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Carbon Monoxide Rescues Heme Oxygenase-1-Deficient Mice from Arterial Thrombosis in Allogeneic Aortic Transplantation

Bo Chen,* Lingling Guo,† Chunlan Fan,* Subhashini Bolisetty,* Reny Joseph,* Marcienne M. Wright,* Anupam Agarwal,* and James F. George†

From the Department of Medicine, Nephrology Research and Training Center, and the Department of Surgery,† University of Alabama at Birmingham, Birmingham, Alabama*

Heme oxygenase-1 (HO-1) catalyzes the conversion of heme into carbon monoxide (CO), iron, and biliverdin. In preliminary studies, we observed that the absence of HO-1 in aortic allograft recipients resulted in 100% mortality within 4 days due to arterial thrombosis. In contrast, recipients normally expressing HO-1 showed 100% graft patency and survival for more than 56 days. Abdominal aortic transplants were performed using Balb/cJ mice as donors and either *HO-1*$^{+/+}$ or *HO-1*$^{-/-}$ (C57BL/6×FVB) mice as recipients. Light and electron microscopy revealed extensive platelet-rich thrombi along the entire length of the graft in *HO-1*$^{-/-}$ recipients at 24 hours. Treatment of recipients with CORM-2, a CO-releasing molecule (10 mg/kg of body weight intravenously), 1 hour prior and 1, 3, and 6 days after transplantation, significantly improved survival (62% at >56 days, $P < 0.001$) compared with *HO-1*$^{-/-}$ recipients treated with inactive CORM-2 (median survival 1 day). Histological analyses revealed that CO treatment markedly reduced platelet aggregation within the graft. Adoptive transfer of wild-type platelets to *HO-1*$^{-/-}$ recipients also conferred protection and increased survival. Aortic transplants from either *HO-1*$^{-/-}$ or *HO-1*$^{+/+}$ C57BL/6 donors into *HO-1*$^{+/+}$ (Balb/cJ) mice did not develop arterial thrombosis, surviving more than 56 days. These studies demonstrate an important role for systemic HO-1/CO for protection against vascular arterial thrombosis in murine aortic allotransplantation. ***(Am J Pathol 2009, 175:422–429; DOI: 10.2353/ajpath.2009.081033)***

Heme oxygenase-1 (HO-1) is an inducible enzyme that catalyzes the rate-limiting step in heme degradation, leading to the generation of equimolar amounts of iron, biliverdin, and carbon monoxide (CO). Biliverdin is then converted to bilirubin by biliverdin reductase.[1,2] HO-1 is highly up-regulated in mammalian tissues in response to a wide variety of conditions including vascular injury, ischemia, inflammation, immune injury, oxidative stress, cell cycle dysregulation, and sublethal and lethal cell damage.[3–5] The wide range of inducers of HO-1 provides support for a vital role in maintenance of cellular homeostasis under different pathophysiological conditions including inflammatory diseases such as septic shock and asthma,[6,7] cardiovascular diseases such as myocardial infarction and atherosclerosis,[8,9] ischemia-reperfusion injury in multiple organ systems,[8,10] and transplant rejection.[11,12]

One of the products of HO-1-mediated heme degradation, CO, is known to be toxic at high concentrations due to its high affinity for hemoglobin. However, there is substantial evidence that lower concentrations of CO endogenously generated from the breakdown of heme by HO serves essential regulatory roles in a variety of physiological and pathophysiological processes.[13] Exogenous or endogenous CO can confer some of the cytoprotective effects attributed to HO-1.[14,15]

Transitional metal carbonyls, CO-releasing molecules (CORMs), have been used to deliver CO in a controlled manner without altering carboxyhemoglobin levels.[16–18] A wide range of CORMs containing manganese (CORM-1), ruthenium (CORM-2 and −3), boron (CORM-A1), and iron (CORM-F3) are currently being investigated to facil-

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Address reprint requests to James F. George, Ph.D., Department of Surgery, Room 790 LHRB, or Anupam Agarwal, M.D., Department of Medicine, Room 647 THT, 1530 3rd Avenue South, University of Alabama at Birmingham, Birmingham, AL 35294. E-mail: jgeorge@uab.edu and agarwal@uab.edu.

itate the pharmaceutical use of CO for the prevention of vascular dysfunction, inflammation, ischemia-reperfusion injury, and transplant rejection.[19–23]

Thrombosis is a major complication during multiple vascular pathological conditions during which HO-1 and its byproduct CO could provide significant protection through attenuation of inflammation, endothelial cell damage, and apoptosis, as well as modulation of vascular tone.[6,8,9] However, very little is known regarding the potential roles of HO-1 and CO in modulating platelet-dependent effects after vascular injury in the setting of transplantation. In these studies, we show that expression of HO-1 plays a critical role in the development of post-transplant arterial thrombosis immediately following abdominal aortic transplantation. We tested the hypothesis that CO, a product of the HO-1 reaction, mediates anti-thrombotic effects *in vivo* by inhibition of platelet mediated thrombus formation within the graft. We found that HO-1-deficient mice develop vascular thrombosis following aortic transplantation and that the development of thrombosis can be prevented by systemic administration of CORM-2.

Materials and Methods

Reagents

Purified mouse anti-eNOS/NOS type III was obtained from BD Biosciences. Anti-HO-1 antibody (SPA-896) and anti-HO-2 antibody (SPA-897) were obtained from Stress-Gen Biotechnologies. OptiPrep (60% w/v iodixanol) was obtained from Axon Laboratories AG (Le Mont-sur-Lausanne, Switzerland). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Animals

Balb/cJ mice (H-2^{d}) were purchased from Jackson Laboratories Inc. (Bar Harbor, ME). *HO-1*$^{+/+}$ and *HO-1*$^{-/-}$ mice were maintained in breeding colonies on a C57BL/6 (H-2^{b}) × FVB (H-2^{q}) background.[24] All animals were used at an age of 8 to 12 weeks and maintained on standard rodent chow and allowed free access to water. All surgical procedures were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham.

Surgical Procedure

The transplant procedure was performed using a technique as previously described.[25,26] Briefly, donor and recipient mice were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg body weight). A segment of the donor abdominal aorta was isolated and removed, and preserved in chilled normal saline. The recipient aortic segment below the renal arteries and above the iliac bifurcation was transected between two vascular clamps (B-1 Clamp, Fine Science Tools, Foster City, CA), followed by end-to-end anastomosis of the donor aortic segment into recipient's abdominal aorta using 11–0 nylon interrupted sutures (AROS Surgical, Newport Beach, CA). Hind-limb paralysis was monitored postoperatively and scored from 1 to 4: grade 1 = complete paralysis; grade 2 = moderate to severe hind-limb paralysis; grade 3 = mild hind-limb weakness; grade 4 = no paralysis.

Experimental Groups

Groups were established using Balb/cJ mice as donors and *HO-1*$^{+/+}$ mice or *HO-1*$^{-/-}$ littermates as recipients. Experimental groups were treated with multiple doses of CO-releasing molecule, tricarbonyldichlororuthenium(II) dimer (CORM-2, Sigma-Aldrich) at 10 mg/kg intravenously 1 hour before transplantation, and 1, 3, and 6 days after transplantation. Control groups were treated using the same dose schedule except they received inactive CORM-2 that had been previously depleted of CO (iCORM-2) by exposure to air for 24 hours before use. Within each group, eight animals were followed until death or 8 weeks after transplantation; another five animals were sacrificed at 24 hours after transplantation for analysis. Two additional control groups (n = 5 per group) were performed using HO-1$^{+/+}$ or HO-1$^{-/-}$ mice as donors and Balb/cJ mice as recipients. No immunosuppression was used.

Histology

For determination of histological changes, aortic grafts and major organs including the liver, lungs, heart, kidney, brain and large bowel were isolated and harvested, fixed in 10% neutral buffered formalin (Fisher Scientific), and embedded in paraffin. Five-micrometer serial sections were stained using hematoxylin and eosin. Histomorphometric analyses were performed on images acquired with a DMR Leica microscope (Leica, Bannockburn, IL) and IMAGE PRO software (Media Cybernetics, Silver Spring, MD). The patency of aortic grafts was calculated by subtracting the area of thrombus from the lumen area. The areas were calculated in three to five sections per aortic graft. Immunohistochemical staining for mouse eNOS/NOS type III was performed on paraffin-embedded tissue sections. Negative controls without the primary or secondary antibody were also used as previously described.[27]

Transmission Electron Microscopy

A segment of aortic graft was harvested 24 hours after transplantation and immediately fixed in 2.5% glutaraldehyde in phosphate-buffered saline, postfixed in 4% osmium tetroxide, and embedded in Epon resin. Semithin sections (1–2 μm thick) were used to locate vascular tissue in the adventitia of the graft. Ultrathin sections (50–80 nm thick) were prepared, stained with lead citrate and uranyl acetate, and observed with a Zeiss EMI transmission electron microscope.

Western Blot

Splenic tissue was lysed in a buffer containing a broad spectrum mixture of protease inhibitors (Roche Diagnostics, Indianapolis, IN) and Triton X-100. Immunoblot analysis was performed as described[24] by using anti-HO-1 antibody (1:5000 dilution) and anti-HO-2 antibody (1:2000) followed by incubation with the corresponding peroxidase-conjugated secondary antibody (1:10,000 dilution) for 1 hour. The membranes were reprobed with an anti-actin antibody (1:1000; Sigma) to confirm equal loading.

HO Enzyme Activity

Heme oxygenase activity was measured by bilirubin generation in microsomal preparations from mouse spleen as described previously.[28,29] Spleen microsomes were incubated with rat liver cytosol, a source of bilirubin reductase (3 mg), hemin (20 μmol/L), glucose-6-phosphate (2 mmol/L), glucose-6-phosphate dehydrogenase (0.2 units), and NADPH (0.8 mmol/L) for 1 hour at 37°C in the dark. The formed bilirubin was extracted with chloroform and the change in optical density, 464 to 530 nm, was measured (extinction coefficient, 40 mmol/L^{-1}· cm^{-1} for bilirubin). Enzyme activity was expressed as nmol of bilirubin formed per 60 minutes/mg protein.

Adoptive Transfer of Platelets

Platelets were isolated using the method described previously.[30] OptiPrep (5 volumes) was diluted with 0.85% (w/v) NaCl, 1 mmol/L ethylenediamine tetraacetic acid, and 20 mmol/L HEPES-NaOH, pH 7.4 (22 volumes) to produce a 1.063 g/ml solution. In a 15-ml centrifuge tube, 0.8 ml of whole mouse blood was layered over an equal volume of the 1.063 g/ml solution and centrifuged at 350 × *g* for 15 minutes at 20°C in a swinging-bucket rotor (Eppendorf centrifuge 5810R). Platelets were harvested from the band just above the interface and counted in a Neubauer hemocytometer. After washing three times with phosphate-buffered saline, platelets from HO-$1^{+/+}$ mice (1×10^7/g of body weight) or equal volume of vehicle (saline) were injected into HO-$1^{-/-}$ recipient mice through the tail vein 30 minutes before receiving a Balb/cJ abdominal aortic graft.

Statistical Analysis

All data are presented as the mean ± SEM. Statistical analysis was performed by using analysis of variance and the Student-Neuman-Keuls post-test analysis. Survival was determined by the Kaplan-Meier method and the log-rank test was used to analyze the differences among groups. Statistical significance was defined as $P < 0.05$.

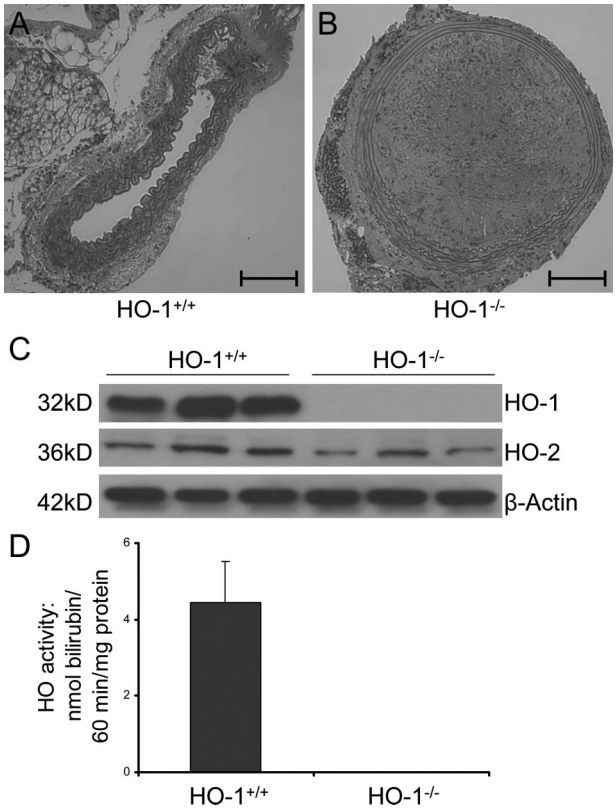

Figure 1. Thrombus formation in allogeneic abdominal aortic grafts in *HO-$1^{-/-}$* and *HO-$1^{+/+}$* mice at 24 hours after transplantation. **A:** Allogeneic aortic grafts from Balb/cJ mice were 100% patent in *HO-$1^{+/+}$* recipient animals ($n = 5$), but in **B**, dense arterial thrombi were found in allogeneic aortic grafts in *HO-$1^{-/-}$* mice ($n = 5$). H&E staining. Original magnification for **A** and **B**, ×200; scale bar = 100 μm. **C:** Genotyping of the *HO-$1^{+/+}$* and *HO-$1^{-/-}$* mice used in the experiments was confirmed by Western blot performed with splenic tissue as described in the methods. **D:** HO enzyme activity in splenic microsomes from *HO-$1^{+/+}$* and *HO-$1^{-/-}$* mice. HO activity was measured by bilirubin generation (nmol/60 minutes/mg protein) as described in *Materials and Methods*.

Results

Arterial Thrombosis and Mortality in HO-1-Deficient Mice Receiving Allogeneic Aortic Grafts

All *HO-$1^{+/+}$* recipients of Balb/cJ aortic allografts survived at least 8 weeks after transplantation, but *HO-$1^{-/-}$* recipients died within 4 days after transplantation ($n = 5$ per group, $P < 0.001$). All deaths were preceded by hind-limb paralysis, which was due to thrombosis of the arterial graft (Figure 1B). *HO-$1^{+/+}$* mice showed patent grafts for >56 days (Figure 1A). The thrombus consisted of stiff, fibrous material consistent with coagulation of blood components or platelets. Histological analysis of vital organs including the liver, heart, lungs, and brain indicated no evidence of thrombus formation or deposition in these organs sufficient to be the cause of death in the HO-$1^{-/-}$ animals. The occlusion of the graft did, however, result in ischemia and necrosis of the large bowel, and the kidneys showed evidence of acute tubular necrosis and thrombus formation in the renal arteries. The blood supply of each of these systems is proximal to the

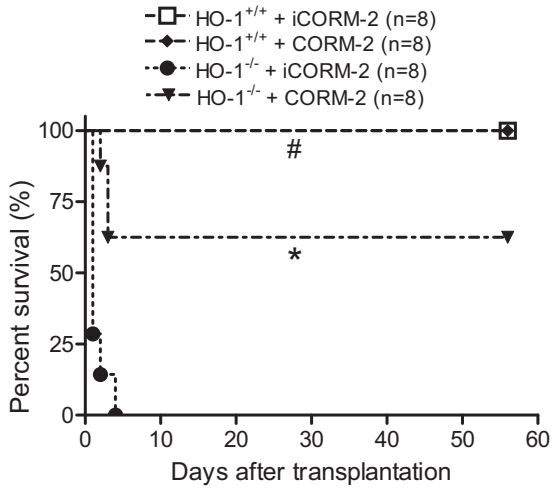

Figure 2. Kaplan-Meier analysis of *HO-1*$^{+/+}$ and *HO-1*$^{-/-}$ recipients of allogeneic aortic grafts following treatment with CORM-2 or iCORM-2. All *HO-1*$^{+/+}$ recipient mice treated with CORM-2 or iCORM-2 survived the entire 8-week follow-up period. In contrast, all eight *HO-1*$^{-/-}$ recipient mice treated with iCORM-2 died with arterial thrombosis within 4 days after transplantation (median survival time = 1 day). Five of eight CORM-2-treated HO-1 knockout mice (62.5%) survived to 8 weeks (median survival time = 56 days, $^{*}P < 0.01$ in comparison with iCORM-2-treated animals). There was no statistical difference between CORM-2-treated group and *HO-1*$^{+/+}$ groups ($^{\#}P = 0.08$).

large thrombus found in the aortic graft. Therefore, it is likely that these abnormalities contributed to death in the HO-1 knockout recipients. Western blots confirmed a lack of HO-1 expression in the *HO-1*$^{-/-}$ mice and showed no compensatory increase in HO-2 expression (Figure 1C). Splenic HO enzyme activity was not detectable in *HO-1*$^{-/-}$ mice (Figure 1D).

Carbon Monoxide-Rescued HO-1-Deficient Recipients by Preventing Arterial Thrombosis

Since arterial thrombosis only occurred in *HO-1*$^{-/-}$ mice and CO, a product of HO-1, has been implicated in venous thrombosis,[31] we determined whether administration of CO using CORM-2, a CO-releasing molecule, would ameliorate the thrombotic complications following surgery. As shown in Figure 2, five of eight CORM-2-treated *HO-1*$^{-/-}$ mice (62.5%) survived at least 8 weeks (median survival time >56 days). In comparison, control *HO-1*$^{-/-}$ graft recipients treated at the same concentration and volume with iCORM-2, which does not release CO, quickly developed arterial thrombosis, as indicated by hind-limb paralysis and all died within 4 days (median survival time = 1 day, $P < 0.01$).

Histological analyses indicated no arterial thrombosis at 24 hours following abdominal aortic transplantation in the allogeneic grafts of *HO-1*$^{+/+}$ recipient animals treated with either CORM-2 or iCORM-2 (Figure 3A and B). In contrast, allografts from *HO-1*$^{-/-}$ mice treated with iCORM-2 showed histological evidence of arterial thrombosis within 24 hours after transplantation with occlusion of the lumen (Figure 3C) and CORM-2 treatment resulted in patent grafts with no occlusion (Figure 3D). Morphometric analysis revealed that the mean patency of the

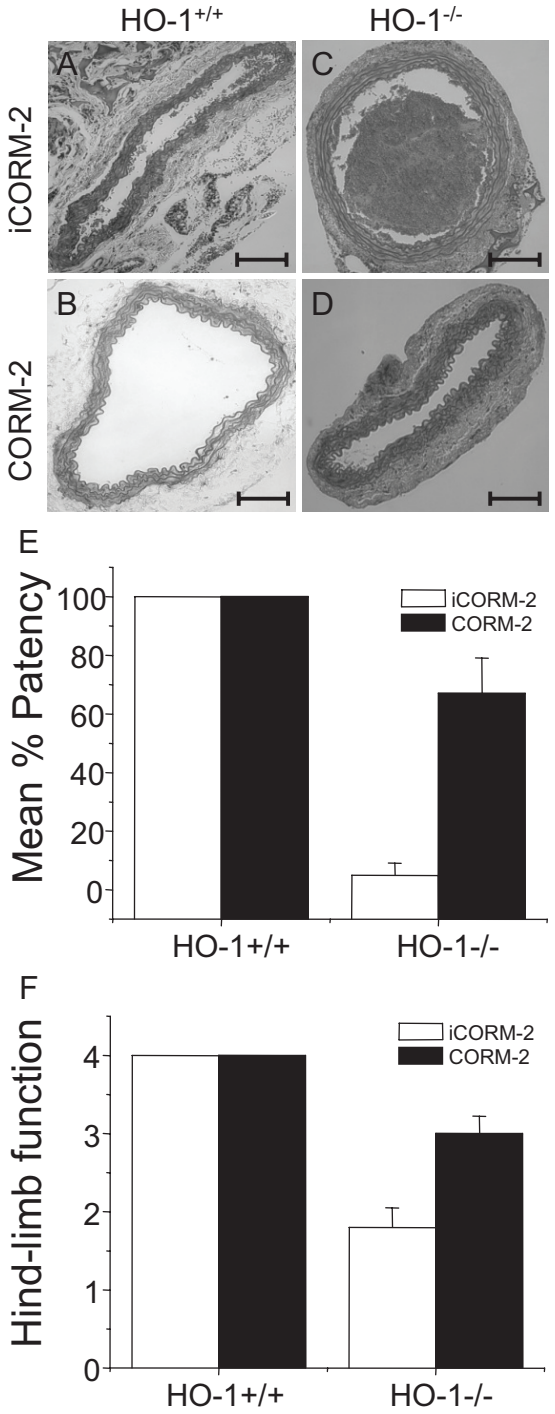

Figure 3. Histological evaluation of aortic grafts. No arterial thrombus can be seen 24 hours after transplantation in the allogeneic grafts of *HO-1*$^{+/+}$ recipient animals treated with either iCORM-2 (**A**, $n = 5$) or CORM-2 (**B**, $n = 5$). **C:** All iCORM-2-treated *HO-1*$^{-/-}$ recipients contained arterial thrombi at 24 hours after transplantation ($n = 5$) H&E staining; original magnification, ×200; scale bar = 100 μm. **D:** *HO-1*$^{-/-}$ mice treated with CORM-2 showed little or no arterial thrombi at 24 hours after transplantation ($n = 5$). **E:** Mean patency of allogeneic aortic grafts in *HO-1*$^{+/+}$ and *HO-1*$^{-/-}$ recipient animals treated with iCORM-2 or CORM-2. CORM-2 treatment significantly increased the mean patency of grafts in *HO-1*$^{-/-}$ recipients (67 ± 12.2%) in comparison with *HO-1*$^{-/-}$ mice treated with iCORM-2 (5.0 ± 4.8%, $P < 0.01$). **F:** Hind-limb function at 24 hours after transplantation. The severity of hind-limb paralysis was graded using a scoring system as described in *Materials and Methods*.

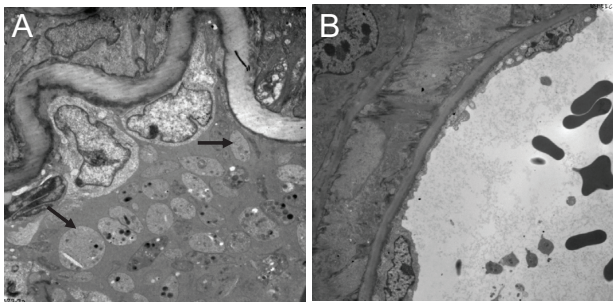

Figure 4. Thrombus formation and endothelial cell morphology in allogeneic aortic grafts in *HO-1*$^{-/-}$ recipients. Transmission electron microscopy of allogeneic aortic grafts 24 hours after transplantation in *HO-1*$^{-/-}$ recipients with iCORM-2 (**A**) or CORM-2 (**B**) treatment. **Arrows** denote platelets. Original magnification for **A**, ×4500; **B**, ×3000.

aortic grafts in the *HO-1*$^{-/-}$ CORM-2-treated group was 67 ± 12.2% in comparison with 5.0 ± 4.8% in *HO-1*$^{-/-}$ iCORM-2-treated recipients (Figure 3E, $P < 0.01$).

As shown in Figure 3F, little or no hind-limb paralysis was observed in the CORM-2-treated animals compared with the iCORM-2 group (hind-limb function score 3.0 ± 0.22 versus 1.8 ± 0.25, $P < 0.01$). As shown in Figure 2 and Figure 3F, all *HO-1*$^{+/+}$ recipient mice, whether treated with CORM-2 or iCORM-2, survived for the full 8-week follow-up period without hind-limb paralysis, indicating that the effects observed in the *HO-1*$^{-/-}$ mice could not be attributed to CORM-2 or iCORM-2 treatment.

Aortic Allograft Endothelial Cell Cytoarchitecture and eNOS Expression in CORM-2-Treated HO-1-Deficient Recipients

To address the mechanism of protection against vascular thrombosis by CORM-2, we hypothesized that CO released from CORM-2 or from HO-1 expression may reduce the extent of endothelial cell damage caused by ischemia/reperfusion injury during surgery. Aortic grafts were harvested 24 hours following allogeneic transplantation. Electron microscopy revealed that the endothelial cell layer appeared to be similar in *HO-1*$^{-/-}$ recipients treated with CORM-2 or iCORM-2, with a largely intact endothelial cell layer and occasional small regions of denudation (Figure 4, A and B). However, in iCORM-2-treated *HO-1*$^{-/-}$ mice, a proteinaceous matrix and platelets were seen adhering to the endothelium. Immunohistochemical staining indicated that the graft endothelium in *HO-1*$^{+/+}$ and *HO-1*$^{-/-}$ mice receiving either CORM-2 or iCORM-2 maintained similar levels of eNOS expression (Figure 5). These results suggested that the differences observed in graft thrombosis as a function of the presence or absence of HO-1 were likely due to a systemic

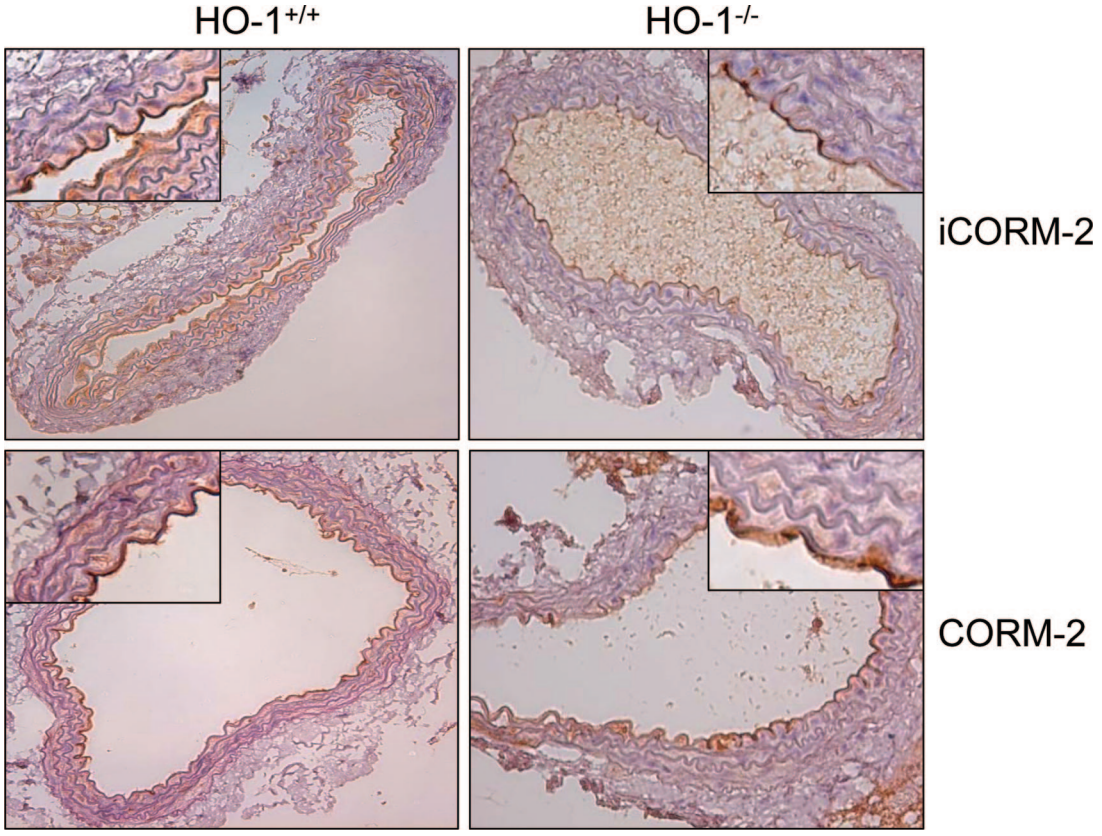

Figure 5. Endothelial nitric oxide synthase (eNOS) expression in aortic grafts in CORM-2 and iCORM-2-treated *HO-1*$^{+/+}$ and *HO-1*$^{-/-}$ recipients. Immunohistochemical staining of eNOS in allogeneic aortic grafts at 24 hours following transplantation in *HO-1*$^{+/+}$ (**left**) and *HO-1*$^{-/-}$ (**right**) recipients treated with iCORM-2 (**top**) or CORM-2 (**lower**). Original magnification, ×200; **inset**, ×400.

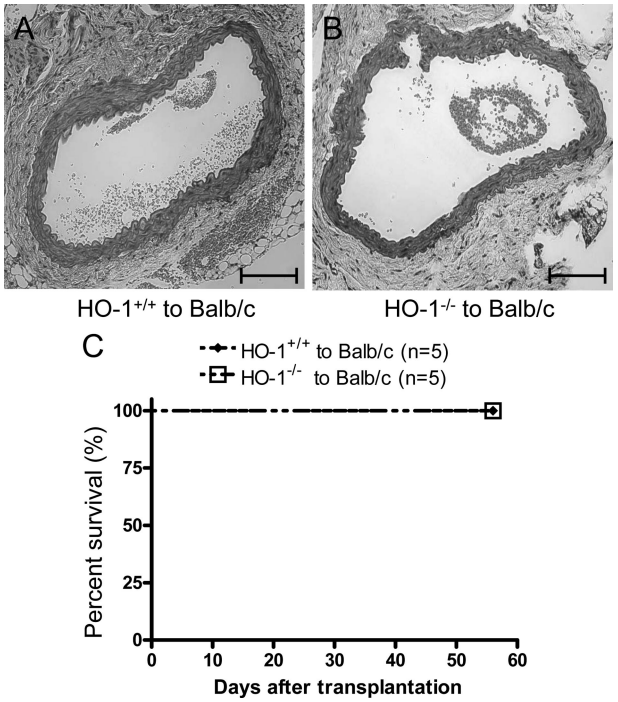

Figure 6. Aortic transplantation from *HO-1*$^{+/+}$ or *HO-1*$^{-/-}$ donors to Balb/cJ recipients. **A** and **B,** H&E staining of allogeneic aortic grafts 8 weeks after transplantation. Inflammation and fibrosis of the adventitia can be found in both groups. Intimal hyperplasia, narrowing and denucleation of the media were not significant. Original magnification , ×200; scale bar = 100 μm. **C:** Kaplan-Meier survival analysis of these two groups. All Balb/cJ recipients receiving either *HO-1*$^{+/+}$ or *HO-1*$^{-/-}$ donors (n = 5 per group) survived to 8 weeks following transplantation.

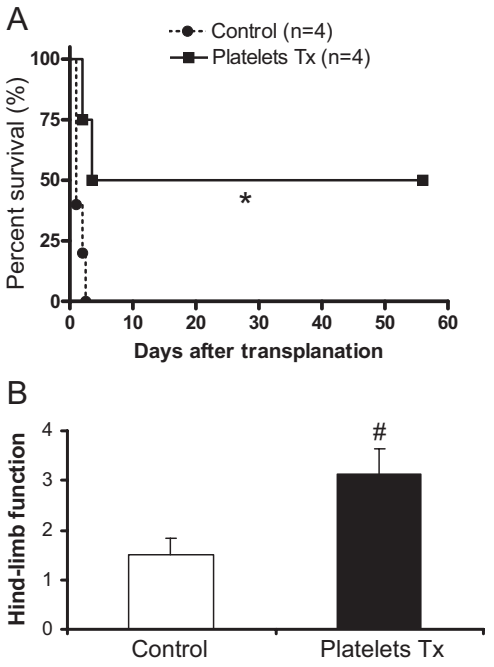

Figure 7. Survival of *HO-1*$^{-/-}$ recipients receiving wild-type platelets and hind-limb function at 24 hours after transplantation. **A:** Kaplan-Meier survival analysis of *HO-1*$^{-/-}$ aortic allograft recipients treated with *HO-1*$^{+/+}$ platelets (solid line) and of control *HO-1*$^{-/-}$ recipients that did not receive platelets but an equal volume of saline (dashed line, $^{*}P < 0.01$ versus treated group). **B:** Hind-limb function at 24 hours after transplantation. $^{\#}P < 0.05$ versus control group.

response to the graft by the host, rather than the graft endothelium per se.

To test this conclusion, we determined the role of local HO-1 expression in the arterial graft on the development of thrombus formation. We transplanted *HO-1*$^{+/+}$ or *HO-1*$^{-/-}$ mouse aortic grafts to *HO-1*$^{+/+}$ Balb/cJ recipients (n = 5/group) and found that local expression of HO-1 had no measurable effect on thrombosis (Figure 6, A and B) or survival (Figure 6C).

Adoptive Transfer of Wild-Type Platelets Prolonged Survival of HO-1-Deficient Aortic Graft Recipients

Based on our findings that systemic CO is important, the presence of large aggregates of platelets in the vascular lumen, and on previous studies by others that platelet aggregation is inhibited by CO,[32,33] we hypothesized that a lack of HO-1 expression in platelets may be a key factor in the development of post-transplant thrombosis in the *HO-1*$^{-/-}$ mice receiving *HO-1*$^{+/+}$ Balb/cJ aortic grafts. To test this hypothesis, we performed adoptive transfer of *HO-1*$^{+/+}$ platelets to *HO-1*$^{-/-}$ mice via tail vein injection before aortic transplantation. As shown in Figure 7A, two of four *HO-1*$^{-/-}$ mouse recipients survived to 8 weeks ($P < 0.05$, compared with *HO-1*$^{-/-}$ mice without platelet transfusion). Accordingly, the *HO-1*$^{-/-}$ recipients that received wild-type platelets also showed significantly less hind-limb paralysis after transplantation (Figure 7B, $P < 0.05$).

Discussion

We have previously shown that the protective effects of systemic administration of interleukin-10 are mediated through a HO-1-dependent mechanism in a Dark Agouti to Lewis rat model of aortic transplantation.[34] To confirm this finding we embarked on further studies to test the role of HO-1 in the protective effect of interleukin-10 on the development of vascular lesions in a mouse aortic transplantation model using *HO-1*$^{-/-}$ mice. Surprisingly, we found that all of the *HO-1*$^{-/-}$ recipient animals, irrespective of interleukin-10 or control treatment, died within 4 days with significant thrombosis located in the allogeneic aortic grafts. In contrast, wild-type recipients showed 100% graft patency and survival for >56 days. This report describes our findings that treatment of HO-1-deficient recipients with CORM-2, a CO-releasing molecule, markedly reduced local platelet aggregation in the allografts and resulted in significantly improved survival in comparison with *HO-1*$^{-/-}$ recipients treated with iCORM-2. These results indicate that application of CO significantly ameliorates thrombus formation in the absence of HO-1. This requirement for CO or HO-1 is systemic because transplantation of aorta from wild-type mice into *HO-1*$^{-/-}$ mice produced similar results. In addition, adoptive transfer of wild-type platelets into HO-1 deficient recipi-

ents also resulted in increased survival and improved hind-limb function compared with control animals.

Thrombosis is a critical event in vascular diseases associated with myocardial infarction and stroke, and in venous thromboembolic disorders, which are a major cause of morbidity and mortality in postoperative patients.[35] Thrombus formation involves the interaction of vascular wall injury and circulating blood cells including leukocytes and platelets. Following endothelial cell damage, tissue factors are released and collagen in the subendothelial matrix becomes exposed, which then triggers platelet activation and aggregation, resulting in thrombus formation.[36] Mice with complete HO-1 deficiency by genetic deletion[37,38] or mice in which HO activity has been inhibited by chemical means (eg, tin protoporphyrin-IX),[36,39] exhibit an accelerated thrombotic response to vascular injury compared with wild-type mice. In this report, we showed accelerated thrombus formation in the setting of allogeneic aortic transplantation, a model that differs from previously reported murine models of vena cava thrombosis and carotid arterial thrombosis induced by oxidant, electric, or photochemical injury.[31,36,38,40]

HO-1 is well known for protective effects against cellular damage, smooth muscle cell proliferation, and vascular constriction caused by vascular inflammation and ischemia-reperfusion injury.[8–10] Previous studies have addressed the role of HO-1 expression and/or CO in modulation of vascular endothelial cell damage, which is a key factor in thrombus formation following hypoxia or oxidant injury,[38,41] irradiation-induced apoptosis,[42] and interleukin-18-dependent inflammation.[43] HO-1 expression or endogenously generated CO inhibits production of proinflammatory cytokines such as tumor necrosis factor-α, interleukin-1, and monocyte chemoattractant protein-1, most likely through inhibition of NF-κB activation.[13,20] These same mechanisms also limit the expression of adhesion molecules such as E- and P-selectin, and the expression of tissue factor and plasminogen activator inhibitor-1, which can facilitate intravascular thrombus formation.[38] In this study, we did not observe significant differences in the morphology of vascular endothelial cells within allografts in HO-1 wild-type and deficient recipients treated with either iCORM-2 or CORM-2. Moreover, experiments in which we transplanted HO-1 wild-type or deficient aortic grafts to Balb/cJ recipients suggested the expression of HO-1 by local vascular endothelial cells had no effect on thrombosis or survival. Therefore, protection of the endothelium by HO-1 or its products may not be the primary mechanism of defense against thrombosis.

Previous studies have shown that platelets possess functional HO-1,[44] and hemin, an inducer of HO-1, dramatically suppresses platelet activation *in vitro* and prevents platelet-dependent thrombosis *in vivo*.[36,37,39] True et al found that platelet counts, bleeding time, platelet aggregation and prothrombin time were not significantly different in HO-1 wild-type and deficient mice,[38] suggesting that HO-1 may not be essential for megakaryopoiesis or platelet production. In our experiments, all of the *HO-1*$^{-/-}$ recipient animals receiving allogeneic aortic grafts died within 4 days after transplantation due to platelet-dependent thrombosis. In contrast, adoptive transfer of wild-type platelets and CO administration rescued HO deficient animals from acute thrombus formation indicating that loss of HO-1 expression in platelets might play an important role in the accelerated thrombosis that contributed to poor survival of the HO-1-deficient recipients.

To deliver CO, we used CORM-2, a chemical compound used by several investigators to demonstrate cytoprotection by CO in models of microbial sepsis, liver inflammation, and renal ischemia-reperfusion injury,[6,20,22] In this model, systemic administration of CO was required to protect aortic grafts from thrombosis. Similar protection was also produced by administration of HO-1-expressing wild-type platelets.

The role of CO in platelets is not clearly understood. CO has been previously reported to attenuate platelet aggregation following elevation of cGMP-mediated activation of guanylate cyclase.[32,45] In contrast, Chlopicki et al found CO inhibits platelet aggregation by a mechanism independent of soluble guanylate cyclase, possibly by affecting calcium-activated potassium channels, cytochrome *P450*, the mitochondrial respiratory chain, or p38MAPK.[33] CO can selectively promote phosphorylation of vasodilator-stimulated phosphoprotein , which is a critical actin motor protein required for platelet aggregation.[46] Apart from direct effects on platelet function, it is possible that platelets serve as a source of CO, leading to direct vascular relaxation by CO or CO-dependent suppression of plasminogen activator inhibitor-1.[37,47] In addition, wild-type platelets could ameliorate thrombosis through selective delivery of HO-1, biliverdin, or CO to the graft site.

In summary, we demonstrate that HO-1 expression plays an important role in prevention of vascular arterial thrombosis in murine aortic allotransplantation, and systemic administration of a byproduct of HO-1 activity, CO, or adoptive transfer of HO-1-expressing platelets rescues HO-1-deficient recipients from thrombosis after transplantation. We suggest these findings may offer mechanistic insights into the therapeutic effects of HO-1 induction or CO administration in organ transplantation.

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