

# Absence of Heme Oxygenase-1 Exacerbates Myocardial Ischemia/Reperfusion Injury in Diabetic Mice

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**Increased production of reactive oxygen species contributes to the etiology of diabetes complications. Pathophysiological stimuli that increase oxidative stress upregulate heme oxygenase (HO)-1, a cytoprotective heme-degrading enzyme. We hypothesized that HO-1 may be important in myocardial injury that is exacerbated by diabetes. To test this hypothesis, the left anterior descending coronary arteries of nondiabetic and diabetic wild-type (HO-1<sup>+/+</sup>) and HO-1 null (HO-1<sup>-/-</sup>) mice were ligated for 1 h followed by 24 h reperfusion. The absence of HO-1 significantly increased myocardial infarct size (36.4 ± 2.0 vs. 21.4 ± 1.8% in HO-1<sup>+/+</sup> mice), while cardiac-specific overexpression of HO-1 protected against myocardial ischemic injury in diabetic mice. Despite similar high blood glucose levels, diabetic HO-1<sup>-/-</sup> mice had fourfold higher oxidative stress and larger infarcts (56.0 ± 2.8%) than diabetic HO-1<sup>+/+</sup> mice (30.8 ± 6.1%). Moreover, hyperglycemia increased the mortality of HO-1<sup>-/-</sup> mice (31.3%) after ischemia/reperfusion injury, and 55% of diabetic HO-1<sup>-/-</sup> mice had mural thrombi in the left ventricles. The increased mortality of diabetic HO-1<sup>-/-</sup> mice may be in part due to formation of left ventricular mural thrombi. Our data demonstrate that the absence of HO-1 renders animals more susceptible to myocardial ischemia/reperfusion damage and diabetes worsens the injury. *Diabetes* 54: 778–784, 2005**

**C**oronary artery disease leading to myocardial infarction and heart failure is a chronic complication of diabetes, accounting for >75% of hospitalizations in diabetic patients (1–3). In addition, myocardial infarction accounts for nearly 50% of all deaths in patients with diabetes (4). The mortality rate of diabetic patients after acute myocardial infarction resulting from ischemia/reperfusion injury is approximately twice that of nondiabetic patients (5–7).

Several reports have suggested that increased oxidative stress contributes to the development and progression of diabetes complications (8–10). Heme oxygenase (HO)-1,

the inducible isoform of the HO family of proteins, is induced by a variety of stimuli that increase oxidative stress (11,12). By degrading the oxidant heme and generating the antioxidant bilirubin and anti-inflammatory molecule carbon monoxide (CO), HO-1 protects cells from death due to pathophysiological stress and oxidative injury (13–18). In diseases that are initiated by stress, such as atherosclerotic lesion formation and vascular remodeling, lack of endogenous HO-1 exacerbates lesion formation (19). Given its cytoprotective role in many cell types and organs, there is growing interest in the role of HO-1 in diabetes (20–23). HO-1 protects islet cells from apoptosis and improves in vivo function after transplantation (20). HO-1 also protects diabetic rats from endothelial cell dysfunction by preventing endothelial cell sloughing (24).

Myocardial ischemia/reperfusion injury results in greater damage in the diabetic (*db/db*) mouse hearts than nondiabetic controls (25). While Nishio et al. (26) reported HO-1 induction in diabetic rat hearts, the functional role of HO-1 in diabetes complications of myocardial infarction has not been examined. We have shown that in response to hypoxia, which leads to pulmonary hypertension, HO-1 null (HO-1<sup>-/-</sup>) mice develop severe right ventricular dilatation and infarction due to oxidative damage (13). This prior study demonstrated the detrimental consequences of HO-1 deficiency in the heart in response to pathological stress. HO-1 has a critical role in cardiac homeostasis in response to oxidative stress-induced injury (16,18,27) and that hyperglycemia increases the production of reactive oxygen species in diabetes (28,29). We hypothesized that in the absence of HO-1, cardiomyocytes may be more susceptible to ischemia/reperfusion damage and diabetes may exacerbate the myocardial injury. To test our hypothesis in vivo, we induced diabetes in wild-type and HO-1<sup>-/-</sup> mice by streptozotocin (STZ) and subjected the nondiabetic control and diabetic animals to a myocardial infarction model to determine if the absence of HO-1 renders mice more prone to myocardial damage.

## RESEARCH DESIGN AND METHODS

Wild-type (HO-1<sup>+/+</sup>) and HO-1<sup>-/-</sup> mice were obtained by breeding HO-1 heterozygous mice (129Sv and BALB/c mixed genetic background) (13). Cardiac-specific HO-1 transgenic mice (FVB genetic background) were as previously described (16). This study used the TG.H transgenic line with high HO-1 exogenous expression levels (16). Wild-type littermates were used as controls. Animal experiments were performed in accordance with NIH (National Institutes of Health) guidelines, and protocols were approved by the Harvard Medical Area Standing Committee on Animals.

**STZ-induced diabetes in mice.** At 6 weeks of age, male mice were treated with STZ (Sigma) at a dose of 50 mg/kg body wt in 20 mmol/l citrate buffer, pH 4.5, by five daily intraperitoneal injections (30). Control mice were injected with citrate buffer alone. Fasting glucose levels were measured from tail vein

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H&E, hematoxylin and eosin; HO, heme oxygenase; STZ, streptozotocin.

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blood using the ACCU-CHEK blood glucose meter (Roche, Indianapolis, IN). Diabetes was confirmed by the presence of hyperglycemia ( $>200$  mg/dl).

**Northern blot analysis.** Total RNA was isolated from mouse hearts using RNA-Bee RNA isolation reagent (Tel-Test, Friendswood, TX), and Northern blot analysis was performed as described (13). The filters were hybridized with a random-primed  $^{32}$ P-labeled HO-1 cDNA probe (31). Equal loading was verified by hybridizing the filters to a  $^{32}$ P-labeled oligonucleotide complementary to 28S ribosomal RNA.

**Mouse model of myocardial infarction.** Four weeks after STZ administration, control and diabetic mice were subjected to myocardial ischemia and reperfusion injury (16). During the operation, the animals were kept warm by using heat lamps. In brief, myocardial ischemia was achieved by ligating the anterior descending branch of the left coronary artery. Regional ischemia was confirmed by visual inspection of pale color in the occluded distal myocardium. After occlusion for 1 h, the ligature was released, allowing reperfusion of the formerly ischemic myocardium. Blood flow was confirmed by visualization of the return of a bright red color in the previously pale region. After the chest wall was closed, the animal was kept warm on a  $37^{\circ}\text{C}$  warming plate until recovery from anesthesia (1–2 h). Hearts were harvested after 24 h reperfusion.

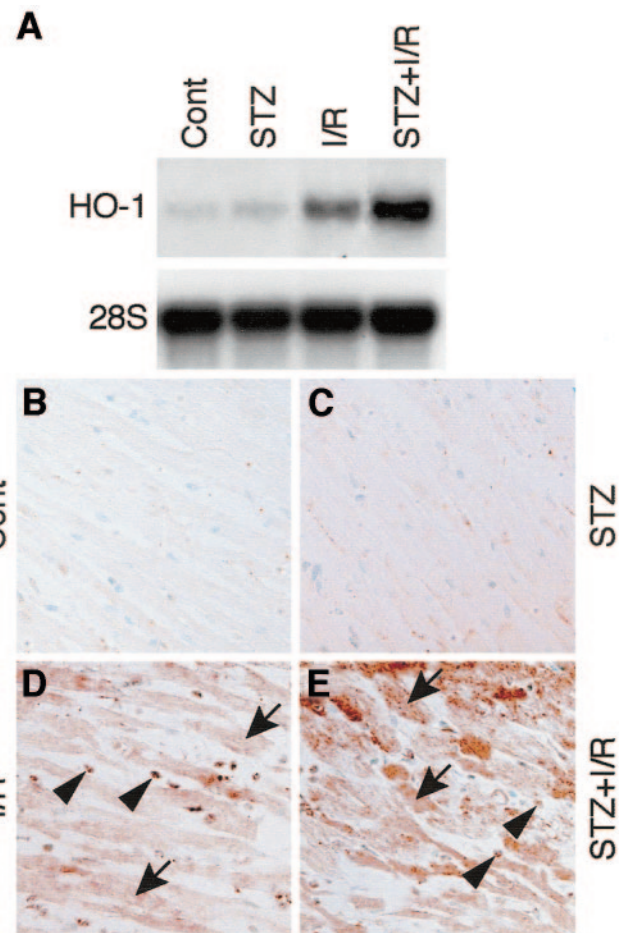
**Assessment of area at risk and infarct size.** To assess myocardial infarct size after 1 h ischemia and 24 h reperfusion, the left coronary artery was occluded with a suture at the same site as the initial ligation (16). To demarcate the ischemic area at risk, 1% Evans blue dye was perfused into the aorta and coronary arteries (with distribution throughout the ventricular wall proximal to the site of coronary artery ligation) to stain the nonischemic area blue (32,33). Hearts were excised and sliced into five ( $\sim 1$  mm) cross sections below the ligation. Heart sections were incubated in a 1% triphenyltetrazolium chloride solution to stain viable myocardium red and the infarct area pale. The infarct area (pale), the area at risk (not blue), and the total left ventricular area from both sides of each section were measured using NIH Image software and averaged. The thickness of each section was measured using dial calipers. The left ventricular area, area at risk, and infarct area of each section were multiplied by the thickness of the section and then totaled from all five sections. The ratio of area at risk to left ventricular area and the ratio of infarct area to area at risk were calculated and expressed as a percentage, as previously described (16).

**Histological analysis and immunohistochemistry.** Ventricles from HO-1 $^{+/+}$  and HO-1 $^{-/-}$  mice were fixed in Methyl Carnoy's solution (60% methanol, 30% chloroform, and 10% acetic acid) at  $4^{\circ}\text{C}$  for 5 h, then in 70% ethanol overnight, and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E). To detect HO-1 expression and inflammatory cells, sections were stained with a polyclonal HO-1 antiserum (SPA-895, diluted 1:400; StressGen Biotechnologies, Victoria, BC, Canada) and a mouse CD45 (leukocyte common antigen) antibody (diluted 1:1,000; BD Biosciences Pharmingen, San Diego, CA) at  $4^{\circ}\text{C}$  overnight. Sections were counterstained with methyl green. To detect oxidation-specific lipid protein adducts, sections were stained with MAL-2 antibody (diluted 1:100; provided by J. Witztum, University of California, San Diego, La Jolla, CA) (13,16,34). To assess inflammatory cell and oxidative stress accumulation, the areas of ischemic myocardium (obtained at  $\times 100$  magnification) were measured by computerized planimetry, and the areas staining positive for CD45 and MAL-2 were measured by colorimetric analysis (13,16,35). The respective positive staining area was divided by the ischemic myocardial area and multiplied by 100.

**Statistical analysis.** Data are presented as means  $\pm$  SE. Statistical significance was determined by a Student's *t* test. A *P* value  $\leq 0.05$  was accepted as statistically significant.

## RESULTS

**HO-1 induction by myocardial ischemia/reperfusion in diabetic mice.** To investigate the myocardial expression of HO-1 in diabetic mice, we used an animal model of diabetes. We induced diabetes in mice by injection of STZ and examined HO-1 expression after 28 days. Northern blot analysis showed that STZ injection ( $n = 3$ ) did not substantially increase HO-1 expression in the heart relative to vehicle-treated controls ( $n = 4$ ) (Fig. 1A,  $118 \pm 14\%$  STZ vs. Cont). Our results are different from the fourfold induction observed in rat hearts after STZ injection (26). It is possible that the kinetics of HO-1 induction by STZ may differ between mouse and rat hearts. To evaluate the combined effects of diabetes and myocardial infarction on HO-1 expression, vehicle-treated control and STZ-induced

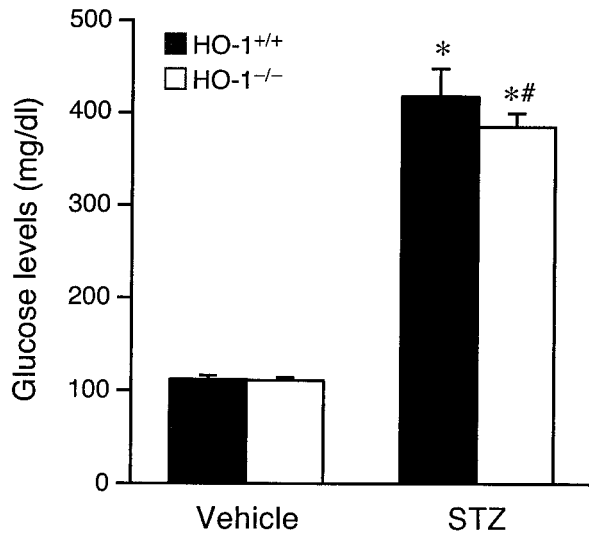


**FIG. 1.** Diabetes and myocardial ischemia/reperfusion injury induce HO-1 expression in the heart. Mouse hearts were harvested 4 weeks after vehicle or STZ injection for Northern and immunohistochemical analyses. **A:** Northern blot analysis of mouse heart RNA. Cont, vehicle-treated control; STZ, STZ-induced diabetes; I/R, control mice subjected to 1 h myocardial ischemia/24 h reperfusion; STZ+I/R, STZ-induced diabetic mice subjected to 1 h ischemia/24 h reperfusion. The blots were hybridized with a random-primed  $^{32}$ P-labeled HO-1 cDNA probe that hybridizes to a 1.8-kb HO-1 message. The blots were subsequently hybridized with a  $^{32}$ P-labeled 28S oligonucleotide to verify equivalent loading. Ventricular sections from vehicle-treated control (**B**) or after STZ injection (**C**), control mice subjected to 1 h myocardial ischemia/24 h reperfusion (**D**), and STZ mice subjected to 1 h ischemia/24 h reperfusion (**E**) were stained with an HO-1 antiserum. Brown staining indicates HO-1 expression. Arrows indicate cardiomyocyte staining, and arrowheads indicate inflammatory cell staining. Original magnification  $\times 200$ .

diabetic mice were subjected to 1 h ischemia and 24 h reperfusion to experimentally induce myocardial infarction. In vehicle-treated mice, myocardial ischemia/reperfusion increased HO-1 expression by fourfold (Fig. 1A). Diabetes further enhanced HO-1 expression in the heart after ischemia/reperfusion ( $n = 2$ ) to a sixfold higher level than control (Fig. 1A, STZ+I/R). In contrast, HO-2 expression was not changed (data not shown).

Consistent with Northern analysis (Fig. 1A), immunostaining with an HO-1 antibody revealed that HO-1 protein was barely detectable in the heart from vehicle-treated control mice (Fig. 1B). STZ slightly increased HO-1 levels in the cardiomyocytes 28 days after treatment (Fig. 1C). Ischemia/reperfusion strongly induced HO-1 protein expression in the cardiomyocytes (Fig. 1D, brown, arrows) and infiltrated inflammatory cells (Fig. 1D, arrowheads).





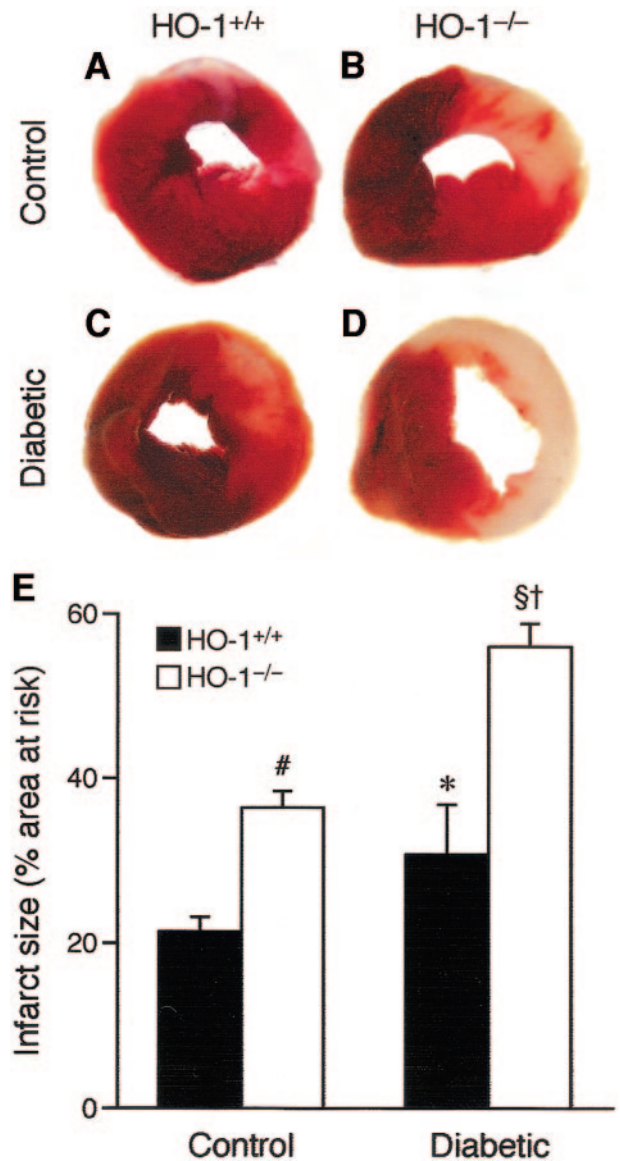
**FIG. 2.** No alterations in blood glucose levels in HO-1<sup>-/-</sup> mice after STZ injection. Fasting blood glucose levels were measured 4 weeks after treatment in HO-1<sup>+/+</sup> mice (■) treated with vehicle ( $n = 10$ ) or STZ ( $n = 9$ ) and HO-1<sup>-/-</sup> mice (□) treated with vehicle ( $n = 11$ ) or STZ ( $n = 16$ ). Error bars indicate SEs. \* $P < 0.05$  vs. vehicle controls; # $P = 0.34$  vs. STZ-induced diabetic HO-1<sup>+/+</sup> mice.

Furthermore, intense brown staining in the hearts from diabetic mice after ischemia/reperfusion indicated elevated HO-1 protein levels in the cardiomyocytes and inflammatory cells (Fig. 1E, arrows and arrowheads, respectively). These data suggest that HO-1 induction may protect against myocardial injury.

**STZ-induced hyperglycemia in both wild-type and HO-1 null mice.** To assess the functional role of HO-1 in diabetic myocardial infarction, we first induced diabetes in HO-1<sup>+/+</sup> and HO-1<sup>-/-</sup> mice. Under basal physiological conditions, the fasting blood glucose levels were not different between HO-1<sup>+/+</sup> ( $121 \pm 4$  mg/dl,  $n = 19$ ) and HO-1<sup>-/-</sup> mice ( $117 \pm 3$  mg/dl,  $n = 27$ ;  $P = 0.44$  vs. wild type). Fasting blood glucose levels in HO-1<sup>+/+</sup> mice increased to  $418 \pm 30$  mg/dl ( $n = 9$ ) 28 days after STZ, as compared with vehicle-treated controls ( $112 \pm 4$ ,  $n = 10$ ;  $P < 0.05$ ) (Fig. 2, black bars). In response to STZ injection, HO-1<sup>-/-</sup> mice also developed elevated fasting blood glucose levels ( $385 \pm 15$  mg/dl,  $n = 16$ ) in comparison with vehicle-treated HO-1<sup>-/-</sup> mice ( $111 \pm 3$ ,  $n = 11$ ;  $P < 0.05$ ) (Fig. 2, white bars). STZ induced hyperglycemia to a similar degree in HO-1<sup>+/+</sup> and HO-1<sup>-/-</sup> mice ( $P = 0.34$ ) (Fig. 2).

**Absence of HO-1 increases myocardial infarct size.** To test whether an absence of HO-1 renders animals more prone to cardiac damage, we subjected HO-1<sup>+/+</sup> and HO-1<sup>-/-</sup> mice to ischemia/reperfusion injury. In HO-1<sup>+/+</sup> mice, 1 h ischemia and 24 h reperfusion resulted in  $21.4 \pm 1.8\%$  ( $n = 7$ ) infarction of the left ventricular area at risk (Fig. 3A and E). In contrast, HO-1<sup>-/-</sup> mice had larger infarcts (Fig. 3B) that were 15% greater ( $36.4 \pm 2.0\%$ ,  $n = 6$ ) than HO-1<sup>+/+</sup> mice ( $P < 0.005$ ) (Fig. 3E). These results indicate that in normoglycemic mice, HO-1 protects against myocardial injury.

**Diabetes exacerbates myocardial infarction in the absence of HO-1.** To determine the effects of diabetes in the setting of HO-1 deficiency in myocardial infarction, we subjected diabetic HO-1<sup>+/+</sup> and HO-1<sup>-/-</sup> mice to ischemia/



**FIG. 3.** Absence of HO-1 increases myocardial infarct size in diabetic mice. Myocardial infarcts from control (vehicle-treated) HO-1<sup>+/+</sup> (A;  $n = 7$ ) or HO-1<sup>-/-</sup> (B;  $n = 6$ ) mice and diabetic HO-1<sup>+/+</sup> (C;  $n = 7$ ) or HO-1<sup>-/-</sup> (D;  $n = 7$ ) mice were assessed by Evans blue and triphenyltetrazolium chloride staining after 1 h ischemia/24 h reperfusion. Representative sections are shown. The Evans blue-perfused area, which is not at risk, stained blue, while viable myocardium stained red and infarcted myocardium appeared pale. Original magnification  $\times 15$ . E: Infarct sizes from HO-1<sup>+/+</sup> (■) and HO-1<sup>-/-</sup> (□) hearts were assessed as percent area at risk (infarcted area divided by risk area and multiplied by 100). # $P < 0.005$  vs. control HO-1<sup>+/+</sup> mice; \* $P = 0.08$  vs. control HO-1<sup>+/+</sup> mice; § $P < 0.005$  vs. control HO-1<sup>-/-</sup> mice; † $P < 0.005$  vs. diabetic HO-1<sup>+/+</sup> mice.

reperfusion injury. In HO-1<sup>+/+</sup> mice, hyperglycemia slightly increased infarct size of HO-1<sup>+/+</sup> mice to  $30.8 \pm 6.1\%$  ( $n = 7$ ) (Fig. 3C and E) compared with control HO-1<sup>+/+</sup> mice, although it did not reach statistical significance ( $P = 0.08$ ) (Fig. 3E, black bars). An absence of HO-1 in diabetic mice significantly exacerbated myocardial injury (Fig. 3D). Diabetic HO-1<sup>-/-</sup> mice had much larger infarcts ( $56.0 \pm 2.8\%$ ,  $n = 7$ ) than diabetic HO-1<sup>+/+</sup> mice ( $P < 0.005$ ) or control HO-1<sup>-/-</sup> mice ( $P < 0.005$ ) (Fig. 3E).

**Myocardial ischemia/reperfusion increases mortality in diabetic HO-1<sup>-/-</sup> mice.** There were no obvious histological differences in the hearts from HO-1<sup>+/+</sup> and HO-1<sup>-/-</sup>

TABLE 1  
Mortality rate in HO-1<sup>+/+</sup> and HO-1<sup>-/-</sup> mice after myocardial ischemia/reperfusion injury

Genotype	Group	No. of mice		Mortality (%)
		Total	Death*	
HO-1 <sup>+/+</sup>	Control	12	1	8.3
	Diabetes	13	2	15.4
HO-1 <sup>-/-</sup>	Control	11	1	9.1
	Diabetes	16	5	31.3

\*Death indicates mice that survived 1 h ischemia but died during 24 h reperfusion.

mice under normal physiological conditions (13) or 28 days after STZ injection (data not shown). In response to myocardial injury (1 h ischemia/24 h reperfusion), vehicle-treated HO-1<sup>+/+</sup> and HO-1<sup>-/-</sup> mice had similar mortality (8.3% and 9.1%, respectively; Table 1). Hyperglycemia increased the mortality of HO-1<sup>+/+</sup> mice almost twofold to 15.4% after ischemia/reperfusion. In contrast, 5 of the 16 (31.3%) diabetic HO-1<sup>-/-</sup> mice that survived 1 h myocardial ischemia died during the subsequent 24 h reperfusion (Table 1).

**Exacerbation of myocardial injury in diabetic HO-1<sup>-/-</sup> mice.** To investigate the cause of increased mortality in diabetic HO-1<sup>-/-</sup> mice after ischemia/reperfusion, we examined ventricular sections by histological analysis. H&E staining revealed that ischemia/reperfusion caused cardiomyocyte damage, indicated by disruption of cardiomyocytes, in nondiabetic control HO-1<sup>+/+</sup> mice (Fig. 4A, arrow). Hyperglycemia slightly increased cardiomyocyte injury in similar ischemic areas in HO-1<sup>+/+</sup> mice (Fig. 4C, arrow). In comparison, an absence of HO-1 exacerbated myocardial damage in the ischemic areas in both control and diabetic HO-1<sup>-/-</sup> mouse hearts (Fig. 4B and D, arrows). In addition to myocyte damage, CD45-positive mononuclear inflammatory cell infiltration was evident within the infarcts of control HO-1<sup>+/+</sup> hearts after ischemia/reperfusion (Fig. 4E, brown). In similar ischemic areas, diabetic HO-1<sup>+/+</sup> hearts had comparable levels of inflammatory cell infiltration (1.8 ± 0.3%) with control HO-1<sup>+/+</sup> hearts (1.6 ± 0.1%). Interestingly, a similar degree of inflammatory cell infiltration was observed in the ischemic myocardium from control HO-1<sup>-/-</sup> (2.0 ± 0.1%) and diabetic HO-1<sup>-/-</sup> (2.1 ± 0.1%) mice subjected to ischemia/reperfusion (Fig. 4F and H). We next assessed oxidative stress in the ischemic hearts by MAL-2 immunostaining, which recognizes oxidation-specific lipid-protein adducts. In HO-1<sup>+/+</sup> mice, oxidative stress was 2.1 ± 0.1% of the ischemic area in control compared with 3.3 ± 0.1% in diabetic hearts. Diabetes increased oxidative stress by nearly twofold in HO-1<sup>-/-</sup> mouse hearts (from 8.3 ± 0.7 to 15.2 ± 1.5%). Loss of HO-1 resulted in an approximate fourfold increase in oxidative stress in the settings of either control or diabetes after ischemia/reperfusion injury, which led to the highest oxidative stress levels in diabetic HO-1<sup>-/-</sup> mouse hearts.

**Ischemia/reperfusion induces mural thrombi in diabetic HO-1<sup>-/-</sup> mice.** Histological analysis revealed extensive myocardial damage and inflammatory cell infiltrates in diabetic HO-1<sup>-/-</sup> mice subjected to ischemia/reperfusion; however, it is likely that additional factors contribute to the higher mortality in these mice. Surprisingly, after ischemia/reperfusion, we observed that three of four

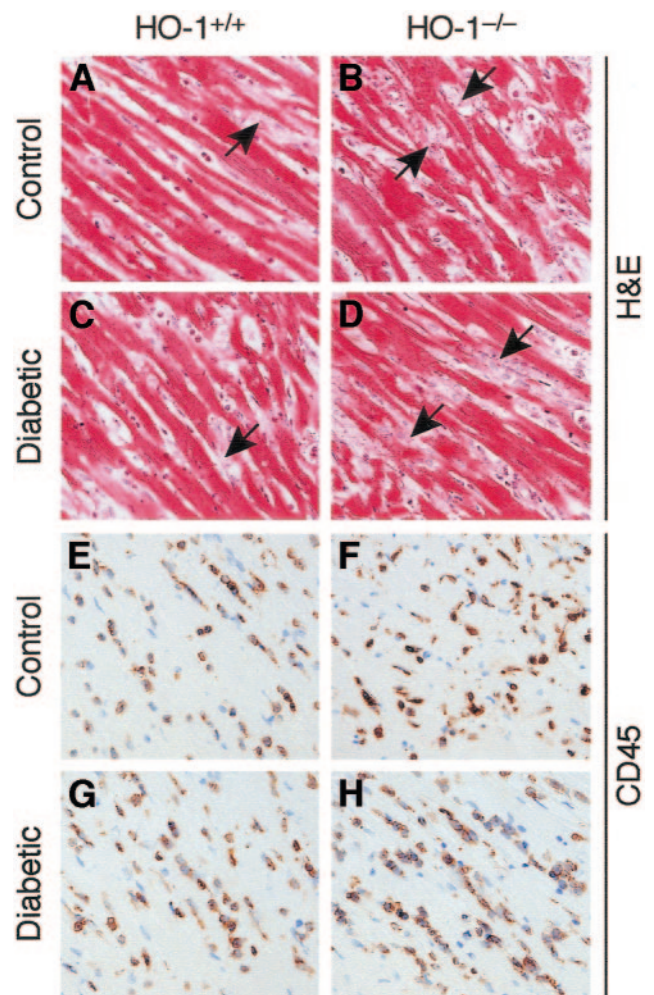
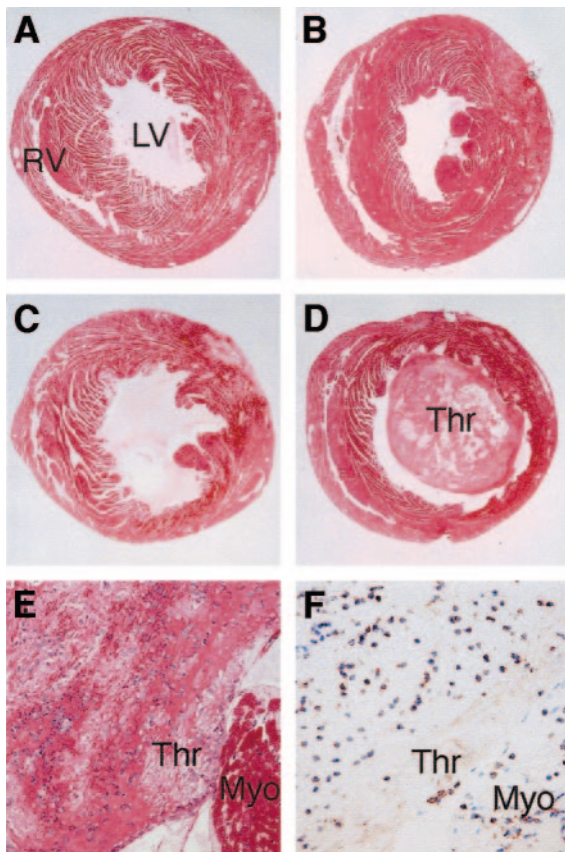


FIG. 4. Diabetes exacerbates myocardial injury in the absence of HO-1. H&E-stained left ventricular sections from similar ischemic areas from control HO-1<sup>+/+</sup> (A; n = 2) or HO-1<sup>-/-</sup> (B; n = 2) mice and diabetic HO-1<sup>+/+</sup> (C; n = 2) or HO-1<sup>-/-</sup> (D; n = 4) mice after ischemia/reperfusion. Arrows indicate disruption of cardiomyocytes. CD45-stained left ventricles from vehicle-treated HO-1<sup>+/+</sup> (E) or HO-1<sup>-/-</sup> (F) mice and diabetic HO-1<sup>+/+</sup> (G) or HO-1<sup>-/-</sup> (H) mice after ischemia/reperfusion. CD45-positive cells stained brown. Original magnification ×200.

hearts from diabetic HO-1<sup>-/-</sup> mice harvested for histological analysis contained large mural thrombi, which adhered to the infarcted left ventricles (Fig. 5D). Additionally, we observed mural thrombi in three of seven hearts evaluated for infarct size. In total, mural thrombi developed in 55% of diabetic HO-1<sup>-/-</sup> mice that survived 1 h ischemia and 24 h reperfusion. In contrast, no mural thrombi were present in the hearts from control and diabetic HO-1<sup>+/+</sup> or control HO-1<sup>-/-</sup> mice subjected to ischemia/reperfusion (Fig. 5A–C). Ventricular sections revealed extensive inflammatory cell infiltration within the thrombi (Fig. 5E and F).

**Cardiac-specific overexpression of HO-1 reduces myocardial infarct size in diabetic mice.** To investigate the role of HO-1 in protecting against myocardial ischemic injury in diabetes, we induced diabetes in wild-type and cardiac-specific HO-1 transgenic mice (TG.H line, FVB strain [16]) and subjected them to experimental myocardial infarction. In diabetic wild-type mice, 1 h ischemia/24 h reperfusion resulted in 52 ± 5% (n = 7) infarction of the left ventricular area at risk (Fig. 6). In contrast, the infarct



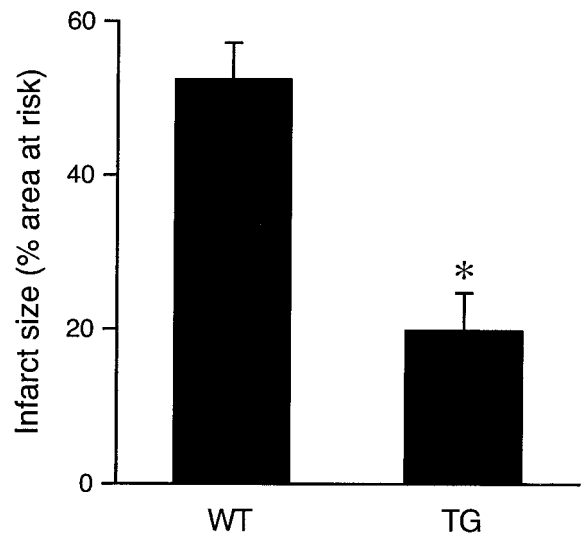


**FIG. 5.** Left ventricular mural thrombi formation in diabetic HO-1<sup>-/-</sup> mice in response to ischemia/reperfusion. Heart cross sections at the papillary muscle level from control HO-1<sup>+/+</sup> (A; *n* = 2) or HO-1<sup>-/-</sup> (B; *n* = 2) mice and diabetic HO-1<sup>+/+</sup> (C; *n* = 2) or HO-1<sup>-/-</sup> (D; *n* = 4) mice after ischemia/reperfusion were stained with H&E. LV, left ventricle; RV, right ventricle; Thr, thrombus. Original magnification  $\times 15$ . E: Higher magnification of D showing thrombus and the underlying myocardium (Myo). F: CD45 staining (brown color) in the thrombus and myocardium.

size of diabetic HO-1 transgenic mice was  $20 \pm 5\%$  (*n* = 6), significantly less than that of diabetic wild-type mice ( $P < 0.005$ ) (Fig. 6), indicating that HO-1 overexpression lessens the diabetes complication of ischemia/reperfusion injury.

## DISCUSSION

Hyperglycemia increases oxidative stress, which contributes to the development of diabetes complications (8,9,36). Oxidative stress and inflammation in turn exacerbate myocardial ischemia/reperfusion injury (37,38), leading to myocardial infarction—a major diabetes complication. HO-1 and its reaction products have been shown to have both antioxidative and anti-inflammatory properties (16,39,40), indicating a protective role of HO-1 in diabetes complications of myocardial infarction. Using HO-1 heterozygous mouse hearts in isolated perfused heart preparations, Yoshida et al. (41) showed that a reduction of HO-1 worsens the ischemic injury. By gain-of-function experiments, we have previously demonstrated that cardiac-specific overexpression of HO-1 protects against ischemia/reperfusion injury in vivo (16). However, the effect of an absence of HO-1 in myocardial injury in vivo has not been elucidated in the settings of either normo- or hyperglycemic conditions. By using homozygous HO-1 null mice,



**FIG. 6.** Cardiac-specific overexpression of HO-1 decreases myocardial infarct size in diabetic mice. Diabetic wild-type (WT; *n* = 7) and cardiac-specific HO-1 transgenic mice (TG.H line) (TG; *n* = 6) mice (both of FVB genetic background) were subjected to 1 h myocardial ischemia and 24 h reperfusion. Myocardial infarct sizes were assessed as percent area at risk (infarcted area divided by risk area and multiplied by 100). \* $P < 0.005$  vs. wild-type mice.

we demonstrated that an absence of HO-1 exacerbated myocardial injury, particularly in concert with diabetes.

Myocardial ischemia/reperfusion induced HO-1 expression in the heart (Fig. 1), suggesting that this induction may function to reduce or limit the myocardial damage and that an absence of HO-1 may exacerbate the injury. Consistent with this hypothesis, the first major finding was that HO-1<sup>-/-</sup> mice had larger infarcts than wild-type mice after experimental myocardial infarction (Fig. 3A and B).

Diabetes elevated cardiac HO-1 expression in response to ischemia/reperfusion (Fig. 1), thus implicating a protective role of HO-1 in diabetic myocardial infarction. The larger infarct of diabetic than control HO-1<sup>+/+</sup> hearts suggested that the extent of HO-1 upregulation may not be sufficient to completely protect against the damage elicited by a combination of diabetes and ischemia/reperfusion; therefore, other factors may also contribute to the myocardial injury. Nevertheless, the second major finding in this study was that despite a similar degree of hyperglycemia after STZ injection in both HO-1<sup>+/+</sup> and HO-1<sup>-/-</sup> mice (Fig. 2), diabetic HO-1<sup>-/-</sup> mice had more severe myocardial infarction (Fig. 3). These results demonstrate that upregulation of HO-1 protects against diabetic myocardial infarction. Supporting our findings, an AA genotype of T(-413)A polymorphism in the promoter region of the HO-1 gene was found to associate with reduced incidence of myocardial infarction, possibly due to the higher expression level of HO-1 (42). The protective effect of HO-1 in the diabetic heart was further supported by our findings that diabetic HO-1 transgenic mice had reduced infarct size compared with that of diabetic wild-type mice (Fig. 6). It is worth noting that the infarct size of diabetic FVB wild-type mice was larger than that of diabetic 129sv and BALB/c mixed-background wild-type mice, likely due to strain differences.

The molecular mechanisms by which HO-1 confers myocardial protection are still under investigation. Interest-

ingly, we found that loss of HO-1 did not substantially increase inflammatory infiltration after ischemia/reperfusion (Fig. 4E–H). This is somewhat unanticipated, given the anti-inflammatory properties of HO-1. One possibility is that accelerated inflammation is a major effect of myocardial reperfusion injury (38); the limited HO-1 induction in the cardiomyocytes (Fig. 1D and E) may not be able to overcome the overwhelming influx of inflammatory cells. Despite a similar degree of inflammatory cell infiltration, the absence of HO-1 increased oxidative stress levels in diabetic mice after myocardial ischemia/reperfusion injury. These data suggest that the antioxidant properties of HO-1 contribute to the protection against myocardial injury.

In addition to exaggerated infarct size, we observed an additional twofold higher mortality in diabetic HO-1<sup>-/-</sup> than in diabetic HO-1<sup>+/+</sup> mice after ischemia/reperfusion injury. Of major significance was the observation that 55% of diabetic HO-1<sup>-/-</sup> mice that survived 1 h ischemia and 24 h reperfusion developed mural thrombi (Fig. 5), despite patent coronary arteries (data not shown). Interestingly, mortality rates of diabetic patients with acute myocardial infarction remain 1.5–2 times higher than those in nondiabetic patients (7). It was suggested that this increased mortality rate is in part due to the tendency toward thrombosis in diabetic patients (7). It remains to be determined whether long-term diabetes reduces HO-1 expression in the heart, which in turn contributes to the higher mortality after myocardial infarction in diabetic patients.

Interestingly, the formation of mural thrombi in the left ventricle of diabetic HO-1<sup>-/-</sup> mice after ischemia/reperfusion injury was similar to what we observed in HO-1<sup>-/-</sup> mice that developed right ventricular mural thrombi in response to chronic hypoxia (13). We previously reported that in response to a similar degree of pulmonary hypertension in wild-type and HO-1<sup>-/-</sup> mice after chronic hypoxia (10% oxygen environment for 5–7 weeks), HO-1<sup>-/-</sup> mice had increased oxidative injury and developed right ventricular infarcts with attached mural thrombi (13). These previous data suggest that in the absence of HO-1, right ventricular cardiomyocytes have a maladaptive response to hypoxia-induced pulmonary hypertension. The myocardial ischemia/reperfusion model we used in the present study does not induce pulmonary hypertension (32). The elevated oxidative stress and larger infarcts with thrombi in the left ventricles of diabetic HO-1<sup>-/-</sup> mice suggest a similar maladaptive response of left ventricular cardiomyocytes to ischemia/reperfusion injury. HO-1 and its reaction product CO have been shown to inhibit platelet aggregation (43,44) and to suppress thrombi growth in vivo under conditions of increased oxidative stress (45). The high incidence of mural thrombi might result from increased susceptibility to thrombus formation in the absence of HO-1. Inhibition of HO-1 activity promotes the formation of platelet-fibrin thrombi (45,46). Alternatively, the left ventricular thrombi formation may be secondary to larger infarcts and the subsequent more significant left ventricular dysfunction and regional wall motion abnormalities. Taken together, it is conceivable that in the absence of HO-1, ischemia/reperfusion in the setting of diabetes resulted in excessive oxidative damage in the left myocardium. The concerted effects of severe myocardial

infarction and the susceptibility to thrombosis may eventually lead to increased mortality.

In summary, we demonstrated in the present study, by loss-of-function experiments, that HO-1 plays an essential protective role in the adaptation of the heart to diabetes complications of myocardial infarction. Taken together, our findings may provide a new target for developing therapeutic strategies to treat/prevent diabetes complications of myocardial infarction.

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