Original Article

Carbon Monoxide Releasing Molecule Accelerates Reendothelialization after Carotid Artery Balloon Injury in Rat*

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Abstract

Objective This study was aimed to investigate the effects of carbon monoxide releasing molecule (CORM-2), a novel carbon monoxide carrier, on the reendothelialization of carotid artery in rat endothelial denudation model.

Methods Male rats subjected to carotid artery balloon injury were treated with CORM-2, inactive CORM-2 (iCORM-2) or dimethyl sulfoxide (DMSO). The reendothelialization capacity was evaluated by Evans Blue dye and the immunostaining with anti-CD31 antibody. The number of circulating endothelial progenitor cells (EPCs) was detected by flow cytometry. The proliferation, migration, and adhesion of human umbilical vein endothelial cells (HUVECs) were assessed by using [^{3}H]thymidine, Boyden chamber and human fibronectin respectively. The expressions of protein were detected by using western blot analysis.

Results CORM-2 remarkably accelerated the re-endothelialization 5 d later and inhibited neointima formation 28 d later. In addition, the number of peripheral EPCs significantly increased in CORM-2-treated rats than that in iCORM-2 or DMSO-treated rats after 5 d later. *In vitro* experiments, CORM-2 significantly enhanced the proliferation, migration and adhesion of HUVECs. The levels of Akt, eNOS phosphorylation, and NO generation in HUVECs were also much higher in CORM-2 treated group. Blocking of PI3K/Akt/eNOS signaling pathway markedly suppressed the enhanced migration and adhesion of HUVECs induced by CORM-2.

Conclusion CORM-2 could promote endothelial repair, and inhibit neointima formation after carotid artery balloon injury, which might be associated with the function changes of HUVECs regulated by PI3K/Akt/eNOS pathway.

Key words: Carbon monoxide; Reendothelialization; Neointima formation; Vascular injury

Biomed Environ Sci, 2015; 28(4): 253-262 *doi: 10.3967/bes2015.036* *ISSN: 0895-3988*

www.besjournal.com (full text) *CN: 11-2816/Q*

INTRODUCTION

Although drug-eluting stent (DES) can prevent vascular smooth muscle cells (VSMCs) proliferation and decrease restenosis rate after percutaneous coronary intervention (PCI)[1], problems still exist in clinical practice, such as late in-stent thrombosis and prolonged intensive anti-platelet therapy[2-3]. Many clinical trials and animal experiments indicated that

*This study was supported by grants from the National Natural Science Foundation of China (No. 30770899 and 81370309) and Natural Science Foundation of Guangdong Province (No. S2012010009659).

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accelerated endothelial restoration was an important therapeutic method for inhibiting thrombosis, neointima formation and improving vascular homeostasis[1,4-5].

It is noteworthy that vascular diseases are initiated from endothelium injury, and endothelial function is closely associated with the development of atherosclerosis. The capacity of luminal endothelial repair is a key modulator of injured vascular homeostasis. Carbon monoxide (CO), traditionally recognized as toxic gas for mammalians, is the endogenous product of heme catalyzed by heme oxygenase-1 (HO-1)[6]. It has been recognized as a gasotransmitter with many important protective properties and beneficial regulatory effects on the pathogenesis of various vascular diseases. Endogenous HO-1-derived CO suppressed the formation of foam cells, attenuated high fat-induced aortic atherosclerotic plaque progression[7-8]. Inhaling low concentration CO rescued the pro-thrombotic phenotype in HO-1$^{-/-}$ mice[9], and significantly prevented injury-induced vascular restenosis in rodents and pigs[10-11]. However, it is still unclear whether CO can influence the reendothelialization after vascular injury.

Although recent studies indicated that exogenously applied CO could provide comprehensive beneficial and therapeutic effects, the release pattern and concentration of administrating CO gas is not easy to control, and carboxyhemoglobin (COHb) might be increased, resulting in the theoretical risk of impairing oxygen delivery to organs and tissues[12]. To resolve this problem, the novel CO-releasing molecules (CORMs) was produced to release CO in a controllable manner under physiological conditions without significant impact on COHb levels[13-14]. In the vasculature, CORMs could protect endothelial cells from cold preservation injury, and inhibit neointima formation after aorta transplantation in rats[15-16]. At cellular level, it has confirmed that CORMs can protect vascular endothelial cells (VECs) through modulating leucocyte-VECs interaction and suppressing lipopolysaccharide (LPS) or tumor necrosis factor alpha (TNF-α)-induced VECs apoptosis[17-18]. Moreover, CORMs can stimulate SDF-1-mediated proangiogenic response in cultivated mature VECs[19]. Accordingly, we hypothesized that CORMs might provide a noninvasive manner to enhance the endothelium repair after injured artery.

MATERIAL AND METHODS

Animals

Male Sprague-Dawley (SD) rats (250 to 280 g) were from the Center of Experiment Animal of Sun Yat-Sen University. All the experimental procedures and protocols were approved by the Animal Care and Use Committee of Sun Yat-Sen University and complied with the guideline for caring of laboratory animals developed by of the Ministry of Science and Technology of the People Republic of China.

Rat Carotid Artery Balloon-Injury Model

Male rat carotid artery balloon injury model was established according to the method in our laboratory previously described[20]. In briefly, a 2F Fogarty arterial embolectomy catheter (Edwards Lifesciences, Irvine, CA, USA) inflated with 300 μL saline was introduced into the common carotid artery of the rat through the external carotid artery to denude endothelium. The adherent tissue in right common carotid artery was cleared off, and the left common carotid artery without inserting the catheter was set as control. Then CORM-2 (Sigma-Aldrich, St Louis, MO, USA) (10 mg/kg), iCORM-2 or DMSO (negative control) were respectively injected via peritoneal cavity every day after the surgery. The rats were fed with normal diet after the surgery completed.

Cells Culture and Pharmacologic Reagents

HUVECs were isolated and cultured according to the procedures in our laboratory previously described[21]. Cultured cells were identified as ECs by their morphology and the presence of VIII-related antigen detected with an indirect immunocyto-chemistry assay. HUVECs were used at passages 2-4. CORM-2 was added with different concentrations (0-20 μmol/L) and iCORM-2 (0-20 μmol/L) or DMSO was respectively set as parallel control.

In vitro experiments with PI3K or eNOS inhibition were performed according to the following experimental protocol. HUVECs were preincubated with inhibitors for 1 h before CORM-2 stimulation. 10 μmoL of LY-294002 (Sigma-Aldrich, St Louis, MO, USA) and 100 μmoL of L-NAME (Sigma-Aldrich, St Louis, MO, USA) were used to inhibit PI3K and eNOS respectively.

Histology and Immunohistochemistry

Male rats were sacrificed and carotid arteries

were collected at scheduled time points after balloon injury. To quantify intima to media area (I/M) ratio, the sections staining with hematoxylin-eosin (HE) were digitized and analyzed under a microscope (Eclipse TI; Nikon, Japan) with Image-Pro Plus software version 6.0. The average values of five sections in every artery were determined.

To assess the extent of the reendothelialization in injured arteries 5 d later, the animals received an i.v. injection of 5% Evans blue diluted in saline 10 min before sacrifice, followed by perfusion with 4% paraformaldehyde in saline for 5 min. Then, the common carotid arteries (1 cm) were collected from the carotid bifurcation, and the arterial tissues were longitudinally opened. The blue-stained area were quantified with Image-Pro Plus software version 6.0 and represented as the percentage of total area of arterial segment subjected to denudation. And the ratio of blue-stained area to total area meant the endothelial denudation percentage. In order to further evaluating the level of the reendothelialization, paraffin-embedded tissue sections were deparaffinized, incubated with primary antibody to CD31 (BD Pharmingen, San Diego, CA, USA) in a humid chamber, followed by Alex Flour 594 secondary antibody (Molecular Probes, Carlsbad, CA, USA). Eight to ten images were taken for each injured vascular and analyzed.

Fluorescence-activated Cell Sorting

Peripheral blood (100 μL) was also collected 5 d later and incubated with anti-rat CD34-FITC, Flk-1-PE antibodies (BD Pharmingen, San Diego, CA, USA) for 30 min. At room temperature, and blood cells were hemolysed by using ACK lysis buffer to eliminate erythrocytes and through two washes with PBS, the immunofluorescence-labeled cells were fixed with 2% paraformaldehyde and analyzed by using quantitative flow cytometry with FACS flow cytometer (Becton Dickinson) and Cell Quest Software counting 10,000 cells per sample.

Elisa Test

VEGF and SDF-1 concentrations in serum samples were determined by using Quantikine kits for rat VEGF and SDF-1 (R&D systems, Minneapolis, MN, USA), respectively.

Western Blot Analysis

Injured carotid arteries and HUVECs were harvested by lysis buffer. The protein concentration was determined by using Bradford assay. Extracted protein were moved on SDS-PAGE gels and transferred to polyvinylidene fluoride membranes. Following antibodies were used: rabbit anti-SDF-1 antibody (Santa Cruz Biotechnology, CA, USA), rabbit anti-phospho-Akt and Akt, rabbit anti-phospho-AMPK and AMPK, rabbit anti-phospho-PKA and PKA antibody (Cell Signaling Technology, CA, USA), then HRP-conjugated anti-rabbit antibody.

Cell [^{3}H]thymidine Incorporation Assay

To assess HUVECs proliferation, the [^{3}H]thymidine incorporation assay was performed as previously described. In brief, the cells were plated onto 24-well plates (4×10^4 cells/well) and grown to 70%-80% confluence (about 24 h). The cells were then incubated with fresh medium and exposed to CORM-2 for 24 h. Then, the cells were metabolically labeled with 1 μCi/mL [methyl-^{3}H]thymidine (DuPont/NEN, Boston, MA, USA) for 3 h. Incorporated [methyl-^{3}H]thymidine was measured in a liquid scintillation counter.

Cell Migration Assay

The migration of HUVECs was assessed with Boyden chamber assays. A total of 2×10^4 HUVECs were trypsinized, resuspended in 250 μL base medium, and pipetted in the upper chamber of a Boyden chamber (Costar Transwell® assay, 8 μm pore size, Corning, NY, USA). The chamber was placed in a 24-well culture dish containing 500 μL base medium with CORM-2 or vehicles. After 24 h incubation, the transmigrated cells were counted by independent investigators.

Cell Adhesion Assay

The confluent HUVECs were incubated with CORM-2, as described above, for 24 h. Pretreated cells were gently detached with 0.5 mmol/L EDTA in PBS and replated on a 6 mm culture dish coated with human fibronectin (20 μg/mL) and incubated with CORM-2 for another 30 min. The adherent cells were washed with PBS, and fixed with 4% formaldehyde. By using digital camera, the images from 5 fields were randomly taken. The adherent HUVECs were counted by independent investigators.

NO Production

The cell culture medium (100 μL) of each group was collected. As the stable metabolite of NO, the level of nitrite was estimated with Greiss method

(Sigma-Aldrich, St Louis, MO, USA). The formation of nitrite (NO_2^-) and nitrate (NO_3^-) was detected in cell culture supernatants. This assay determined the total NO based on the enzymatic of conversion of NO_3^- to NO_2^- by nitrate reductase, and detection of nitrite as an azo dye product of the Greiss reaction. The results are presented as μmoL NO of NO_3^-/NO_2^- per liter of medium.

Statistical Analysis

The results are expressed as mean±SD. Statistical comparison of single parameters between two groups were performed with paired Student's *t* test. Kruskal-Wallis One-Way ANOVA was used to compare the means of multiple groups, followed by Dunn's test. Significance level was set established at $P<0.05$.

RESULTS

CORM-2 Accelerated Reendothelialization in Injured Artery

To evaluate the effect of CORM-2 on the reendothelialization, the carotid endothelial recovery after balloon injury was assess by using Evans Blue staining. Non-endothelialized lesions were marked by blue staining, whereas the reendothelialized area appeared white (Figure 1A). CORM-2 treatment promoted the reendothelialization in the balloon-injured arterial segments 5 d later, and the reendothelialization area in the CORM-2-treated rats was significantly larger than that in DMSO and iCORM-2 treated rats (45.6%±5.8%, 18.7%±2.3%, and 17.3%±3.4%, $P<0.01$, $n=6$, respectively) (Figure 1A and 1B). Furthermore, vessels were collected at 5, 7, 14 d after injury and the immunostaining with anti-CD31 antibody in the transverse section of the vessels revealed that the $CD31^+$ length of intact lumen surface was readily observed in uninjured vessels (Figure 1C). In the animals treated with iCORM-2 and DMSO, the endothelial cell (EC) monolayer was absent on day 5 after balloon injury (Figure 1C), but fully restored on day 14 (data not shown). In contrast, CORM-2-treated rats showed a partial restoration of the EC monolayer on day 5 (Figure 1C), consistent with the result from Evans Blue dye experiment.

CORM-2 Inhibited Neointima Formation after Arterial Injury

Enhanced endothelial repair leads to impaired neointima formation. Accordingly, in this study, whether CORM-2-induced endothelial repair can inhibit neointimal hyperplasia was evaluated. Twenty eight days after the surgery, prominent neointima developed in the DMSO and iCORM-2-treated group, whereas neointima formation was significantly suppressed in the CORM-2-treated animals (Figure 2A). Quantitative analysis revealed that the intima to media area-ratio (I/M ratio) in the CORM-2-treated group (0.57±0.24, $n=7$, $P<0.05$, Figure 2B) was significantly smaller than that in the DMSO and iCORM-2-treated groups on day 28 (1.27±0.24 and 1.35±0.45, $n=7$). However, the difference in medial thickness were not significant (data not shown).

CORM-2 Facilitated Mobilization of EPCs

The effect of CORM-2 on circulating EPCs population was also evaluated. CD34 and Flk-1 double-stained ($CD34^+/Flk\text{-}1^+$) cells were identified as

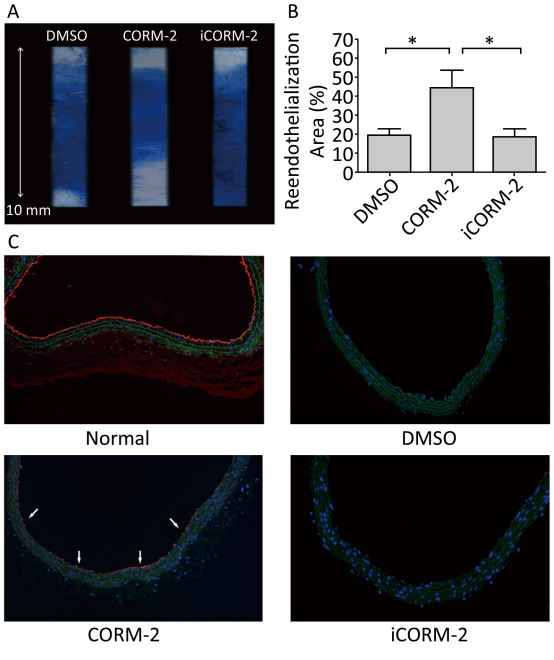

Figure 1. CORM-2 facilitated reendothelialization after balloon injury. A, Evans blue dye was injected 5 d later via tail vein 10 min before euthanasia. Endothelium denudation areas were marked by blue staining, whereas the reendothelialized areas appeared white. B, Quantifying the reendothelialized areas by computer-assisted morphometry ($n=6$, $P<0.05$). Compared with CORM-2 group: $^*P<0.05$. C, ECs identified by immunostaining with anti-CD31 antibody 5 d after arterial injury (×200).

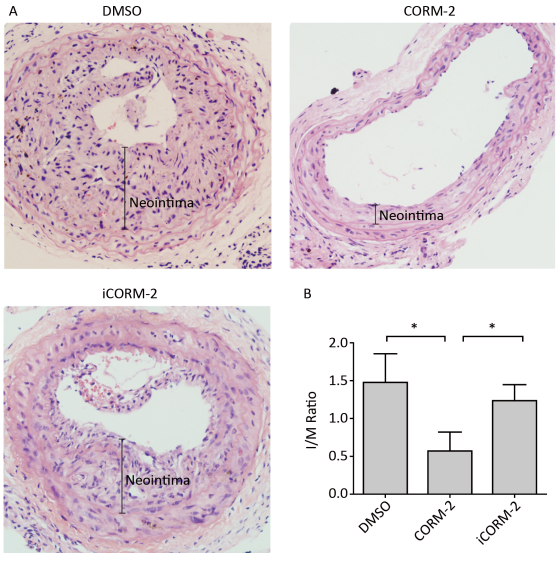

Figure 2. CORM-2 inhibited neointima formation after vascular injury. The rats were subjected to balloon injury of carotid artery and then treated with CORM-2, iCORM-2, or DMSO per day until animal euthanasia 28 d later. A, Morphometric analysis of hematoxylin and eosin-stained sections (×200). B, Quantifying I/M ratio of injured arteries. Compared with normal group: $^{*}P<0.05$.

EPCs. Flow cytometry analysis showed that the count of CD34+/Flk-1+ cells was much higher in CORM-2 treated group than that in DMSO and iCORM-2 treated groups (4.2-fold and 3.5-fold, n=7 each group, $P<0.05$, respectively) (Figure 3A and 3B).

Because VEGF and SDF-1 were the major chemotactic cytokines involved in EPC-mediated neovascularization and reendothelialization[22], the effect of CORM-2 on the serum levels of SDF-1 and VEGF was evaluated. As shown in Figure 3C, the serum levels began to increase 1 d later (peaked on day 5), and it was significantly higher in CORM-2 treated group than DMSO and iCORM-2 treated group. In addition, the SDF-1 levels in all the groups decreased to baseline level 28 d later. However, the VEGF level showed no significant changes in all the groups at different times mentioned above (Figure 3D). Accordingly, we also detected the SDF-1 and VEGF proteins expression in injured carotid arteries as indicated by Western blot analysis. The protein expression changes of SDF-1 was similar to the serum levels (increase 1 d later, peaked on day 5 and decreased to baseline level on day 28), but it was also significantly higher in CORM-2 treated group than in DMSO and iCORM-2 treated groups (Figure 3E

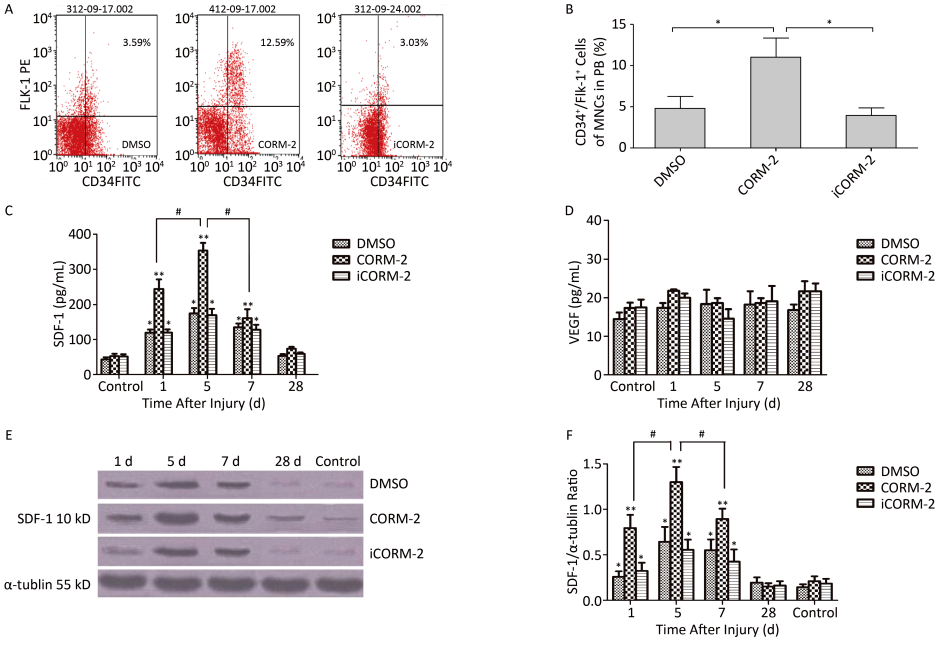

Figure 3. Effects of CORM-2 on the expression of SDF-1 and VEGF and the number of circulating EPCs. Rats were subjected to balloon injury followed by intraperitoneal injection of CORM-2, iCORM-2, and DMSO. A, On day 5, the peripheral blood samples were collected for flow cytometry analysis with antibodies to CD34 and Flk-1. B, Quantification analysis of $CD34^{+}/Flk\text{-}1^{+}$ cells in peripheral blood after vascular injury. C-D, Serum SDF-1 and VEGF levels determined by ELISA. E, The proteins expression of SDF-1 in injured carotid arteries. F, Quantification analysis of SDF-1 protein expression. Compared with control group: $^{*}P<0.05$, $^{**}P<0.01$; Compared with CORM-2 group on day 1 and 7: $^{\#}P<0.01$.

and 3F). The protein expression of VEGF in injured tissues also showed no change in all the groups (data not shown).

CORM-2 Enhanced Proliferation, Adhesion and Migration of HUVECs

It was demonstrated in arterial injury experiments that non-injured endothelium near to injured area played pivotal role in endothelial regeneration[23]. Therefore, we focused on the regulation effects of CORM-2 on HUVECs functions. Similar to *in vivo* observation, CORM-2 at the concentration from 0 to 10 μmol/L significantly enhanced HUVECs migration activity, increased DNA synthesis of HUVECs and fibronectin-mediated cell number in a dose-dependent manner, and significantly increased DNA synthesis and fibronectin-mediated cell number of HUVECs over control groups (Figure 4A-4C). However, 20 μmol/L CORM-2 exerted less pronounced effects on above mentioned functional activities of HUVECs compared with 15 μmol/L CORM-2 (Figure 4A-4C).

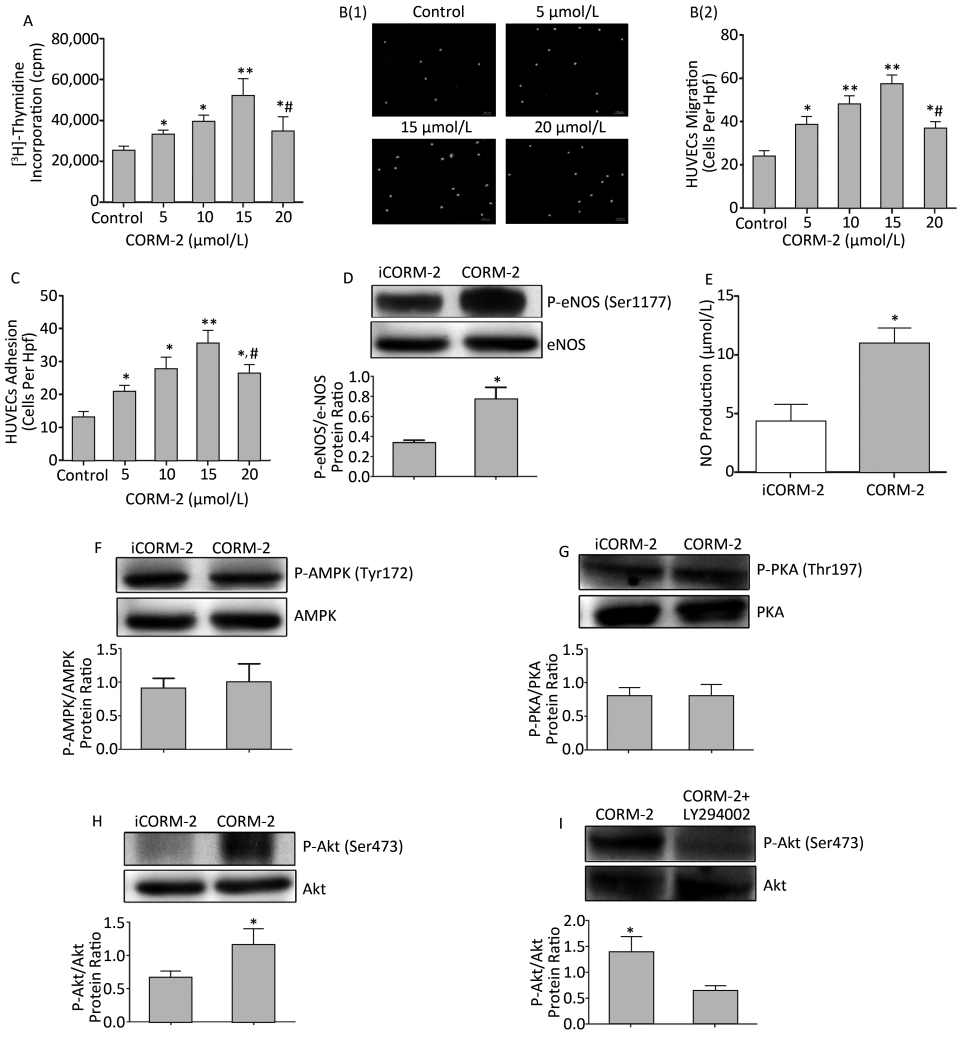

Figure 4. Effects of CORM-2 on *in vitro* HUVECs functions. A, Quantification analysis of HUVECs proliferative activity by [^{3}H]thymidine incorporation after incubation with CORM-2 for 24 h. B, Representative photograph (×200) and quantification analysis of CORM-2-induced migration of HUVECs. C, Quantification analysis of CORM-2-induced adhesion in HUVECs. D, Representative photograph and quantification analysis of eNOS phosphorylation of HUVECs. E, Total NO production in the medium of HUVECs stimulated with CORM-2 or iCORM-2. F-H, Representative photograph and quantification analysis of the phosphorylation of AMPK (F), PKA (G), Akt (H), Akt+LY294002 (I). Compared with normal group: $^{*}P<0.05$, $^{**}P<0.01$; Compared with CORM-2 group at 15 μmol/L: $^{\#}P<0.01$.

PI3K/Akt/eNOS Signal Pathway Participated in Regulating HUVECs Functions and NO Generation Induced by CORM-2

As we know, many biological functions of the vascular endothelium are modulated by eNOS-derived NO, including migration and adhesion, so eNOS-derived NO is recognized as the important signaling molecular in vascular biology. Previous studies confirmed that there was a cross-talk between the gases CO and NO, especially in the vasculature[24-25]. Thus, we hypothesized that eNOS activation and NO generation might play a crucial role in the enhanced functions induced by CORM-2 *in vitro*. In our study, it was observed that the exposure to 10 μmol/L CORM-2 for 30 min remarkably increased eNOS phosphorylation and NO generation in HUVECs (Figure 4D and 4E). It is well known that eNOS is downstream kinase of AMP-activated protein kinase (AMPK), protein kinase A (PKA), PKG and PKB (Akt)[26-28]. Thus, we further investigated the possible effect of above signaling pathways in CORM-2-induced phosphorylation of eNOS in HUVECs, and found that AMPK, PKA, and PKG activation did not increased (Figure 4F and 4G). Interestingly, Akt activation by CO was shown in an inconsistent manner in the different cellular models. One study reported that in human ECs that CO inhibited Akt activation in human ECs[29], whereas another study showed that CO increased Akt activity in ischemia/reperfusion injury-induced heart of rat[30]. Here, we wanted to assess this effect in our model. It was observed that treatment with 10 μmol/L CORM-2 for 30 min induced a rapid Akt phosphorylation (Figure 4H), which was abrogated by the selective PI3K inhibitor (LY294002) (Figure 4I). In addition, blocking PI3K with LY294002 could nearly abolish CORM-2-induced eNOS phosphorylation and NO generation in HUVECs (Figure 5A and 5B). Similarly, the combination of PI3K and eNOS inhibitions could also achieve the parallel response (Figure 5A and 5B).

We further investigated whether CORM-2-activated PI3K/Akt/eNOS signaling pathway contributes to enhanced functions of HUVECs *in vitro*. Unsurprisingly, we found that PI3K or eNOS inhibition significantly reduced HUVECs migration (Figure 6A) and fibronectin-mediated adhesion (Figure 6B) capacities in response to 10 μmol/L CORM-2, which was support the hypothesis of interaction of CO and NO *in vitro* in the regulation of migration, adhesion of HUVECs, and suggested that CO can not only increases Akt phosphorylation but also drive activation of eNOS and influence its activity to generate NO. We believed that CO obviously triggered the activation of PI3K/Akt/eNOS pathway, which enhanced *in vitro* functions of HUVECs.

DISCUSSION

In this study, it was observed that CORM-2 inhibited neointima formation and accelerated reendothelialization after artery injury. At the same time, CORM-2 treatment also augmented the number of circulating EPCs and up-regulated serum

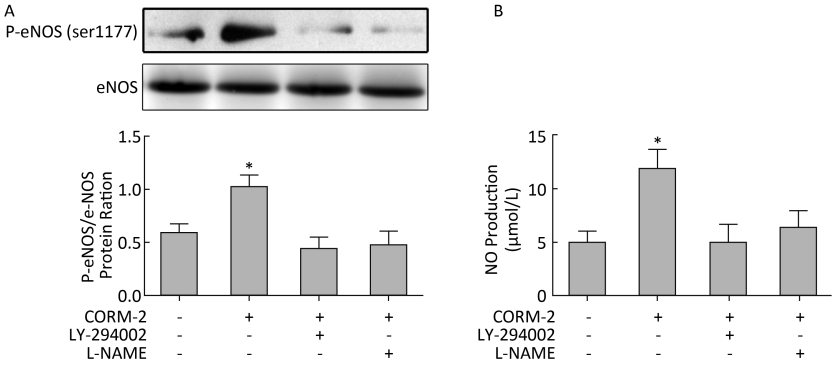

Figure 5. Effects of PI3K/Akt signaling pathway in CORM-2-induced phosphorylation of eNOS as well as NO generation in HUVECs. A, Representative photograph and quantification analysis of eNOS phosphorylation of HUVECs. B, Total NO production in the medium of HUVECs stimulated with CORM-2 or CORM-2 combined with Akt inhibitor (LY-294002) and eNOS inhibitor (L-NAME). Compared with CORM-2 group: $^{*}P<0.05$.

SDF-1 level and its expression in injured arteries. In addition, *in vitro* experiments found that CORM-2 could promote the phosphorylation of Akt and eNOS, and enhance HUVECs functions including proliferation, migration and adhesion. After treatment with PI3K inhibitor or eNOS inhibitor, the migration and adhesion of HUVECs were marked attenuated. It suggested that HUVECs function changes induced by CORM-2 were mediated by PI3K/Akt/eNOS signaling pathway.

PCI treatment is commonly performed as a therapy for coronary artery disease. However, coronary arteries in-stent thrombosis after PCI is a critical clinical problem[31]. Many clinical trials and animal experiments suggested that reendothelialization played a pivotal role in preventing in-stent thrombosis, so it is very important to find a method to promote injured vascular repair. Our study found that reendothelialization area was much larger in CORM-2 group than that in DMSO and iCORM-2 groups 5 d later. On day 28, CORM-2 group showed significantly reduced intimal thickeness and increased luminal area. Previously, Study from Wegiel et al. also found that systemic administration of inhaled CO (250 ppm) enhanced endothelial repair in wire-mediated vascular injury[24]. Wu et al. also observed probucol, a pharmacological inducer of endogenous CO, could increase reendothelialization and decrease neointimal thickening after aortic balloon injury in a rabbit dietary-probucol model[33]. These results supported the hypothesis of CO in preventing negative vascular remodeling by promoting endothelium repair.

Increasing evidences confirmed the beneficial effect of bone marrow-derived EPCs on vascular repair, and circulating EPCs mobilized from bone marrow could be recruited to injured vascular wall and incorporated into nascent endothelium. Previous studies have confirmed that systemic up-regulation of HO-1 expression or pharmacological induced HO-1-derived CO can mobilize EPCs from bone marrow into circulation and participate in reendothelialization[32-33]. Our flow cytometry data showed that CORM-2 also increased the number of $CD34^+/Flk\text{-}1^+$ cells, indicating that exogenous CO might directly promote EPCs mobilization or proliferation. Indeed, the study of Deshane et al showed that the treatment with CORM-2 could enhance SDF-1-induced EPCs migration, a key event in reendothelialization[19]. Another report showed that exogenous administration of CO could augment the number of EPCs colonies, and induce the differentiation of EPCs into mature ECs[24]. All of these results proved that EPCs mediated the protective effect of CO on injured vascular.

As VEGF and SDF-1 are two major angiogenic factors involving EPCs mediated neovascularization and reendothelialization, several earlier findings showed that up-regulation of HO-1 in cardiac enhanced the induction of SDF-1 and VEGF in ischemic heart[22,34]. An administration of CO led to higher circulating levels of SDF-1 and VEGF[32]. Moreover, blocked SDF-1 and VEGF with neutralizing antibodies significantly inhibited HO-1/CO-induced $c\text{-}kit^+$ stem cells recruitment in mycadial infarction areas, and attenuated neovascularization-mediated cardiovascular protection[22], supporting the theory that VEGF and SDF-1 are involved in HO-1/CO-mediated cardiovascular repair. In order to explore whether CO has similar effect in this study, further experiment was conducted to test the role of CORM-2. The result showed that injection of CORM-2 for 5 d consecutively led to the markedly elevated serum level of SDF-1 rather than VEGF. In parallel, the protein expression of SDF-1 in injured vessel was also significantly increased on day 5 after balloon injury, indicating that SDF-1 could mediate

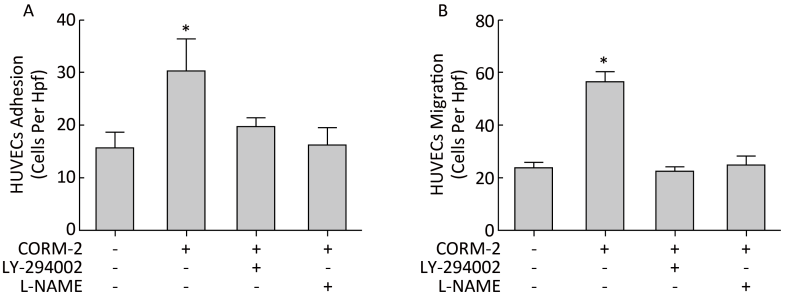

Figure 6. PI3K/Akt/eNOS signaling blockade inhibits *in vitro* functions of HUVECs treated with CORM-2. A-B, Quantification analysis of adhesion (A) and migration (B). Compared with CORM-2 group: $^*P<0.05$.

the enhanced vascular protection by CO. Previous studies on various diseases have revealed multiple pathways contributing to protective effects of CO. CO has been shown to activate transcriptional factors HIF-1α, STAT, SP-1, and signaling molecular MAPK family, Akt and sGC[6]. A study confirmed that activator protein 2α (AP-2α) is a potential transcriptional factor of SDF-1 gene expression, Akt-dependent upregulation of AP-2α is essential for CO-induced SDF-1 expression involving in myocardial repair[35]. However, whether Akt/AP-2α pathway mediates CO-induced SDF-1 expression needs further evaluation.

It is well known that endothelialization was mainly due to endothelial repair of mature ECs near to injured area[23,5]. Our Evans blue staining images showed that blue area disappeared step by step from boundary of the injury region and white area became larger and larger, indicating that ECs moved from border area to wound area. In addition, our *in vitro* experiments showed that the proliferation, migration and adhesion of HUVECs were significantly enhanced by CORM-2 in a dose-dependent manner, but this phenomenon was not observed in DMSO and iCORM-2-treated groups. Li et al. previously reported a similar phenomenon that adding CO into human microvessel ECs could exert proangiogenic response in Matrigel Matrix plus, and capillary sprouts from ECs was impaired due to HO-1 knockout, but it was reversed by the administration of CORM[36]. From these data, we can conclude that CO exerts multiple beneficial effects on ECs functions, and these data also support our *in vivo* observation that CORM-2 promotes vascular repair after arterial injury.

Previous studies showed that eNOS-derived NO played a critical role on CO-mediated restoring homeostasis[24]. In the heart, CO protected against cardiac ischemia-reperfusion injury and increased eNOS activity. In the lung, CO could reverse established pulmonary arterial hypertension and its induced-right ventricular hypertrophy, which was abrogated in eNOS knockout mice. These results indicated that eNOS might also be an important target for CO in ECs. In this study, it was observed that CO enhanced ECs proliferation, migration and adhesion, activated eNOS phosphorylation and increased NO production. In addition, inhibiting PI3K or eNOS could reduce *in vitro* migration, adhesion of ECs induced by CORM-2, and attenuate CORM-2-induced NO generation. These data suggested that PI3K/Akt/eNOS signaling pathway might participate in regulating CO-induced *in vitro* ECs functions. Beside, some studies showed that many stimulation or drug intervention such as adiponectin, statin and shear stress could also activate eNOS phosphorylation, which could be also regulated by AMPK and PKA[26,37]. However, it was not observed that CORM-2 could activate AMPK and PKA in this study, indicating that eNOS phosphorylation induced by CORM-2 was regulated by PI3K/Akt pathway rather than AMPK, PKA pathway.

In conclusion, this study confirmed for the first time that CORM-2, a novel lipid soluble molecule of CO, could promote post-injury endothelium repair and enhance ECs proliferation, migration and adhesion partly via activating PI3K/Akt/eNOS signaling pathway, indicating that CORM-2 administration prior to PCI might exert the clinical vascular protective benefit by promoting reendothelialization, and PI3K/Akt/eNOS signaling pathway might act as the potential important intervention target in endothelial repair after vascular injury. It is necessary to conduct more studies to further confirm the observations mentioned above.

Received: October 24, 2014;
Accepted: February 26, 2015

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