Vascular Heme Oxygenase-1 Induction Suppresses Microvascular Thrombus Formation In Vivo

N. Lindenblatt, R. Bordel, W. Schareck, M. D. Menger, B. Vollmar

Objective—By heme degradation, heme oxygenase-1 (HO-1) provides endogenous carbon monoxide and bilirubin, both of which play major roles in vascular biology. The current study aimed to examine whether induction of HO-1 and its byproducts modulate the process of microvascular thrombus formation in vivo.

Methods and Results—In individual microvessels of mouse cremaster muscle preparations, ferric chloride-induced thrombus formation was analyzed using intravital fluorescence microscopy. When mice were pretreated with an intraperitoneal injection of hemin, a HO-1 inducer, immunohistochemistry and Western blot protein analysis of cremaster muscle tissue displayed a marked induction of HO-1. In these animals, superfusion with ferric chloride solution induced arteriolar and venular thrombus formation, which, however, was significantly delayed when compared with thrombus formation in animals without HO-1 induction. The delay in thrombus formation in hemin-treated mice was completely blunted by tin protoporphyrin-IX, a HO-1 inhibitor, but not by copper protoporphyrin-IX, which does not inhibit the enzyme. Coadministration of the vitamin E analogue Trolox in HO-1–blocked animals almost completely restored the delay in thrombus formation, implying that, besides CO, the antioxidant HO pathway metabolite bilirubin mainly contributes to the antithrombotic property of HO-1. This was further supported by the fact that bilirubin was found as effective as hemin in delay of ferric chloride-induced thrombus formation. Animals with HO-1 induction revealed reduced P-selectin protein expression in cremaster muscle tissue, which most probably presented the molecular basis for delayed thrombus growth.

Conclusion—Local induction of HO-1 activity may be of preventive and therapeutic value for clinical disorders with increased risk of thrombotic events. (Arterioscler Thromb Vasc Biol. 2004;24:1-6.)

Key Words: thrombosis ■ platelet ■ leukocyte ■ fluorescence microscopy ■ heme oxygenase ■ carbon monoxide ■ bilirubin ■ P-selectin

Thromboembolism is still a major problem in almost all fields of clinical medicine, resulting in tissue infarction and ischemic necrosis. Besides specific plasma protein components such as von Willebrand factor and fibrinogen, platelets, leukocytes, and endothelial cells are among the cellular components critical for this process.1,2 In thrombus formation associated with hemostasis or thrombotic disease, blood platelets first undergo a rapid transition from a circulating to a rolling state, using the GPIb-IX-V complex and PSGL-1 for interaction with the activated endothelium via surface-expressed endothelial P-selectin.1,3 This causes platelet activation with subsequent firm adhesion leading to shear resistant platelet deposition.1 The expression of P-selectin on the platelet surface after platelet stimulation and alpha granule secretion allow for interaction and recruitment of leukocytes and monocytes to thrombotic sites with fibrin deposition for thrombus stabilization.4

The microvascular endothelium is equipped with a number of mechanisms that prevent thrombus formation in the circulatory system. Among others, it harbors endothelium-derived factors such as nitric oxide and prostacyclin, exerting vasodilatory and anti-adhesive actions.5 Analogous to nitric oxide, carbon monoxide (CO) has been implicated as a biological second messenger with endothelium-derived relaxing activity and antiinflammatory properties.6,7 Heme oxygenases are the rate-limiting enzymes in the catabolism of heme into bilirubin, free iron, and CO and exist in different isoforms.8,9 HO-2 is thought to be constitutive, whereas HO-1 is inducible by various stimuli, such as cytokines and oxidants.7 Besides CO, the HO byproduct bilirubin additionally exerts antioxidant effects. Further, heme degradation by HO leads to ferritin synthesis with sequestration of iron, thus preventing its participation in subsequent oxidant stress-induced injury.7 With the view that free radical generation and consequent oxidative stress have a distinctive role in the pathogenesis of thrombotic events,10 this study aimed to examine whether HO-1 induction could attenuate microvascular thrombus formation in vivo.
Methods

Mice Cremaster Muscle Preparation

On approval by the local government, all experiments were performed in accordance with the German legislation on protection of animals and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council). Male C57BL/6J mice with a body weight of 20 to 25 g were anesthetized by an intraperitoneal injection of ketamine (90 mg/kg body weight and xylazine (25 mg/kg body weight), and a polyethylene catheter was placed into the right jugular vein, serving for application of fluorescent dyes.

For the study of vascular thrombus formation, we used the opened cremaster muscle preparation as originally described by Baez in rats and transferred by our group to mice. A midline incision of the skin and fascia was made over the ventral aspect of the scrotum and extended up to the inguinal fold and to the distal end of the scrotum. The incised tissues were retracted to expose the cremaster muscle sack that was maintained under gentle traction to carefully separate the remaining connective tissue by blunt dissection from around the cremaster sack. Then, the cremaster muscle was incised while avoiding cutting the larger anastomosing vessels. Hemostasis was achieved with 5-0 threads, serving also to spread the tissue. After dissection of the vessel connecting the cremaster and the testis, the epididymis and testis were put to the side of the preparation. The preparation was performed on a transparent pedestal to allow microscopic observation of the cremaster muscle microcirculation by transillumination and epi-illumination techniques.

After the preparation of the cremaster muscle, the animals were allowed to recover from surgical preparation for 15 minutes. Then, induction of thrombus formation was performed in randomly chosen venules (n = 1 to 2 per preparation) and arterioles (n = 1 to 2 per preparation).

In Vivo Thrombosis Model

After intravenous injection of 0.1 mL 5% fluorescein isothiocyanate-labeled dextran (MW 15,000; Sigma, Deisenhofen, Germany) and subsequent circulation for 30 seconds, the cremaster muscle microcirculation was visualized by intravital fluorescence microscopy using a Nikon microscope (E600-FN; Nikon, Tokyo, Japan). The microscopic procedure was performed at a constant room temperature of 21°C to 23°C. The epi-illumination setup included a 100-W HBO mercury lamp and an illuminator equipped with a blue filter (465 to 495 nm > 505 nm excitation/emission wavelengths). Microscopic images were recorded by a charge-coupled device videocamera (FK 6990 IQ-S; Pieper, Schwerte, Germany) and stored on videotapes for off-line evaluation. Using a x20 water immersion objective (Plan Fluor ×20/0.75; Nikon), resting blood flow was monitored in individual arterioles (diameter range 30 to 50 μm) and venules (diameter range 60 to 80 μm), followed by superfusion with ferric chloride (30 μL of a 25 mmol/L-solution; Sigma) for induction of microvascular thrombosis. Recording of vessels was discontinued after blood flow in the vessel ceased for at least 60 seconds because of complete vessel occlusion. Because rapid spreading of ferric chloride solution allowed us to study only 1 to 2 arterioles and venules within each preparation, left and right cremaster muscles were prepared for analysis of thrombotic vessel occlusion within each animal.

Analysis included the time periods until sustained cessation of blood flow caused by complete vessel occlusion. Microcirculatory analysis further included the determination of vessel diameter and blood cell velocity before thrombus induction with calculation of vascular wall shear rates, based on the Newtonian definition γ = 8πμV/D (V represents the red blood cell centerline velocity divided by 1.6, according to the Baker-Wayland factor, and D represents the individual inner vessel diameter).

Experimental Groups and Protocol

Eighteen hours before the experiment, mice (n = 7) were administered hemin (50 μmol/L per kg body weight intraperitoneally) for HO-1 induction. Control animals received equivalent volumes of the vehicle DMSO (n = 7). For blockade of HO-1, hemin-treated animals additionally received tin protoporphyrin (SnPP-IX; 50 μmol/L per kg body weight intraperitoneally) 18 hours before the experiment (n = 4). Additional hemin-treated animals received the copper protoporphyrin-IX (CuPP-IX; 50 μmol/L per kg body weight) 18 hours before the experiment (n = 3). To address the role and impact of CO versus bilirubin, hemin/SnPP-IX–treated animals additionally received Trolox (20 mg/kg intraperitoneally) 15 minutes before induction of thrombus formation (n = 4). In an additional series of animals (n = 4), cremaster muscle preparations of hemin/SnPP-IX–treated animals were topically exposed to 10 μmol/L bilirubin, followed by superfusion with ferric chloride, as described.

To address the participation of P-selectin in thrombogenesis, P-selectin–deficient mice (Jackson Laboratory; C57BL/6J-Selp<sup>−/−</sup>, n = 5) were used for ferric chloride-induced microvascular thrombus formation. Wild-type C57BL/6J mice (n = 5) served as controls.

Additional hemin-treated and hemin/SnPP-IX–treated mice (n = 3 to 4 each) exclusively served for withdrawal of arterial blood by left ventricular puncture for subsequent analysis of plasma bilirubin (Hitachi 704; Boehringer Mannheim, Mannheim, Germany).

Chemicals

The HO-1 inducer hemin (Fluka, Steinheim, Germany) was dissolved in DMSO to a final concentration of 5 μmol/mL. Tin protoporphyrin-IX (SnPP-IX; Frontier Scientific, Lancashire, UK), a HO-1 inhibitor, and copper protoporphyrin-IX (CuPP-IX; Frontier Scientific, Lancashire, UK), which does not inhibit HO-1, were dissolved in 8.4% sodium-bicarbonate and phosphate-buffered saline to achieve a final concentration of 5 μmol/mL. The solutions were stored at a maximal temperature of 8°C in the dark and used within the next hour. The water-soluble vitamin E analogue Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxonic acid; Sigma-Aldrich, Steinheim, Germany) was dissolved in phosphate-buffered saline to a final concentration of 20 mg/mL. All solutions were freshly prepared on the day of the experiment according to the manufacturers’ directions. Dose and application modes of drugs were chosen in accordance to a previously published work of our group.

Bilirubin (Sigma-Aldrich) was dissolved in deionized water (1 mg/mL), adjusted to a pH of 8.4 by addition of 2 N sodium hydroxide, and administered at a final concentration of 10 μmol/L, as described by others.

Western Blot Analysis of HO-1 and P-selectin

For whole protein extracts and Western blot analysis of HO-1 and P-selectin, cremaster muscle tissue was homogenized in lysis buffer (10 mmol/L Tris pH 7.5, 10 mmol/L NaCl, 0.1 mmol/L EDTA, 0.5% Triton-X 100, 0.02% NaN<sub>3</sub>, 0.2 mmol/L PMSF), incubated for 30 minutes on ice, and centrifuged for 15 minutes at 10,000g. Before use, all buffers received a protease inhibitor cocktail (1:100 v/v; Sigma). Protein concentrations were determined using the bicinchoninic acid protein assay (Sigma) with bovine serum albumin as standard; 20 μg protein/lane were separated discontinuously on sodium dodecyl sulfate polyacrylamide gels (12%) and transferred to a polyvinylidene fluoride membrane (Immoblon-P; Millipore, Eschborn, Germany). After blockade of nonspecific binding sites, membranes were incubated for 2 hours at room temperature with rabbit polyclonal anti-HO-1 (1:2000; StressGen Biotechnologies, Victoria, BC, Canada) and goat polyclonal anti-P-selectin (1:100; Santa Cruz Biotechnology, Heidelberg, Germany) followed by peroxidase-conjugated goat anti-rabbit Ig antibody (1:1000; Cell Signaling Technology, based on Germany) or donkey anti-goat Ig antibody (1:4000; Santa Cruz Biotechnology, Heidelberg, Germany) as secondary antibody. Protein expression was visualized by means of luminol-enhanced chemiluminescence (ECL plus; Amersham Pharmacia Biotech) and exposure of the membrane to a blue light-sensitive autoradiography film (Kodak BioMax Light Film; Kodak-Industrie, Chalon-sur-Saone, France). Signals were densitometrically assessed (Gel-Dokumentationssystem E.A.S.Y. Win32; Herolab GmbH, Wiesloch, Germany).
Histology and Immunohistochemistry

At the end of each experiment, the cremaster muscle was fixed in 4% phosphate-buffered formalin for 2 to 3 days and embedded in paraffin. From the paraffin-embedded tissue blocks, 4-μm sections were cut and stained with hematoxylin–eosin for histological analysis. For immunohistochemical demonstration of HO-1, sections collected on poly-L-lysine–coated glass slides were treated by microwave for antigen unmasking. Rabbit polyclonal anti-HO-1 (1:200; StressGen Biotechnologies) was used as primary antibody and incubated for 90 to 120 minutes at room temperature, followed by an alkaline–phosphatase conjugated goat anti-rabbit antibody (1:25; Cell Signaling Technology) and development using new fuchsin as chromogen. The sections were counterstained with hematoxylin and examined by light microscopy (Biomed; Leitz, Wetzlar, Germany).

Statistical Analysis

After proving the assumption of normality and equal variance across groups, differences between groups were assessed using 1-way ANOVA followed by the appropriate post-hoc comparison test. All data were expressed as means ± SEM and overall statistical significance was set at P<0.05. Statistics were performed using the software package SigmaStat (Jandel Corporation, San Rafael, Calif).

Results

Western Blot Analysis and Immunohistochemistry of Cremaster Muscle Tissue

As illustrated by Western blot analysis of mice cremaster muscle tissue, hemin injection induced a marked increase in HO-1 protein expression (Figure 1). Concomitant application of SnPP-IX, but not CuPP-IX, caused a further increase of HO-1 protein expression, presumably caused by the ability of SnPP-IX to act as a potent inhibitor of HO-1 enzymatic activity (Figure 1). Hemin treatment caused some elevation in bilirubin levels (4.7±0.3 μmol/L versus 4.1±0.1 μmol/L in DMSO-treated control animals), which in turn was inhibited by coadministration of SnPP-IX (3.8±0.3 μmol/L).

Immunohistochemistry of cremaster muscle tissue of DMSO-treated control animals exhibited little, if any, immunoreactivity of HO-1 (Figure 2A). In contrast, cremaster muscle tissue of hemin-treated animals displayed marked immunoreactivity of HO-1 at vascular sites and, although less pronounced, within the muscle tissue (Figure 2B). Vascular smooth muscle cells and, particularly, arteriolar and venular endothelia constituted the major site of prominent HO-1 expression (Figure 2B). Hemin-treated animals further exhibited reduced P-selectin protein expression when compared with DMSO-treated controls (Figure 3).
The effect of vascular HO-1 expression was assessed in an in vivo thrombosis model. Superfusion of microvessels with ferric chloride solution caused a complete thrombotic occlusion of the individually exposed vessel.

At baseline, ie, before thrombus induction, animals of either of the groups did not differ with respect to velocity and wall shear rates in arterioles and venules (Table ). Quantitative analysis of ferric chloride-induced thrombus formation in controls, ie, DMSO-treated animals, revealed a complete occlusion of arterioles and venules after 143±1100617 seconds and 130±1100625 seconds, respectively (Figures 4 and 5). Hemin-induced HO-1 expression caused a significant delay in microvascular thrombus formation. Arteriolar and venular vessel lumen were found clogged at an average time of 670±11006110 seconds and 647±129 seconds (Figures 4 and 5). Accordingly, within the first 100 seconds on ferric chloride superfusion, arteriolar and venular blood cell velocity slowed down by −32% and −47% in the DMSO-treated animals, whereas in animals with hemin-induced HO-1 expression, velocity remained almost unchanged (−9% and −3%). Treatment of HO-1–expressing animals with SnPP-IX, but not CuPP-IX, restored the potential of ferric chloride solution to induce thrombus formation, resulting in vessel occlusion time periods almost equivalent to those observed in the hemin-untreated, DMSO-exposed control animals (Figures 4 and 5). In hemin/SnPP-IX–treated animals, the red blood cell velocity profile was found in line with the kinetics of thrombus formation, exhibiting a decrease in blood cell velocity of −24% and −31% in arterioles and venules. In contrast, velocities in hemin/CuPP-IX–treated animals decreased by

| Table: Blood Flow Velocity (μm/s) and Wall Shear Rates (γ; s⁻¹) in Mice Cremaster Muscle Microvessels Before Thrombus Induction by Ferric Chloride Superfusion |
|--------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|
|                                | Arterioles                      | Venules                        |                                |                                |                                |
|                                | Acceleration Caused by Gravity  |                                | Acceleration Caused by Gravity  |                                |                                |
| Velocity                       | by Gravity                      |                                | Velocity                       | by Gravity                      |                                |
| DMSO                           | 950±189                        | 170±32                         | 524±73                         | 72±11                          |
| Hemin                          | 1070±158                       | 186±23                         | 534±107                        | 65±11                          |
| Hemin/SnPP-IX                  | 1531±338                       | 266±44                         | 640±114                        | 65±14                          |
| Hemin/CuPP-IX                  | 888±127                        | 182±41                         | 457±62                         | 49±10                          |
| Hemin/SnPP-IX+Trolox           | 1064±154                       | 197±33                         | 500±90                         | 52±8                           |
| Hemin/SnPP-IX+bilirubin        | 1229±90                        | 227±21                         | 599±72                         | 87±17                          |

Values are given as means±SEM. 
Blood flow velocity given in μm/s.
Wall shear rates given as γ and s⁻¹.

Figure 4. Time of complete occlusion of arterioles on superfusion with ferric chloride solution in animals that were treated with either DMSO, hemin, hemin/SnPP-IX, hemin/CuPP-IX, hemin/SnPP-IX+Trolox, or hemin/SnPP-IX+bilirubin. Means±SEM. *P<0.05 versus DMSO; #P<0.05 versus hemin/SnPP-IX.

Figure 5. Time of complete occlusion of venules on superfusion with ferric chloride solution in animals that were treated with either DMSO, hemin, hemin/SnPP-IX, hemin/CuPP-IX, hemin/SnPP-IX+Trolox, or hemin/SnPP-IX+bilirubin. Means±SEM. *P<0.05 versus DMSO; #P<0.05 versus hemin/SnPP-IX.
only $-9\%$ (arterioles) and $-10\%$ (venules) within the first 100 seconds on ferric chloride superfusion.

To differentiate between the contribution of CO and bilirubin in delay of thrombus formation, animals with hemin-induced HO-1 induction were pretreated with SnPP-IX and Trolox. Interestingly, administration of Trolox in HO-1–blocked animals almost completely restored the delay in kinetics of thrombus growth, as observed in hemin-treated animals (Figures 4 and 5) and partly prevented the initial decrease in arteriolar and venular blood flow velocity ($-16\%$ and $-7\%$). In line with this, topical application of cremaster muscle preparations of hemin/SnPP-IX–treated animals with bilirubin exerted antithrombogenic actions similar as observed in hemin-treated animals (Figures 4 and 5), implying that HO-1 exerts its antithrombogenic potential through the metabolite bilirubin rather than CO.

To further evaluate whether reduced P-selectin expression could be a mechanistic basis for the observed antithrombogenic actions of hemin, P-selectin–deficient mice were studied with respect to thrombus formation on ferric chloride superfusion. Thrombotic vessel occlusion was found markedly delayed in mice deficient for P-selectin expression, as given by arteriolar and venular occlusion times of $998\pm122$ seconds and $1152\pm147$ seconds, when compared with P-selectin–competent C57BL/6J wild-type animals ($207\pm55$ seconds and $232\pm63$ seconds).

**Discussion**

The vessel wall releases potent platelet-inhibiting substances, such as prostacyclin and nitric oxide, to limit the extent of platelet activation and thrombus formation. Better insight into the role of endogenous anti-thromboembolic substances is of major importance to improve our understanding of clinical disorders in which thromboembolic processes play a role. The current study provides, for the first time to our knowledge, in vivo evidence that microvascular thrombus formation is significantly delayed in microvessels expressing HO-1 and suggests the role of HO-1 pathway metabolites, ie, CO and bilirubin, in this scenario.

**Methodological Considerations**

The tool we used for induction of HO-1 was the application of hemin. This naturally occurring substrate for HO has been implicated in the attenuation of the inflammatory response. Scavenging various oxidants, biliverdin, and its reduced product bilirubin exert potent antioxidant and anti-adhesive properties.

Numerous studies using HO-1 inducers or gene transfer could demonstrate that tissues expressing high levels of HO-1 are less susceptible to noxious stimuli, such as proinflammatory reagents and oxidant stress. In line with this, animals deficient for HO-1 have chronic inflammation and enhanced sensitivity to oxidative stress.

The increased production of CO and biliverdin/bilirubin has been implicated in the attenuation of the inflammatory response. Scavenging various oxidants, biliverdin, and its reduced product bilirubin exert potent antioxidant and anti-adhesive properties. Among these putative actions of the HO-1 pathway products, the most relevant is a delay of thrombus formation in hemin-treated animals is completely inhibited by SnPP-IX, but not by CuPP-IX, underscores the specificity of the HO pathway in maintenance of vessel patency. Considering that blood cell–vessel wall interactions during thrombus formation primarily involve P-selectin on activated endothelial cells, we suggest that the antithrombotic action of HO-1 is mainly related to an inhibitory action on P-selectin in the vasculature, as indicated herein by the reduced P-selectin protein expression in Western blot analysis of hemin-treated animals. This view is further underlined by a study demonstrating by the dual radiolabeled monoclonal antibody technique that pretreatment with hemin attenuated the increased P-selectin expression of endothelial cells normally elicited by lipo polysaccharide.

Moreover, using real-time laser confocal video microscopy, Hayashi et al were able to monitor a reduction of H$_2$O$_2$-elicited venular P-selectin expression in the hemin-treated versus the hemin-untreated mesentery. Finally, experiments of the present study in P-selectin–deficient mice, demonstrating a marked delay in thrombus formation, additionally underscore the reduced P-selectin expression as a mechanistic basis for the antithrombogenic actions of hemin.

To further distinguish the roles of CO and bilirubin in this model, we studied ferric chloride-mediated thrombus induction by administration of the antioxidant Trolox in HO-1–blocked animals. This regimen significantly prolonged thrombus growth, implying that bilirubin mainly mediates the antithrombotic property of the HO pathway. In line with others, we additionally examined whether direct treatment of cremaster muscle preparations with products of the HO reaction, ie, bilirubin, exerts antithrombogenic action. Interestingly, superfusion of bilirubin at a final concentration of 10 µmol/L was capable to prolong thrombotic vessel occlusion to an extent, similar to that seen in hemin-treated animals. Although it is difficult to finally assess whether direct superfusion with 10 µmol/L bilirubin equals bilirubin release in mice undergoing HO-1 induction by hemin treatment, HO-1 seems to exert its antithrombogenic potential mainly through the metabolite bilirubin.

However, besides bilirubin, CO also has been shown to mimic anti-adhesive properties elicited by hemin-induced HO-1 expression to an extent, which depends on the individual inflammatory stimulus used. Moreover, a recent investigation reported on the paradoxical rescue from ischemic lung injury by inhaled CO, based on its ability to derepress fibrinolysis via modulation of plasminogen activator inhibitor. Thus, it is also reasonable to speculate that reduced
expression of plasminogen activator inhibitor and thus limited accrual of microvascular fibrinogen might, at least in part, account for the observed antithrombotic activity of HO-1.

In summary, vascular HO-1 with release of CO, particularly of bilirubin, attenuates thrombus formation, most probably via modulation of P-selectin expression on endothelial cells. Thus, local induction of HO-1 activity may be of preventive and therapeutic value for clinical disorders with increased risk of thrombotic events.

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References

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