. A cDNA fragment encoding the full length AP2α was generated by reverse transcription (RT)-PCR using RNA isolated from HeLa cells and subcloned into pFLAG-CMV-2 expression vector. The identity and orientation of the subcloned DNA fragments were verified by DNA sequencing. Hemagglutinin (HA)-tagged wild type (WT)-, constitutive active (CA)- and dominant negative (DN)-AKT constructs were provided by Dr. JY Chen in Institute of Biomedical Sciences, Academia Sinica. The lentiviral vectors bearing mouse Lamin A/C specific shRNA (target sequence 5′-GCTTGACTTCCAGAAGAACAT-3′), mouse AP-2α specific shRNA (target sequence 5′-GAGTTGCTTGACCCACTTCAA-3′) and negative control shRNA (target sequence 5′-CAAATCACAGAATCGTCGTAT-3′) were provided by the National RNAi Core Facility of Academia Sinica, Taiwan.

Cell culture
Rat embryonic myoblast H9C2 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 100 units/ml of penicillin, and 100 μg/ml of streptomycin. Neonatal cardiomyocytes were isolated from Sprague-Dawley rats (1-2 days old) and cultured in DMEM containing 10% FBS, and 0.1 mmol/L bromodeoxyuridine as described previously. For virus infection, H9C2 cells or primary cardiomyocytes (4 x 10^5) in 3.5-cm dishes were incubated with 100 MOI of Adv or Adv-HO-1 in serum free medium for 2 h, followed by incubation in medium containing 5% FBS for additional 22 h. For CO exposure, cells cultured in 3.5-cm dishes were changed to medium containing 1% FBS and placed in a chamber containing indicated concentrations of CO or treated with indicated concentrations of tricarbonyldichlororuthenium (II) dimer (CORM-2) for various
The inactive CORM-2 (iCORM-2) was prepared by pre-incubation in DMEM at 37°C overnight prior to use.

**Quantitative reverse-transcription-polymerase chain reaction (RT-PCR)**

Total RNA was extracted using Trizol reagent and 2 μg of RNA was reverse transcribed to cDNA using Superscript II reverse transcriptase (18080-051, Invitrogen). Real-time PCR was performed with LightCycler® FastStart DNA MasterPLUS SYBR Green reagent I (03515885001, Roche Applied Science). The primer sequences used for amplification of rat SDF-1 were: sense, 5’-ATGGACGCCAAGGTCG-3’; and antisense, 5’-CGGGTCAATGCACACT-3’. For rat AP-2α were: sense, 5’-AGTCTAAGAATGGAGGGC-3’; and antisense, 5’-TGGGAATGTGGTGCGGT-3’. For rat GAPDH were: sense, 5’-ACTCCATTCACTCCACCTTTT-3’; and antisense, 5’-TTACTCCTGGAGGCCATGT-3’. The primer sequences used for amplification of mouse SDF-1 were: sense, 5’-CCTCCCAACCATTGT-3’; and antisense, 5’-CGGAAGGCGTTTATGC-3’. For mouse AP-2α were: sense, 5’-CTAAACCACAGCATTCC-3’; and antisense, 5’-GTACGTGTTTTGGCG-3’. For mouse GAPDH were: sense, 5’-TGAAGGCGTTGTAACGCAATTG-3’; and antisense, 5’-TCTCGTGTTTACACCCATCA-3’.

**ELISA**

SDF-1α protein level in cell culture medium was determined using SDF-1α Quantikine ELISA kit (MCX120, R & D systems) according to the manufacturer’s instruction. To determine SDF-1α level in heart tissue, the left ventricular tissue was homogenized with buffer containing 50 mmol/L Tris-HCl pH 7.4, 100 mmol/L NaCl, 1% NP-40, 0.1% SDS and protease inhibitor cocktail (539134, Calbiochem), followed by centrifugation at 14,000 ×g for 10 min at 4°C. Supernatant was removed and used for assay.

**HO activity measurement**

H9C2 cells were treated with or without 50 μmol/L of CORM-2 in the absence or presence of tin protoporphyrin IX (SnPP) (10 μmol/L) for 24 h. Cells were then rinsed twice with ice-cold phosphate-buffered saline (PBS) and harvested by centrifugation at 1,000 x g for 5 min at 4°C. The whole cell lysate was prepared and HO activity was determined as described previously. Briefly, 500 μg of protein lysates were incubated in 200 μl of buffer containing 0.1 mol/L K₃PO₄, pH 7.4, 1.5 mg of rat liver cytosol, 50 μmol/L hemin, 1 mmol/L
NADPH, 2 mmol/L glucose-6-phosphate and 0.3 units of glucose-6-phosphate dehydrogenase. The mixture was incubated in the dark for 1 h at 37 °C. Bilirubin was then extracted with 1 ml chloroform and determined by the absorbance difference between 464 and 530 nm with an extinction coefficient of 40/mM*cm.

**Intracellular reactive oxygen species (ROS) detection**

The intracellular ROS was detected using the fluorescent probe 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H$_2$DCFDA) (C6827, Molecular Probes). Briefly, H9C2 cells were loaded with 10 μmol/L CM-H$_2$DCFDA in the dark for 1 h at 37°C. Cells were washed once with Hank's balanced salt solution, incubated in prewarmed growth medium for additional 30 min at 37°C, and examined by fluorescence microscope.

**Western blot analysis**

Cultured cells were rinsed twice with ice-cold PBS and directly lysed in 1 x SDS sample buffer. For the preparation of tissue lysates, the left ventricular tissue was homogenized with buffer containing 50 mmol/L Tris-HCl pH 7.4, 100 mmol/L NaCl, 10 mmol/L NaF, 1 mmol/L Na$_3$VO$_4$, 1 mmol/L Na$_4$P$_2$O$_7$, 1% NP-40, 0.1% SDS, and protease inhibitor cocktail, followed by centrifugation at 14,000 ×g for 10 min at 4 °C. The supernatant was removed and the protein concentration determined. Protein samples were subjected to electrophoresis on 10% SDS-polyacrylamide gels, and transblotted onto polyvinylidene difluoride membrane (Millipore Corporation). The membranes were blocked with 5% nonfat milk in PBS containing 0.1% Tween 20 (PBST) at room temperature for 1 h, followed by incubation with anti-HO-1, anti-AP-2α (ab52222, Abcam); anti-AKT (9272, Cell Signaling Technology), anti-phospho AKT (9271S, Cell Signaling Technology); anti-HIF-1α (sc-53546, Santa Cruz); or anti-GAPDH (sc-25778, Santa Cruz) antibody diluted in PBST buffer containing 1% nonfat milk at room temperature for another 1 h. After three washes with PBST buffer, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody diluted in PBST containing 1% nonfat milk at room temperature for 1 h, followed by three washes with PBST. Antigens were then detected using enhanced chemiluminescence system (Pierce).

**Promoter assay**
Promoter assay was performed as described previously. Briefly, H9C2 cells seeded in 12-well plates at a density of $8 \times 10^4$ cells/well were transfected in triplicates with 1μg of tested promoter construct and 0.1μg of pRL-TK plasmid carrying renilla luciferase gene as an internal control using Lipofectamine 2000. After 22 h, cells were changed to medium containing 1 % FBS and treated with 50 μmol/L of CORM-2 for additional 18 h. Cell lysates were then prepared and both firefly and renilla luciferase activities were measured using dual-luciferase reporter assay system (E1910, Promega). In some experiments, H9C2 cells were transfected with plasmids bearing various AKT and AP2α constructs, or siRNA as indicated together with the promoter constructs for 22 h prior to CORM-2 treatment and luciferase assay.

**SiRNA knockdown experiment**
The specific siRNA targeting rat AP-2α ( sense sequence #1 :5'-CCAGAUAACUGUAUUAtt-3'; sense sequence #2: 5'-GGGCGAUUUAAGAAAtt-3') and a control siRNA ( sequence : 5'-UUCUGAAGCUGUCAGUtt-3') were synthesized by MD Bio Inc. Taiwan. For promoter assay, H9C2 cells seeded in 12-well plates at a density of $8 \times 10^4$ cells/well were transfected with 20 pmol of AP-2α siRNA or control siRNA together with the tested promoter constructs as described above. For Western blot analysis and quantitative RT-PCR experiments, H9C2 cells seeded in 6-cm dishes at a density of $5 \times 10^5$ cells/dish were transfected with 100 pmol of AP-2α siRNA or control siRNA with Lipofectamine 2000 for 22 h. Cells were then treated with 50 μmol/L of CORM-2 for 18 h prior to further experiments.

**Chromatin immunoprecipitation (ChIP) assay**
ChIP was performed using an EZ ChIP™ kit (17-371, Upstate). Briefly, H9C2 cells ($6.5 \times 10^6$) were treated with or without 50 μmol/L CORM-2 for 1 h at 37°C, followed by incubation with 1% formaldehyde for 10 min at room temperature. The reaction was then quenched with 0.125 mol/L glycine and washed twice with ice-cold PBS. The cells were resuspended in 300 μl of lysis buffer (50 mmol/L Tris-HCl, pH 8.1, 10 mmol/L EDTA, 1% SDS, and protease inhibitor cocktail).and sonicated using a Misonix Sonicator S-4000 in cold water (100-watt, six pulses at 30 s each with 1 min of rest between each pulse). After centrifugation at 12,000 x g for 5 min, 100 μl of the supernatant containing the sheared cross-linked chromatin was diluted with 900 μl of 16.7 mmol/L Tris-HCl, pH 8.1, 1.2 mmol/L EDTA, 0.01% SDS, 1.1% Triton X-100,
167 mmol/L NaCl, and protease inhibitor cocktail and precleared with 30 μl of prewashed protein G-agarose for 1 h with rotation. After centrifugation at 600 x g for 1 min, the supernatant was incubated with 2 μg of normal rabbit IgG or anti-AP-2α antibody (ab52222, Abcam) overnight at 4 °C with rotation, then 60 μl of protein G-agarose was added, and incubation continued for 1 h. The protein G-agarose was spun down and then washed sequentially once each with low salt buffer, high salt buffer, and LiCl washing buffer and twice with Tris-EDTA buffer. The protein-DNA complex was eluted from the protein G-agarose by incubation with 100 μl of 1% SDS and 0.1 mol/L NaHCO₃ at room temperature for 15 min, followed by centrifugation at 600 x g for 1 min. The elution step was repeated once more. Then 8 μl of 5 mol/L 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 and used for PCR. The primers used for amplification of the rat SDF-1 promoter were: sense, 5'-GAGCATTTGTAGTCCAAGGAGCC-3'; and antisense, 5'-GGCGGTGTCTGAAGTGGCT-3'.

**Animal operation**

The animal experimental protocol was approved by the Institutional Animal Care and Utilization Committee of Academia Sinica, Taiwan. Wild type (HO-1⁺/⁺) and HO-1⁻/⁻ mice in B6/129SV mixed genetic background were originally obtained from Dr. SF Yet in National Health Research Institutes of Taiwan, and bred in our animal facility. C56BL/6J mice were obtained from the National Laboratory Animal Center of Taiwan. To avoid the potential influence of estrous cycle in female mice, male mice were used for the experiments. Male mice (8-12 weeks) were anesthetized by i.m. injection of a combination of ketamine (80 mg/kg of body weight), xylazine (20 mg/kg) and atropine (1.6 mg/kg). The left anterior descending coronary artery was ligated with an 8-0 nylon suture. Occlusion was confirmed by the pale anterior wall of the left ventricle (LV). The chest cavity was then closed with 6-0 suture. Sham-operated mice underwent the same surgical procedure except the coronary ligation. For experiments with CO-treatment, mice receiving coronary ligation for 7 days were randomly divided into 2 groups. One group of mice remained in normal air condition and another group of mice were exposed to 250 ppm CO gas for 1 h per day for 6 consecutive days. After the last CO exposure, some mice from each group were sacrificed and their hearts were collected for RNA isolation, tissue lysate preparation or tissue sectioning.
The remaining mice in both groups were subjected to echocardiography at 4 weeks after coronary ligation. In experiment to test the effect of CORM-2, mice receiving coronary ligation for a week were subjected to i.v. injection of saline, CORM-2 or inactive CORM-2 (4mg / kg body weight/day) for a period of 6 days and their heart functions were examined at 4 weeks after coronary ligation. An ultrasound unit (Philips, IE33) with a 17 MHz linear transducer was used for echocardiography. The animals were anesthetized using 2.5% isoflurane at beginning, after animals were sedative then maintained anesthesia with 1% during echocardiographic examination. Long and short axis M-mode at the level of the papillary muscle was used for measurement of diastolic and systolic diameter of LV, as well as the wall thickness. Pulsed wave Doppler was performed at the LV outflow tract, right ventricular outflow tract, and the mitral valves for recording the flow pattern and velocity. For in vivo shRNA-mediated gene knockdown experiments, the lentiviral vectors bearing control shRNA or AP-2α shRNA (1×10⁹ virus particles) plus 10 ng of substance P were injected at five sites around the infarct borders of mice right after coronary ligation. After 7 days, mice receiving the same lentivirus were randomly divided into 2 subgroups and treated with or without CO gas as described above. Total 180 mice were used in this study, and the mortality rate of the infarcted mice was about 13% after coronary ligation.

**Histological analysis and Immunohistochemistry**

The infarct size and collagen content of the heart were assessed in Masson trichrome-stained sections. MetaMorph imaging software (Universal Imaging Corp.) was used for calculating the circumference of LV, infarct length, LV area, and collagen deposition area. Infarct size was represented by infarct length/circumference of LV and collagen content was expressed as collagen area/area of LV as described previously.⁵ For immunohistochemical staining, heart cryosections were blocked with 5% normal goat serum in PBS for 1 h, followed by incubation with anti-CD11b (14-0112-81, eBioscience), anti-Gr-1 (14-5931-81, eBioscience), anti-c-kit (sc-168, Santa Cruz), and anti-CD31 (sc-1506, Santa Cruz) antibodies as indicated at 37°C for 1h. After three washes with PBS, sections were incubated with Alexa Fluor 568-conjugated secondary antibody (Molecular Probes) for another 1 h at room temperature in dark, followed by three washes. Nuclei were stained with DAPI reagent (Sigma). The numbers of positive-stained cells and vascular density were quantified morphometrically by examining two randomly selected fields in the peri-infarct area of each section. Four sections from each animal were
examined.

**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.

**References**