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Induction of Heme Oxygenase-1 Is a Beneficial Response in a Murine Model of Venous Thrombosis

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The induction of heme oxygenase-1 (HO-1) may protect against tissue injury. The present study examines the induction of HO-1 in a murine model of venous thrombosis and explores the downstream consequences of this induction. In a model of stasis-induced thrombosis created by ligation of the inferior vena cava, HO-1 expression is markedly induced. Such expression occurs primarily in smooth muscle cells in the venous wall and in leukocytes infiltrating the venous wall and clot. To determine the significance of HO-1 induction in venous thrombosis, this model was imposed in HO-1^{+/+} and HO-1^{-/-} mice. The initial clot size did not differ in either group by day 2, but was significantly larger in HO-1^{-/-} mice by day 10, where an exaggerated inflammatory response in the venous wall was also observed. Following ligation of the inferior vena cava, HO-1^{-/-} mice exhibited increased nuclear factor κ B activation and markedly increased up-regulation of tissue factor, selectins, inflammatory cytokines, and matrix metalloproteinase-9, the latter incriminated in both clot lysis and vascular injury. We conclude that HO-1 deficiency impairs thrombus resolution and exaggerates the inflammatory response to thrombus formation. These findings offer insight into recent observations that polymorphisms in the HO-1 gene may increase the risk for recurrent venous thrombosis and dysfunction of hemodialysis arteriovenous fistulas, the latter caused, in part, by thrombosis. (Am J Pathol 2008; 173:1882–1890; DOI: 10.2353/ajpath.2008.080556)

imately 1 million venous thromboembolic events occur each year in the United States, some 30% of which prove fatal. In addition to such high mortality and its well recognized predilection for recurrence, venous thromboembolic disease is commonly complicated by the post-thrombotic syndrome, and less commonly, by pulmonary hypertension.^{1–3} Understanding the pathogenesis of venous thrombosis, the processes whereby venous thrombosis resolves, and the mechanisms accounting for chronic venous injury are thus important issues, and the focus of substantial investigation.^{4–7} Among the insights uncovered by such studies is the heightened appreciation that a complex, bidirectional relationship exists between thrombosis and inflammation^{4–7}: inflammation predisposes to thrombosis; thrombosis induces an inflammatory response; and inflammation is a critical determinant of clot resolution and chronic venous stasis syndromes.

In the injured kidney, vasculature, and elsewhere, considerable interest currently surrounds the induction of heme oxygenase-1 (HO-1).^{8–15} HO is the rate-limiting enzyme in the degradation of heme, converting the tetrapyrrole ring to biliverdin, a process during which iron is released from the heme ring, and carbon monoxide evolves. HO-1 is an isozyme that is readily inducible by diverse insults, and numerous studies in models of renal, vascular, and other types of tissue injury have demonstrated a protective role for HO-1. The cytoprotective properties of HO-1 are ascribed to its anti-inflammatory, vasorelaxant, antioxidant, and anti-apoptotic effects.^{8–15} That such protective effects of HO-1 may extend to human disease is supported by the observation that the inability to express HO-1 leads to multisystem disease and death in childhood,¹⁶ and that the risk for certain diseases is increased by polymorphisms in the HO-1 promoter characterized by long GT repeats.^{17–19} In a recent prospective study, polymorphisms in the HO-1 gene characterized by long GT repeats were associated

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Venous thromboembolic disease is a major cause of morbidity and mortality in diverse patient populations.^{1,2} Unaltered in its incidence for the past 25 years, approx-

with an increased risk for recurrent venous thrombosis.²⁰ However, this latter study did not determine how such polymorphisms influence HO-1 expression or HO activity, and thus the role of the HO-1 system as a determinant of the risk for venous thrombosis remains unresolved.

Using a long-established and widely employed model of venous thrombosis, and one based on the ligation of the infra-renal inferior vena cava (IVC),^{4,6,7,21–23} the present study addressed whether expression of HO-1 influences venous thrombosis and clot resolution. Ligation of the IVC induces prompt and prominent venous clot formation, inflammation in the clot and venous wall, and, over ensuing days, increasing resolution of the venous clot. The current investigation analyzed the expression of HO-1 in this model, and assessed the functional significance of such HO-1 expression by using genetically altered mice unable to express HO-1.

Materials and Methods

The present study used homozygous HO-1 null mutant mice generated by targeted disruption of the HO-1 gene as described by Poss and Tonegawa,²⁴ and as used in earlier studies by our laboratory.^{25–29} Colonies of mice were maintained by breeding HO-1^{-/-} males with HO-1^{+/-} females. Offspring were genotyped at the time of weaning using PCR to amplify the wild-type and mutant alleles of genomic DNA from tail samples. HO-1^{+/+} mice were used as controls. For all experiments, HO-1^{+/+} and HO-1^{-/-} mice were age-matched and sex-matched, and mice between 16 to 30 weeks of age were used. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee of the Mayo Clinic.

Murine Model of Deep Venous Thrombosis: Ligation of the IVC in Mice

This model was performed as described previously by Myers et al.^{21–23} Mice were anesthetized (pentobarbital, 50 mg/kg, i.p.) and were placed on a temperature-regulated table to maintain body temperature at 37°C. Following a midline laparotomy, the bowel was exteriorized and protected with moist sterile gauze. The fascia surrounding the aorta and IVC was approached and the vessels were carefully separated by blunt dissection. Once separated, the IVC was ligated with 6–0 silk nonabsorbable suture just below the renal veins and any side branches of the IVC were also ligated. After the ligation, the bowel was returned to the body cavity, 1 ml of warm saline was injected into the peritoneum, and the laparotomy incision was closed. Sham-operated mice underwent midline laparotomy and bowel manipulation, with no ligation of the IVC, but were otherwise treated in an identical manner. At designated time points after ligation, the thrombus-containing IVC was carefully removed by cutting the IVC proximally, just below the ligature, and distally, at the lower end of the thrombus. As previously reported,^{21–23}

the thrombus constitutes the bulk of the combined weight of thrombus and IVC, and thus the weight of the thrombus-containing IVC is widely used in studies of this model in mice to reflect the weight of clot; the size of the clot is also assessed by the length of the thrombus and the weight/length ratio of the thrombus. Moreover, the thrombus may not be readily separated from the IVC without traumatizing and tearing the clot and the wall of the IVC, and, notably, for reasons that are unclear, this was especially so in HO-1^{-/-} mice. Thus, in our studies, as in numerous previous studies of this model,^{21–23} assessment of this model used the thrombus-containing IVC along with the IVC from the sham-operated mice.

Measurement of Heme Oxygenase Activity

Heme oxygenase activity was assessed in murine IVC and thrombus-containing IVC using a method previously used by our laboratory.³⁰ This method utilizes the measurement of the production of bilirubin by microsomes, the latter prepared as previously described.³⁰

Immunohistochemical Localization of HO-1 Expression

Immunohistochemical analysis of HO-1 expression was performed on 5 micron sections prepared from formalin-fixed, paraffin-embedded IVCs and thrombi using a rabbit polyclonal antibody (catalog no. SPA-895, StressGen, Ann Arbor, MI) as a primary antibody and using a secondary antibody conjugated to a horseradish peroxidase-labeled polymer (EnVision+, HRP, Rabbit, catalog no. K4003, Dako, Carpinteria, CA).²⁹ Before antibody incubation, tissues were subjected to 40 minutes of antigen retrieval (10 mmol/L citrate buffer, pH 6.0, 98°C) and 5 minutes of serum-free protein block (catalog no. X0909, Dako). Visualization was achieved using diaminobenzidine (DAB+, catalog no. K3468, Dako) as a chromogen and hematoxylin for counterstaining.

Morphometric Determination of the Thickness of the Venous Wall of the IVC

Thickness of the wall of the IVC was determined, as previously described, by measuring the length of 12 sagittal sections evenly spaced around the circumference of and through the wall of the IVC, and obtaining the mean of these 12 values.²⁹

mRNA Expression by Quantitative Real-Time Reverse Transcriptase-PCR

For analysis of gene expression, total RNA was extracted from mouse IVC and thrombus using the TRIzol method (Invitrogen, Carlsbad, CA) and further purified with an RNeasy Mini Kit (Qiagen, Valencia, CA), according to each manufacturer's protocol.²⁶ Two hundred nanograms of purified total RNA were subsequently used in 40 µl reverse transcription reactions (Transcriptor First

Strand cDNA Synthesis Kit, Roche Applied Science, Indianapolis, IN) using random hexamers. The resulting cDNA was used in quantitative real-time PCR analysis as in our earlier study.²⁶ Reactions were performed on an ABI Prism 7900HT (Applied Biosystems, Foster City, CA) using TaqMan Mastermix reagents (part no. 4309169, Applied Biosystems). Probes and primers used for quantification were obtained as assay sets for each target mRNA (TaqMan Gene Expression Assays, Applied Biosystems) and used according to the manufacturer's protocol. Parameters for quantitative PCR were as follows: 10 minutes at 95°C, followed by 40 cycles of amplification for 15 seconds at 95°C, and 1 minute at 60°C. Expression of 18S rRNA was used for standardization of the expression of each target gene.

Western Analysis and Zymography

Protein extracts for zymography and western analysis of MMP-2 and MMP-9 protein were prepared by homogenizing the IVC and thrombus-containing IVC in a lysis buffer (50 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 10 mmol/L CaCl₂, 0.02% NaN₃, and 0.05% Brij35) containing protease inhibitors (Complete, EDTA-Free, Roche Diagnostics, Indianapolis, IN), and centrifuging at 10,000 × *g* to remove insoluble debris. HO-1 western analysis was performed on microsomal fractions prepared as we have described previously.³⁰

Gel zymography was performed as previously used by our laboratory²⁹ on 15 µg of protein in a denaturing buffer (62.5 mmol/L Tris-HCl pH 6.8, 1% SDS, 5% glycerol, and 0.0005% bromophenol blue), separated on a pre-made 10% zymogram gelatin polyacrylamide gel (BioRad Laboratories, Hercules, CA). The gel was then rinsed in 2.5% Triton X-100 for 2 hours with mild agitation and incubated overnight in activation buffer (50 mmol/L Tris-HCl pH 7.4 and 10 mmol/L CaCl₂) at 37°C. The gel was stained with Coomassie Brilliant Blue R-250 (BioRad) and destained until clear bands were visible against a dark background. Band intensities for zymography were measured using densitometry (GS-800 densitometer, BioRad).

Western Analysis was performed as described in our previous studies.^{26,29} Briefly, proteins (25 to 60 µg) were separated on 10% Tris-HCl gels and transferred to polyvinylidene fluoride membranes. Primary antibodies for MMP-2 and MMP-9 (catalog nos. AF1488, and AF909, R&D Systems, Minneapolis, MN), HO-1 (catalog no. SPA-895, StressGen, Ann Arbor, MI), or β-actin (catalog no. 612657; BD Transduction Laboratories, San Diego, CA) were used in overnight incubations at 4°C. Horseradish peroxidase-conjugated secondary antibodies were then used and bands were visualized using an enhanced chemiluminescence method (catalog no. RPN-2106, Amersham, Piscataway, NJ).

Electrophoretic Mobility Shift Assay for Analysis of Nuclear Factor-κB

Extraction of nuclear proteins and nuclear factor (NF)-κB gel shift analysis were performed as described in our

Figure 1. HO-1 mRNA expression following ligation of the inferior vena cava (IVC). HO-1 mRNA was determined by quantitative real-time RT-PCR in the thrombus-containing IVC (IVC-ligated) at days 1 and 2 following ligation of the IVC, and in the IVC in sham-operated (Sham) mice. *n* = 3 and *n* = 3 to 4 in sham-operated and IVC-ligated groups on days 1 and 2 respectively. **P* < 0.05, IVC-ligated group versus sham-operated group for that day.

prior studies.³¹ IVCs from four mice were pooled for each of the sham groups, and IVCs and accompanying thrombi from two mice were pooled for each of the IVC-ligation groups. Nuclear extract (2.5 µg) from each pooled sample was used in the binding reaction. A double-stranded consensus oligonucleotide for NF-κB (catalog no. E3291, Promega, Madison, WI) was used to detect transcription factor binding. Bound and unbound oligo were separated on a 5% TBE nondenaturing polyacrylamide gel (BioRad) and visualized by autoradiography.

Statistics

Data are expressed as Mean ± SEM. Data for HO-1^{-/-} and HO-1^{+/+} mice for a given condition were compared using Student's *t*-test for parametric data, and the Mann-Whitney test for nonparametric data. Results were considered significant for *P* < 0.05.

Results

Expression of HO-1 Following Ligation of the IVC

Using multiple approaches, we demonstrate marked induction of HO-1 following ligation of the IVC. By real-time RT-PCR, as shown in Figure 1, HO-1 mRNA was strongly induced at day 1 and day 2 following ligation of the IVC. This induction of HO-1 mRNA was accompanied by increased expression of HO-1 protein and HO activity (Figure 2). The histological sites of expression of HO-1 were localized by immunohistochemistry. As demonstrated in Figure 3, there was strong induction of HO-1 in the venous wall following ligation of the IVC, which localized to endothelial cells and smooth muscle cells in the venous wall. In addition, leukocytes infiltrating the venous wall and the venous clot in the ligated IVC also expressed HO-1. The wall of the IVC in sham-operated mice re-

Figure 2. Expression of HO-1 protein and HO activity following ligation of the IVC. **A:** HO-1 protein expression was determined by Western analysis in the thrombus-containing IVC (IVC-ligated) at day 2 following ligation of the IVC, and in the IVC in sham-operated mice. **B:** HO activity was determined in the thrombus-containing IVC (IVC-ligated) at day 2 following ligation of the IVC, and in the IVC in sham-operated mice. $n = 5$ and $n = 8$ in sham-operated and IVC-ligated groups respectively. * $P < 0.05$, IVC-ligated group versus sham-operated group.

vealed an endothelial lining only, and endothelial cells in the IVC in sham-operated mice expressed HO-1.

Examination of the Pathobiologic Significance of HO-1 Following Ligation of the IVC

To determine the biological significance of such HO-1 up-regulation, we imposed this model of venous thrombosis in HO-1^{+/+} and HO-1^{-/-} mice. We measured clot size at day 2 and day 10 following ligation of the IVC in HO-1^{+/+} and HO-1^{-/-} mice, as clot size is widely regarded and used as a valid determinant of clot resolution in this model of venous thrombosis.^{21–23,32} As demonstrated in Figure 4, clot size, as measured by clot weight and clot length, was virtually identical at day 2 after ligation of the IVC in HO-1^{+/+} and HO-1^{-/-} mice. However, at day 10, the clot size was markedly greater in the HO-1^{-/-} mice as compared to the HO-1^{+/+} mice as

Figure 4. Clot size in HO-1^{+/+} and HO-1^{-/-} mice at days 2 and 10 following ligation of the IVC. **A:** Clot weight in HO-1^{+/+} and HO-1^{-/-} mice at days 2 and 10 following the ligation of the IVC. **B:** Clot length in HO-1^{+/+} and HO-1^{-/-} mice at days 2 and 10 following the ligation of the IVC. $n = 5$ and $n = 4$ in HO-1^{+/+} and HO-1^{-/-} mice respectively on day 2, and $n = 11$ and $n = 9$ in HO-1^{+/+} and HO-1^{-/-} mice respectively on day 10 after ligation of the IVC. * $P < 0.05$, IVC-ligated HO-1^{-/-} mice versus IVC-ligated HO-1^{+/+} mice at day 10.

determined by the weight and length of the clot (Figure 4), and the weight/length ratio of the clot (Figure 5A). It is also notable that the temporal profile in clot size differed between HO-1^{+/+} and HO-1^{-/-} mice. While the clot size decreased in HO-1^{+/+} mice from day 2 to day 10, the clot size increased in the HO-1^{-/-} mice (Figure 4). Thus, HO-1 deficiency impairs processes that enable clot resolution.

The impaired clot resolution at day 10 following IVC ligation observed in HO-1^{-/-} mice as compared with HO-1^{+/+} mice persisted at a subsequent timepoint. Twenty one days after IVC ligation, and as compared with HO-1^{+/+} mice, HO-1^{-/-} mice exhibited markedly and

Figure 3. Localization of HO-1 protein by immunohistochemistry at day 2 following ligation of the IVC. **A:** Low power view of the IVC in the sham-operated mouse (magnification = original $\times 100$). **B:** Low power view of the IVC in the IVC-ligated mouse (magnification = original $\times 100$). **C:** High power view of the IVC in the sham-operated mouse (magnification = original $\times 400$). **D:** High power view of the IVC in the IVC-ligated mouse (magnification = original $\times 400$). Cells expressing HO-1 appear dark brown in color. HO-1 is expressed by the endothelial cells in the IVC in the sham-operated mice; HO-1 is expressed by endothelial cells and smooth muscle cells in the IVC and in leukocytes infiltrating the venous wall and clot following ligation of the IVC.

Figure 5. Clot weight/length and wall thickness of the IVC in HO-1^{+/+} and HO-1^{-/-} mice at day 10 following ligation of the IVC. **A:** Clot weight/length in HO-1^{+/+} and HO-1^{-/-} mice at day 10 following the ligation of the IVC. $n = 11$ and $n = 9$ in HO-1^{+/+} and HO-1^{-/-} mice respectively. **B:** Wall thickness of the IVC in HO-1^{+/+} and HO-1^{-/-} mice at day 10 following the ligation of the IVC. $n = 5$ in HO-1^{+/+} and HO-1^{-/-} mice. * $P < 0.05$, IVC-ligated HO-1^{-/-} mice versus IVC-ligated HO-1^{+/+} mice.

significantly greater clot weight (4.91 ± 0.27 vs. 22.80 ± 2.18 mg), clot length (3.42 ± 0.19 vs. 6.05 ± 0.24 mm), and clot weight/length ratio (1.44 ± 0.06 vs. 3.78 ± 0.40), $n = 7$ and $n = 6$ in HO-1^{+/+} and HO-1^{-/-} mice respectively. Thus, even at 21 days, there is impaired clot resolution following IVC ligation in HO-1^{-/-} mice as compared with HO-1^{+/+} mice.

We also undertook morphometric and histological studies of the venous clot and venous wall in HO-1^{+/+} and HO-1^{-/-} mice following ligation of the IVC (Figure 5B and Figure 6). The venous wall was significantly thickened in HO-1^{-/-} mice as compared to HO-1^{+/+} mice following ligation of the IVC (Figure 5B). This increased thickness in the venous wall was due to increased numbers of smooth muscle cells, a more prominent inflammatory response, and increased extracellular matrix deposition (Figure 6). The heightened inflammatory response in the venous wall of the IVC in HO-1^{-/-} mice included persisting neutrophilic infiltration, which was absent in the venous wall of the inferior vena cava in HO-1^{+/+} mice subjected to ligation of the IVC.

Due to the exaggerated inflammatory and proliferative responses observed in HO-1^{-/-} mice subjected to ligation of the IVC, we examined the expression of some of the key pro-inflammatory participants incriminated in this model. Following ligation of the IVC, HO-1^{-/-} mice dem-

Figure 7. Expression of P-selectin and E-selectin at day 10 following ligation of the IVC in HO-1^{+/+} and HO-1^{-/-} mice. P-selectin mRNA (**A**) and E-selectin mRNA (**B**) were determined by quantitative real-time RT-PCR in the thrombus-containing IVC (IVC-ligated) in HO-1^{+/+} and HO-1^{-/-} mice at day 10 following ligation of the IVC, and in the IVC in sham-operated mice. For either gene, $n = 5$ and $n = 6$ in HO-1^{+/+} and HO-1^{-/-} mice respectively subjected to sham operation, and $n = 7$ and $n = 5$ in HO-1^{+/+} and HO-1^{-/-} mice respectively subjected to ligation of the IVC. * $P < 0.05$, IVC-ligated HO-1^{-/-} mice versus IVC-ligated HO-1^{+/+} mice for that gene.

onstrated markedly greater mRNA expression for P-selectin and E-selectin (Figure 7), interleukin (IL)-6 and monocyte chemoattractant protein (MCP)-1 (Figure 8), and chemokines (C-X-C motif; CXC) L1 and L12 (KC and SDF1 respectively, Figure 9). Because a sizable number of pro-inflammatory participants are up-regulated by NF- κ B, we evaluated activation of this pro-inflammatory transcription factor. As demonstrated in Figure 10, greater activation of NF- κ B occurred in HO-1^{-/-} mice as compared with HO-1^{+/+} mice following ligation of the IVC.

In addition to pro-inflammatory participants, NF- κ B is a critical transcriptional regulator of MMP-9.³³ MMP-9 is of considerable interest in the pathogenesis of vascular injury in general,^{34,35} and in clot lysis as induced in this model, in particular.^{4,7,36} In HO-1^{-/-} mice, MMP-9 was strikingly up-regulated following ligation of the IVC, as assessed by mRNA and protein expression, and by zymography (Figure 11). In this model, MMP-2 was not differentially expressed in HO-1^{+/+} and HO-1^{-/-} mice (data not shown).

We also determined whether procoagulant species are up-regulated in HO-1^{-/-} mice following IVC ligation. Tissue factor is a critical initiator of, and participant in, thrombus formation in diverse pathophysiologic conditions, and is up-regulated, at least in part, via NF- κ B-dependent mechanisms. We thus evaluated expression of tissue factor. As demonstrated in Figure 12, expression of tissue factor is increased threefold in HO-1^{-/-} mice as

Figure 6. Histological appearances of the thrombus-containing IVC at day 10 following ligation of the IVC in HO-1^{+/+} and HO-1^{-/-} mice. The lumina of the IVC in HO-1^{+/+} mice (**A**) and HO-1^{-/-} mice (**B**) are filled with thrombus. The wall of the IVC is markedly thickened in HO-1^{-/-} mice as compared to HO-1^{+/+} mice due to inflammatory infiltrate, cellular proliferation, and extracellular matrix deposition. All tissues are stained with H&E (magnification = original $\times 200$).

Figure 8. Expression of IL-6 and MCP-1 mRNA at day 10 following ligation of the IVC in HO-1^{+/+} and HO-1^{-/-} mice. IL-6 mRNA (A) and MCP-1 mRNA (B) were determined by quantitative real-time RT-PCR in the thrombus-containing IVC (IVC-ligated) in HO-1^{+/+} and HO-1^{-/-} mice at day 10 following ligation of the IVC, and in the IVC in sham-operated mice. For either gene, $n = 5$ and $n = 4$ in HO-1^{+/+} and HO-1^{-/-} mice respectively subjected to sham operation, and $n = 7$ and $n = 5$ in HO-1^{+/+} and HO-1^{-/-} mice subjected to ligation of the IVC. * $P < 0.05$, IVC-ligated HO-1^{-/-} mice versus IVC-ligated HO-1^{+/+} mice for that gene.

compared to HO-1^{+/+} mice when subjected to IVC ligation. No difference in expression of tissue factor was observed between HO-1^{+/+} and HO-1^{-/-} mice following sham ligation. Such increased expression of tissue factor may foster clot formation despite the up-regulation of potentially lytic mechanisms as provided by increased expression of MMP-9.

Discussion

To the best of our knowledge, the present study is the first to examine the expression and pathobiologic significance of HO-1 in the IVC-ligation model of venous thrombosis, and the first to employ HO-1^{-/-} mice in any model of venous thrombosis. The present findings demonstrate that HO-1 is robustly induced in the venous thrombus and venous wall following ligation of the IVC. While the basis for this induction of HO-1 was not explored in our study, such induction may reflect the effect of inflammation and/or oxidative stress in this model. This induction of HO-1 following venous thrombosis is functionally significant: in mice unable to express HO-1, the clot size progressed with time such that at day 10 following ligation of the IVC, clot size, assessed by three indices (weight,

Figure 10. Activation of NF-κB, as assessed by the electromobility shift assay, at day 10 following ligation of the IVC in HO-1^{+/+} and HO-1^{-/-} mice. In performing this assay, IVCs from four mice were pooled for each of the sham groups, and the thrombus-containing IVCs from two mice were pooled for each of the IVC-ligated groups. The minus sign signifies a control lane that did not contain nuclear extract in the binding reaction.

length, and weight/length ratio), was markedly greater than that observed in wild-type mice. Prior studies have demonstrated the validity of clot size as an index of clot resolution following ligation of the IVC, and, indeed, experts in this field have stated that the size of the clot is the single most important index of resolution.³² Moreover, while the clot size in HO-1^{+/+} mice decreased from day 2 to day 10, a finding consistent with the established features of this venous thrombosis model in wild-type

Figure 9. Expression of CXCL1 and CXCL12 mRNA at day 10 following ligation of the IVC in HO-1^{+/+} and HO-1^{-/-} mice. CXCL1 mRNA (A) and CXCL12 mRNA (B) were determined by quantitative real-time RT-PCR in the thrombus-containing IVC (IVC-ligated) in HO-1^{+/+} and HO-1^{-/-} mice at day 10 following ligation of the IVC, and in the IVC in sham-operated mice. For either gene, $n = 5$ –6 and $n = 6$ in HO-1^{+/+} and HO-1^{-/-} mice respectively subjected to sham operation, and $n = 7$ and $n = 5$ in HO-1^{+/+} and HO-1^{-/-} mice respectively subjected to ligation of the IVC. * $P < 0.05$, IVC-ligated HO-1^{-/-} mice versus IVC-ligated HO-1^{+/+} mice for that gene.

Figure 11. Expression of MMP-9 at day 10 following ligation of the IVC in HO-1^{+/+} and HO-1^{-/-} mice, and as assessed by mRNA, zymography, and western analysis. **A:** MMP-9 mRNA was determined by quantitative real-time RT-PCR in the thrombus-containing IVC (IVC-ligated) in HO-1^{+/+} and HO-1^{-/-} mice at day 10 following ligation of the IVC, and in the IVC in sham-operated mice. $n = 6$ and $n = 4$ in HO-1^{+/+} and HO-1^{-/-} mice respectively subjected to sham operation, and $n = 7$ and $n = 5$ in HO-1^{+/+} and HO-1^{-/-} mice respectively subjected to ligation of the IVC. * $P < 0.05$, IVC-ligated HO-1^{-/-} mice versus IVC-ligated HO-1^{+/+} mice. **B:** Assessment of pro-MMP-9 by zymography. The numbers below each lane represent the individual densitometric reading, and the means of the values for that experimental group were provided below this. **C:** Assessment of pro-MMP-9 protein by western analysis. Equivalency of loading was assessed by immunoblotting for β-actin.

Figure 12. Expression of tissue factor mRNA at day 10 following ligation of the IVC in HO-1^{+/+} and HO-1^{-/-} mice. Tissue factor mRNA was determined by quantitative real-time RT-PCR in the thrombus-containing IVC (IVC-ligated) in HO-1^{+/+} and HO-1^{-/-} mice at day 10 following ligation of the IVC, and in the IVC in sham-operated mice. $n = 6$ in HO-1^{+/+} and HO-1^{-/-} mice subjected to sham operation, and $n = 6$ and $n = 5$ in HO-1^{+/+} and HO-1^{-/-} mice respectively subjected to ligation of the IVC. * $P < 0.05$, IVC-ligated HO-1^{-/-} mice versus IVC-ligated HO-1^{+/+} mice.

mice, the clot size in HO-1^{-/-} mice increased from day 2 to day 10. From these findings we conclude that the deficiency of HO-1 impairs the processes that enable net resolution of the clot.

The effect of genetic deficiency of HO-1 in mice on arterial thrombosis has been analyzed in two prior studies that have used dissimilar chemical insults by which to induce arterial thrombosis.^{37,38} In studies in which arterial thrombosis was induced by the application of ferric chloride to the carotid artery, the occlusion time for arterial thrombosis was not different in HO-1^{+/+} and HO-1^{-/-} mice nor was *ex vivo* blood clotting in collagen-coated capillary tubes altered in HO-1^{-/-} mice as compared to HO-1^{+/+} mice.³⁷ However, under conditions of oxidative stress as induced by hemin, arterial clot formation was hastened in HO-1^{-/-} mice.³⁷ In studies in which arterial thrombosis was induced by the application of photochemical stress to the carotid artery, such arterial thrombosis occurred more rapidly in HO-1^{-/-} mice.³⁸ In these latter studies, HO-1^{-/-} mice did not exhibit any intrinsic defects in hemostasis as assessed either by bleeding time, prothrombin time, or platelet counts. These findings, in conjunction with unaltered clot formation either *in vivo* or *ex vivo* in HO-1^{-/-} mice subjected to the ferric chloride model, indicate that there are no intrinsic defects in hemostatic function in the unstressed state in HO-1^{-/-} mice.^{37,38} From these findings we suggest that the exaggerated clot size we observed in HO-1^{-/-} mice following stasis-induced venous thrombosis may not be ascribed to inherent, *pre-existing*, hemostatic defects present in this mutant strain. Indeed, the size of the established clot at day 2 was not different in HO-1^{+/+} and HO-1^{-/-} mice.

A salient finding in our analysis was the striking up-regulation of key participants in inflammation in the venous thrombosis model in HO-1^{-/-} mice as compared with HO-1^{+/+} mice. Indeed, activation of NF- κ B, the archetypal transcriptional instigator of inflammatory processes, was much more pronounced following ligation of the IVC in HO-1^{-/-} mice as compared with HO-1^{+/+}

mice. As shown by numerous experimental and clinical observations, inflammation predisposes to venous thrombosis and, conversely, venous thrombosis is associated with inflammation as reflected by induction of assorted cytokines.⁴⁻⁷ The significance of inflammation as a determinant of clot resolution, chronic venous injury, and venous remodeling has been evaluated in rodent models of venous thrombosis induced by ligation of the IVC.^{4,6,7} The induction of stasis-induced venous thrombosis is attended by neutrophilic infiltration of the clot within the first few days followed by an influx of monocytes, the latter peaking at day 8. Modulating the inflammatory response influences the resolution of the clot, and the observed effects are partly dependent on when, in relation to clot formation, the inflammatory response is altered. For example, the application of anti-inflammatory strategies including the administration of IL-10,³⁹ or an inhibitor of P-selectin,²³ generally administered before or at the time of initiation of venous thrombosis, or a pre-existing inability to express E-selectin or both P-selectin and E-selectin (as occurs in E-selectin knockout and P-selectin/E-selectin double knockout mice respectively),²¹ can all reduce thrombosis and inflammation. However, the inflammatory response itself enables clot resolution as shown by numerous lines of evidence: the administration of IL-8, a pro-inflammatory and pro-angiogenic cytokine, promotes dissolution of the clot, when administered after stasis-induced thrombosis is imposed⁴⁰; neutropenia impairs clot resolution⁴¹; and mice lacking CXCR2, the receptor for the CXCL2 chemokine, which recruits neutrophils, exhibit larger thrombi following stasis-induced thrombosis.⁴² In addition to neutrophils, monocytes are also critically involved in promoting clot resolution because mice lacking CCR2 (the receptor for MCP-1 that is the dominant chemoattractant for monocytes) have markedly impaired resolution of stasis-induced thrombosis.⁴³ Thus, on the basis of these studies, manipulating the inflammatory milieu may exert dual and contrasting effects on clot resolution.

Substantial information suggests that the mechanism whereby inflammation promotes clot lysis involves MMP-9. For example, increased clot resolution as achieved by IL-8, is associated with increased MMP-9 expression,⁴⁰ and delayed resolution of the clot, as observed in CCR2^{-/-} mice and in CXCR2^{-/-} mice, is associated with diminished expression of MMP-9.^{42,43} We thus examined expression of MMP-9 as a possible basis for impaired clot resolution in HO-1^{-/-} mice. However, in our studies, the impairment in clot resolution in HO-1^{-/-} mice was associated with a striking induction of MMP-9. This would thus suggest that the other adverse effects induced by deficiency of HO-1 override the actions of MMP-9 that promote clot lysis.

We suggest that the exaggerated and unabated pro-inflammatory response induced by HO-1 deficiency underlies the enlargement of the clot size between days 2 and 10, a time period during which clot size decreased in HO-1^{+/+} mice. Inflammation may exert actions that foster clot formation and propagation that more than offset the capacity of inflammation to promote clot lysis, and it is these clot-promoting properties that may prove dominant

in the HO-1 deficiency state. For example, a pro-inflammatory state can activate the endothelium thereby transforming the endothelial phenotype from an anticoagulant to a procoagulant one.⁴⁻⁷ The exaggerated and protracted up-regulation of inflammation that occurs in the HO-1 deficiency state may thus lead to sustained endothelial activation and the persistence of a procoagulant phenotype. Such a phenotypic transformation may promote the generation and propagation of thrombus at a rate that exceeds that at which thrombus is lysed, despite augmentation of the latter by inflammatory processes. Indeed, we observed significant and marked up-regulation of tissue factor in HO-1^{-/-} mice as compared to HO-1^{+/+} mice following IVC ligation. Tissue factor is critically involved in thrombogenesis in a number of pathophysiologic states and may thus contribute to the impaired clot resolution observed in HO-1^{-/-} mice following IVC ligation. Examination of the expression of the plasminogen-plasmin system would also be of interest.

Persistent inflammation in the venous wall may promote chronic scarring of the venous wall.^{44,45} Indeed, it has been pointed out that while therapeutic strategies that are pro-inflammatory may aid clot lysis, the persistence of such inflammatory effects after the thrombus is lysed incurs the risk for chronic venous insufficiency.⁴⁵ MMP-9 is incriminated as at least one of the intermediates whereby sustained inflammation can lead to injury of the arterial and venous vasculature.^{34,35} The markedly increased inflammatory response observed in the venous wall along with the marked induction of MMP-9 in HO-1^{-/-} mice may thus set the stage for chronic venous injury and scarring.

We suggest that our findings may be germane to dysfunction and failure of arteriovenous fistulas used for hemodialysis. Failure of these fistulas is often due, as a terminal event, to thrombotic luminal occlusion of the venous limb, the latter thickened and stenosed by neointima hyperplasia and inflammation.^{46,47} Thrombosed arteriovenous fistulas exhibit, as compared with non-thrombosed fistulas, marked inflammatory responses in the venous wall, and significantly increased expression of MMP-9, the latter localizing, mainly, to leukocytes present in the subintimal area of the venous wall.⁴⁸ It has been suggested that such expression of MMP-9 lyses the extracellular matrix and damages the endothelium such that the latter assumes procoagulant properties that promote thrombosis. Our recent studies demonstrate that HO-1 deficiency exaggerates neointimal hyperplasia in a murine model of an arteriovenous fistula, markedly up-regulates MMP-9 expression, and compromises blood flow through the fistula, leading to premature failure of these arteriovenous fistulas.²⁹ Interestingly, dysfunction and failure of arteriovenous fistulas are more likely to occur in patients exhibiting polymorphisms in the HO-1 gene characterized by long GT repeats.⁴⁹ Long GT repeats in the HO-1 promoter, as shown by others, may lead to reduced expression or inducibility of HO-1.¹⁷⁻¹⁹ We suggest that the exaggerated inflammatory response and the markedly impaired resolution of venous clot, as observed in the present study, may be germane to the premature

cessation of blood flow that we recently observed in arteriovenous fistulas created in HO-1 deficient mice.

In summary, we demonstrate that venous thrombosis as induced by ligation of the IVC leads to marked induction of HO-1, and such induction of HO-1 is a beneficial response: the inability to express HO-1 markedly impairs clot resolution and strikingly exaggerates the inflammatory responses induced by thrombosis. We suggest that these findings may offer insights into the recent observation that polymorphisms in the HO-1 gene, characterized by long GT repeats, may increase the risk for recurrent venous thrombosis²⁰ and for dysfunction of arteriovenous fistulas the latter caused, in part, by thrombosis occurring in the venous limbs of these fistulas.⁴⁹

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