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Induction of Heme Oxygenase-1 Expression Inhibits Platelet-Dependent Thrombosis

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ABSTRACT

Heme oxygenase-1 (HO-1) plays a key role in protecting tissue from oxidative stress. Although some studies implicate HO-1 in modulating thrombosis after vascular injury, the impact of HO-1 on the rate of clot formation *in vivo* is poorly defined. This study examined the potential function of HO-1 in regulating platelet-dependent arterial thrombosis. Platelet-rich thrombi were induced in C57BL/6J mice by applying 10% ferric chloride to the exposed carotid artery. Mean occlusion time of wild-type mice ($n = 10$) was 14.6 ± 1.0 min versus 12.9 ± 0.6 min for *HO-1*$^{-/-}$ mice ($n = 11$, $p = 0.17$). However, after challenge with hemin, mean occlusion time was significantly longer in wild-type mice (16.3 ± 1.2 min, $n = 15$) than *HO-1*$^{-/-}$ mice (12.0 ± 1.0 min, $n = 9$; $p = 0.021$). Hemin administration induced an approximately twofold increase in oxidative stress, measured as plasma thiobarbituric acid reactive substances. Immunohistochemical analysis revealed that hemin induced a robust increase in HO-1 expression within the carotid arterial wall. *Ex vivo* blood clotting within a collagen-coated perfusion chamber was studied to determine whether the accelerated thrombosis observed in *HO-1*$^{-/-}$ mice was contributed to by effects on the blood itself. Under basal conditions, mean clot formation during perfusion of blood over collagen did not differ between wild-type mice and *HO-1*$^{-/-}$ mice. However, after hemin challenge, mean clot formation was significantly increased in *HO-1*$^{-/-}$ mice compared with wild-type controls. These results suggest that, under basal conditions, HO-1 does not exert a significant effect on platelet-dependent clot formation *in vivo*. However, under conditions that stimulate HO-1 production, platelet-dependent thrombus formation is inhibited by HO-1. Enhanced HO-1 expression in response to oxidative stress may represent an adaptive response mechanism to down-regulate platelet activation under prothrombotic conditions. *Antioxid. Redox Signal.* 6, 729–735.

INTRODUCTION

Heme oxygenase (HO) is a microsomal enzyme that plays a key role in protecting mammalian cells against oxidative stress (12, 18, 23, 31). HO catalyzes the degradation of heme, a prooxidant, to carbon monoxide (CO), biliverdin, and iron, by-products with antioxidant and cytoprotective properties. There are three HO isoforms, HO-1, HO-2, and HO-3. Expression of HO-1, a 32-kDa heat shock protein, is induced by a variety of stimuli, including hypoxia, shear stress, cytokines, and reactive oxygen species. HO-1 is found in several organs, including the spleen, liver, and heart. HO-1 also is expressed in the wall of blood vessels, where it may play a pivotal role in the development of vascular diseases, such as atherosclerosis and intimal hyperplasia, by modulating the responses of vascular cells to oxidative stress (9, 16, 34). Although reactive oxygen species have been implicated in the pathogenesis of thrombosis (1), very little is known regarding the potential role of HO-1 in modulating blood clotting after vascular injury. CO produced by HO-1

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enhances the production of guanosine 3′,5′-cyclic monophosphate (cGMP), an inhibitor of platelet activation, by stimulating guanylyl cyclase (5). Unlike wild-type mice, mice with complete deficiency of HO-1 develop right ventricular infarctions and intracardiac thrombi in response to hypoxia (33). In addition, suppression of HO-1 activity promotes the formation of platelet-fibrin thrombi within the arterioles of transplanted hearts (25). These results suggest that HO-1 may play an important role in determining the thrombotic response to vascular injury. However, an analysis of the impact of HO-1 on thrombus formation after direct arterial injury has not been reported. In addition, it is not clear whether the observed effects of HO-1 deficiency on thrombus formation in animal models of right ventricular infarction and cardiac transplantation resulted from effects on the hemostatic system *per se,* or simply from greater vascular injury in the absence of HO-1, which led to enhanced thrombosis. Therefore, the purpose of this study was to examine the impact of HO-1 expression, in both the basal and stimulated state, on platelet-dependent thrombosis after arterial injury. Our results suggest that, under conditions of increased oxidative stress, HO-1 plays an important antithrombotic role *in vivo,* and that this effect is mediated, at least in part, by a direct effect on the blood.

MATERIALS AND METHODS

Animals

The genetic background of mice used in experiments was C57BL/6J, except for the indicated experiments that included 129/Sv mice. HO-1-deficient (*HO-1*$^{-/-}$) mice, generated as described previously (33), were back-crossed ≥10 generations into the C57BL/6J or the 129/Sv genetic background. Genotyping of mice was performed by PCR analysis of tail DNA. All experimental groups were composed of approximately 50% males/50% females. Mice were fed normal chow (Rodent Diet 5001, LabDiet). All animal care and experimental procedures were approved by the University of Michigan Committee on Use and Care of Animals.

Murine arterial thrombosis model

Carotid artery injury and thrombosis were induced in 16–20-week-old pentobarbital-anesthetized mice (weight 22–25 g) by placing a 1 × 2 mm piece of filter paper, saturated with freshly prepared 10% ferric chloride ($FeCl_3$) solution, on the adventitia of the midportion of the surgically exposed carotid artery for 3 min, as described previously (35). Blood flow was monitored with a perivascular flow probe (Model 0.5VB, Transonic Systems). Occlusion time was defined as the interval between the initiation of vascular injury and the onset of stable occlusion, defined as loss of detectable flow for ≥1 min. As indicated, mice received twice daily intraperitoneal injections of hemin (15 mg/kg; Cal-Biochem) or phosphate-buffered saline (PBS) on each of the 3 days before being subjected to carotid artery injury. The operator was blinded to genotype and pharmacologic interventions when performing the carotid injury protocol.

Ex vivo *perfusion chamber*

Clot formation during perfusion of nonanticoagulated blood through a collagen-coated capillary tube under conditions of constant flow and shear stress was performed by modifying a previously described method (2). Mice were anesthetized by intraperitoneal administration of sodium pentobarbital (120 mg/kg) and placed on a heating pad (Fine Scientific Tools). ^{125}I-Human fibrinogen (0.5 μCi; Amersham) was administered via tail vein injection. The inferior vena cava was surgically exposed and cannulated with a 25-gauge butterfly needle that was connected via silastic tubing to a perfusion chamber consisting of two glass capillary tubes (length of each 60 mm, 0.8 mm internal diameter, Drummond Scientific), connected in series, the second of which was coated with human type III collagen (Sigma), as described (2). Tubing and capillary tubes were maintained at 37°C, and a constant flow rate (220 μl/min) through the system was maintained with a peristaltic pump. Blood flowed through the perfusion chamber for 2.0 min, after which the capillary tubes were perfused for 1 min with PBS. Before the animal was euthanized, 50 μl of whole blood was collected. The radioactive counts per minute (cpm) emitted by the perfused, washed collagen-coated capillary tube and by the sample of whole blood were measured in a gamma-counter. The ratio of (capillary tube cpm)/(blood cpm) was calculated and used as a measure of the amount of clot formation. Control experiments confirmed that clot formation within perfused capillary tubes that were not coated with collagen was negligible. In addition, induction of thrombocytopenia in mice with busulfan, as well as pretreatment of animals with the thrombin inhibitor, hiurdin, markedly suppressed clot formation during perfusion of collagen-coated capillary tubes (data not shown).

Immunoassays

HO-1 protein expression *in situ* within carotid arteries was assessed by incubating cross-sections with polyclonal anti-HO-1 antiserum (SPA-895, StressGen Biotechnologies), as described previously (34). Western blot analysis of HO-1 expression in extracts prepared from carotid arteries, platelets, and spleen was performed as described previously (20, 33).

Biochemical assays

Lipid peroxides were measured in pooled plasma as thiobarbituric acid reactive substances (TBARS) with an assay kit from ZeptoMetrix, according to the manufacturer's instructions. Results were expressed as malondialdehyde (MDA) equivalents. Platelet lysates were prepared from pooled blood samples and processed as described previously (30). The concentration of cGMP within the aqueous phase of platelet lysates was measured with a competitive enzyme immunoassay (Cayman), according to the manufacturer's instructions.

Statistics

Group data are presented as means ± 1 standard error (SEM). The Student's *t* test was used to compare groups.

RESULTS

HO-1 inhibits thrombosis under stimulated, but not basal conditions

The mean time required to form a thrombus that completely obstructed carotid artery blood flow was longer in wild-type mice (14.6 ± 1.0 min, $n = 10$) than in *HO-1*$^{-/-}$ mice (12.9 ± 0.6 min, $n = 11$), although the difference between groups was statistically insignificant ($p = 0.17$, Fig. 1). As HO-1 expression is induced by a variety of stimuli (18), similar experiments were performed with mice that had received daily injections of hemin, an inducer of oxidative stress and HO-1 expression (23), for 3 days prior to carotid artery injury. Under these conditions, thrombus formation was significantly faster in HO-1-deficient mice compared with wild-type controls, with a mean occlusion time of 16.3 ± 1.2 min for wild-type mice ($n = 15$) versus 12.0 ± 1.0 min for *HO-1*$^{-/-}$ mice ($n = 9$, $p = 0.02$).

In addition to inducing HO-1 expression, hemin is an HO-1 substrate (32). If hemin itself were prothrombotic, it is possible that thrombosis after hemin administration might be faster in HO-1-deficient mice than in wild-type mice due to a reduced capacity of the former group to metabolize hemin. To examine this possibility, a piece of filter paper saturated with hemin (40 mg/ml) was applied to the surface of the carotid artery for 3 min ($n = 1$ mouse) or for 10 min ($n = 3$ mice), and carotid blood flow was monitored. No evidence of thrombosis was observed in any of the mice during 30 min of flow monitoring. Thereafter, $FeCl_3$-saturated filter paper was applied to the same carotid artery previously exposed to hemin. Mean time to formation of an occlusive thrombus was 17.8 ± 4.8 min ($n = 4$), results similar to those of wild-type mice not exposed to hemin (16.3 ± 1.2 min). These results, and the fact that 3 days of hemin administration did not accelerate thrombus formation in wild-type mice (Fig. 1), suggested that hemin itself did not promote platelet-dependent carotid artery thrombosis.

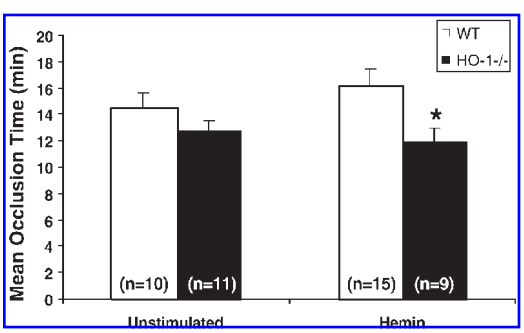

FIG. 1. Mean occlusion times after initiation of vascular injury in wild-type (WT) mice and *HO-1*$^{-/-}$ mice. Under basal (unstimulated) conditions, there was no significant difference between groups. After 3 days of hemin exposure, mean occlusion time of wild-type mice was significantly longer than that of *HO-1*$^{-/-}$ mice. *$p = 0.02$ versus hemin-treated wild-type mice.

Hemin induces oxidative stress and vascular expression of HO-1

To confirm that hemin administration induced oxidative stress, hemin was administered to wild-type mice ($n = 6$; 4 were C57BL/6J, 2 were 129/Sv) for 3 days in the same manner as for the thrombosis experiments. Control mice ($n = 6$; 4 were C57BL/6J, 2 were 129/Sv) received identical injections of PBS. TBARS were twofold higher in pooled plasma prepared from hemin-treated mice (0.72 nmol/ml MDA) compared with pooled plasma prepared from control mice (0.35 nmol/ml MDA). Carotid arteries, platelets, and spleens were retrieved from mice at the time of blood collection. We were unable to detect HO-1 protein in platelet or carotid artery extracts of wild-type mice by western blotting, including after hemin exposure, although HO-1 was readily detected in spleen, where its expression was up-regulated by hemin (data not shown). However, by using the more sensitive method of immunohistochemistry, we were able to demonstrate that arterial wall expression of HO-1 was clearly increased by hemin administration, particularly within the media (Fig. 2).

We attempted to identify potential mechanisms underlying the effect of HO-1 on thrombus formation. CO, a by-product of HO-1 metabolism of heme, stimulates production of cGMP, an inhibitor of platelet aggregation (5). We measured platelet cGMP levels in wild-type mice and *HO-1*$^{-/-}$ mice that were treated with hemin or with vehicle control for 3 days to test the hypothesis that cGMP production would be reduced in platelets from *HO-1*$^{-/-}$ mice. Platelet cGMP was 1.8–2.0-fold higher in wild-type mice treated with hemin versus vehicle (Table 1). However, basal platelet cGMP levels were similar in *HO-1*$^{-/-}$ mice compared with wild-type mice, and the increase in platelet cGMP concentration induced by hemin in *HO-1*$^{-/-}$ mice (1.6-fold) did not appear to be significantly blunted compared with that observed in wild-type mice. Biliverdin also is produced by HO-1 during heme degradation and is immediately converted to bilirubin (8). To examine its potential effects on thrombosis, biliverdin (5 mg/kg; Sigma) was administered to mice by tail vein injection, whereas control mice received an equal volume of vehicle. Fifteen minutes later, $FeCl_3$ was applied to arteries and thrombosis times were measured. The mean occlusion time of biliverdin-treated mice (9.2 ± 0.72 min, $n = 5$) was not significantly different from that of control mice (9.8 ± 1.5 min, $n = 5$), suggesting that biliverdin did not mediate the effect of HO-1 genotype on thrombosis in our model system.

Induction of HO-1 expression decreases clot formation in an ex vivo *perfusion chamber*

Accelerated thrombosis in the absence of HO-1 could result from effects on the blood and/or the blood vessel wall. To determine if HO-1 modulates the thromboreactivity of blood, we used a collagen-coated *ex vivo* perfusion chamber to study clot formation under pathophysiologically relevant conditions, but in the absence of a blood vessel wall. Mean clot formation during 2 min of blood flow through the perfusion chamber in wild-type mice (0.11 ± 0.003, $n = 14$, Fig. 3) did not differ significantly from that observed in *HO-1*$^{-/-}$ mice

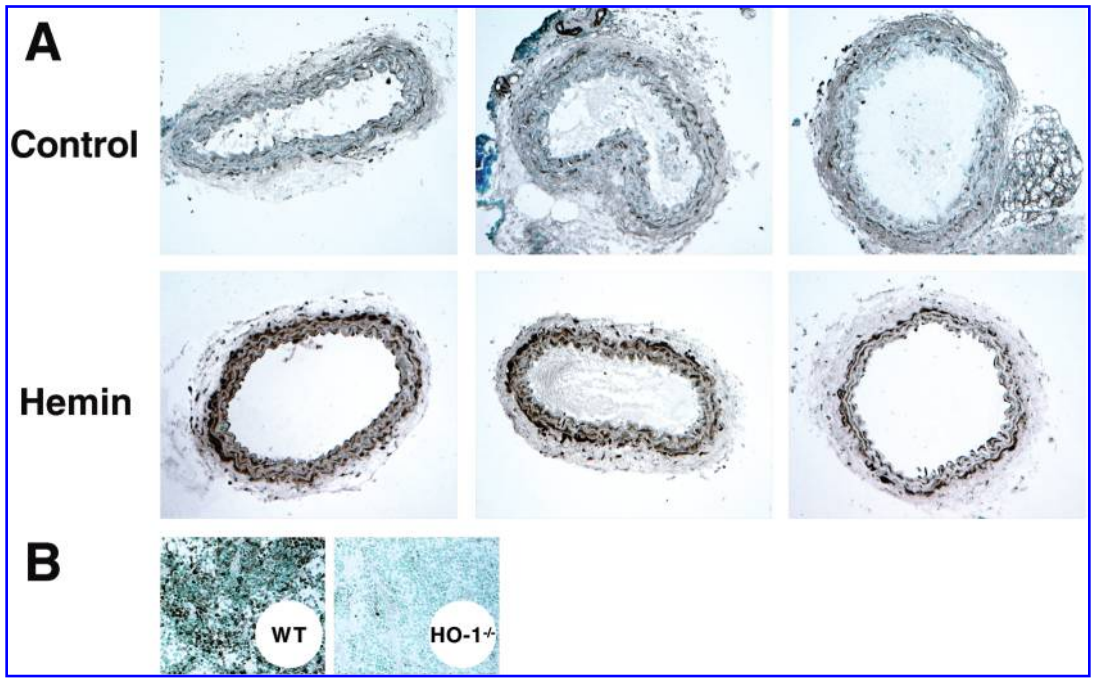

FIG. 2. Induction of arterial HO-1 expression by hemin. (**A**) Hemin or PBS (control) was administered to C57BL/6J wild-type mice for 3 days. Carotid arteries (which were not injured by $FeCl_3$) were retrieved, and HO-1 expression was studied by immunohistochemistry. Arterial cross-sections prepared from three control mice (upper row) and from three hemin-treated mice (lower row) are shown. (B) Immunohistochemical analysis of spleen tissue from a wild-type (WT) mouse and an *HO-1*$^{-/-}$ mouse, demonstrating specificity of the anti-HO-1 antibody.

(0.12 ± 0.006, $n = 14$, $p = 0.11$). However, after 3 days of hemin challenge, mean clot formation was significantly less in wild-type mice (0.049 ± 0.09, $n = 6$) than *HO-1*$^{-/-}$ mice (0.099 ± 0.012, $n = 6$, $p < 0.01$). Therefore, under conditions of increased oxidative stress, *ex vivo* clot formation was accelerated in *HO-1*$^{-/-}$ mice compared with wild-type mice. These results suggested that HO-1 modulates thrombosis, at least in part, by affecting the blood itself.

DISCUSSION

In this study, we examined the role of HO-1 in regulating intravascular clot formation, an early and critical response of arteries to stress. We demonstrated that, under conditions of increased oxidative stress, mice with complete HO-1 deficiency exhibit an accelerated thrombotic response to vascular injury compared with wild-type mice. Our results identify HO-1 as an important inhibitor of thrombus growth *in vivo*. Previous studies addressing the vascular biology of HO-1 focused predominantly on its function in modulating processes such as vasoreactivity, vascular smooth muscle cell proliferation, and atherosclerosis (9, 16, 26, 34). As thrombi contain vasoactive compounds and mitogens, such as thromboxane A_2 and platelet-derived growth factor, it is possible that the capacity of HO-1 to protect against vasoconstriction, neointima formation, and atherosclerosis development may be mediated to some extent by its antithrombotic properties.

Previous studies demonstrated that HO-1 protects against the development of intracardiac thrombi during hypoxia and

TABLE 1. EFFECT OF HEMIN ADMINISTRATION ON PLATELET cGMP LEVELS

Genetic background genotype	*Treatment*	*Platelet cGMP (pmol/10^9 platelets)*	*Ratio of platelet cGMP in hemin- vs. PBS-treated mice*
C57BL6J, wild-type	PBS ($n = 4$)	0.076	2.0
	Hemin ($n = 4$)	0.148	
129/Sv, wild-type	PBS ($n = 2$)	0.026	1.8
	Hemin ($n = 2$)	0.047	
129/Sv, *HO-1*$^{-/-}$	PBS ($n = 2$)	0.035	1.6
	Hemin ($n = 2$)	0.057	

All cGMP measurements were made from pooled platelet samples prepared from the number of mice indicated in column 2.

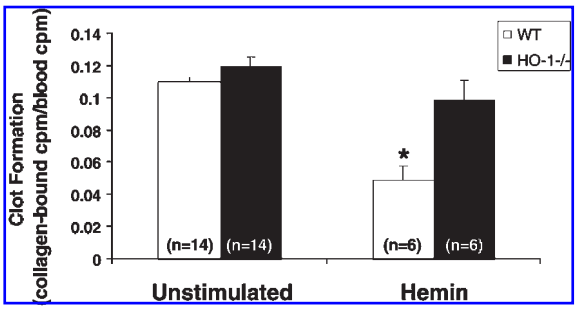

FIG. 3. ***Ex vivo*** **clot formation in wild-type** ***versus HO-1***$^{-/-}$ **mice.** Blood was routed from the inferior vena cava through a collagen-coated capillary tube, and *ex vivo* clot formation was measured. Mice were studied under basal (*i.e.,* unstimulated) conditions or after receiving hemin for 3 days. *$p < 0.01$ versus hemin-treated *HO-1*$^{-/-}$ mice.

arteriolar thrombi after cardiac transplantation (25, 33). However, it was not possible to determine from these studies if alteration of HO-1 expression affected the reactivity of blood itself to thrombotic stimuli, because increased thrombosis could have been driven by an increase in tissue injury that resulted from suppression of HO-1 function. Our *ex vivo* perfusion chamber studies demonstrate that HO-1, when up-regulated by oxidative stress, can alter the clotting tendency of flowing blood, because vascular wall cell injury was not a component of this experimental system. Earlier studies demonstrated that transient exposure of isolated platelets to cultured endothelial or vascular smooth muscle cells inhibited *in vitro* platelet aggregation in an HO-1-dependent manner (25, 29). The thrombi that form in response to $FeCl_3$ arterial injury are platelet-rich (10), and platelet activation drives clot formation during perfusion of blood over a collagen-coated surface. Therefore, our studies suggest that HO-1 functions *in vivo* to suppress platelet activation, at least under conditions that up-regulate HO-1 expression. However, it is also possible that the enhanced thrombotic response observed in our carotid artery experiments could have been contributed to by enhanced vascular wall cell necrosis after $FeCl_3$ injury.

We used hemin in our experiments to induce HO-1 expression, a method used by several other investigators (6, 19, 27). The mechanism by which hemin induces HO-1 is not precisely defined. However, hemin is recognized to induce oxidative stress, a key trigger of HO-1 expression, and we demonstrated that plasma TBARS increased in mice after hemin administration. Hemin catalyzes lipid peroxidation within cell membranes (28). This effect may be mediated by release of iron from hemin, catalyzed either by HOs or by peroxides (3, 13, 17, 36). Free iron can contribute to the production of hydroxyl radicals via the Fenton reaction (15). Hemin may also directly catalyze lipid peroxidation by reacting with membrane lipid peroxides (21).

Although the main goal of our study was to determine if HO-1 can modulate clot formation *in vivo,* we also attempted to identify potential mechanisms underlying HO-1's antithrombotic effect. We demonstrated that HO-1 protein is increased within the vascular wall after hemin exposure, suggesting that arteries can respond to oxidative stress, a potential trigger of thrombosis (1), by up-regulating HO-1 expression. Increased vascular HO-1 expression could lead to enhanced release of CO into the circulation, where it could inhibit platelet function by stimulating the production of cGMP by guanylyl cyclase (5, 25, 29, 31). By this hypothesis, one would anticipate decreased platelet cGMP levels in *HO-1*$^{-/-}$ mice compared with wild-type mice. However, we failed to observe such a difference, although the number of mice in our experimental groups was very small, and these data must be interpreted with caution. Future experiments, in which thrombosis is studied in mice after exposure to exogenous CO or to a guanylyl cyclase inhibitor, should help to define better the *in vivo* significance of cGMP as a downstream mediator of HO-1's effect on thrombosis. Biliverdin, another product of heme degradation by HO-1, is rapidly converted to bilirubin, which has antioxidative properties that potentially could affect thrombus formation (8). Our experiment involving biliverdin administration did not support a key role for biliverdin/bilirubin production in modulating the antithrombotic effects of HO-1 in our model system. However, further experiments are necessary to define better the potential effects of biliverdin production on thrombosis. Effects of HO-1 expression on iron metabolism must also be considered as a potential mechanism underlying our *in vivo* data. It is possible that abnormal accumulation of iron within cells, as has been observed in HO-1 deficiency (22), could have contributed to increased vascular injury or enhanced platelet reactivity in our studies (4, 24). In a previous study, we demonstrated that chronic iron administration increases vascular oxidative stress and accelerates thrombus formation after murine arterial injury (7). Effects of HO-1 deficiency on the expression of other heme oxygenases could potentially affect thrombus formation after vascular injury. However, previous studies suggest that HO-2 expression is not significantly altered in *HO-1*$^{-/-}$ mice (33). Although our studies suggest that HO-1 suppresses clot formation by inhibiting platelet function *in vivo,* they do not definitively establish this as the mechanism underlying HO-1's effect. Other mechanisms are possible, including CO-dependent suppression of plasminogen activator inhibitor-1 expression (11), and potential effects of HO-1 on tissue factor expression (14). Further studies are necessary to identify the mechanisms by which HO-1 modulates thrombus formation after vascular injury. It also will be important to examine the effect on thrombosis of other stimuli of HO-1 expression with more physiologic and pathophysiologic relevance than hemin, such as hypoxia and lipopolysaccharide.

In summary, we have demonstrated that, under basal conditions, HO-1 does not exert a significant effect on platelet-dependent clot formation *in vivo.* However, in response to oxidative stress, platelet-dependent thrombus formation is inhibited by HO-1. This effect is mediated, at least in part, by a reduction in the responsiveness of flowing blood to thrombotic stimuli. Our studies suggest that strategies aimed at enhancing HO-1 expression may be of therapeutic potential in the management and prevention of thrombotic diseases. Furthermore, they suggest that an increase in HO-1 expression in response to oxidative stress may represent an adaptive response mechanism to down-regulate clot formation under prothrombotic conditions.

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ABBREVIATIONS

cGMP, guanosine 3′,5′-cyclic monophosphate; CO, carbon monoxide; cpm, counts per minute; $FeCl_3$, ferric chloride; HO-1, heme oxygenase-1; MDA, malondialdehyde; PBS, phosphate-buffered saline; TBARS, thiobarbituric acid reactive substances.

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