First published March 27, 2015; doi:10.1152/ajpheart.00882.2014.

Induction and functional significance of the heme oxygenase system in pathological shear stress in vivo

Lu Kang,1 Matthew L. Hillestad,2 Joseph P. Grande,3 Anthony J. Croatt,1 Michael A. Barry,4 Gianrico Farrugia,5 Zvonimir S. Katusic,6 and Karl A. Nath1

1Division of Nephrology and Hypertension, Mayo Clinic, Rochester, Minnesota; 2Division of Cardiovascular Disease, Mayo Clinic, Rochester, Minnesota; 3Division of Infectious Diseases, Mayo Clinic, Rochester, Minnesota; 4Division of Gastroenterology and Hepatology, Mayo Clinic, Rochester, Minnesota; and 5Departments of Anesthesiology and Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic, Rochester, Minnesota

Submitted 9 December 2014; accepted in final form 19 March 2015

INSULTS TO THE VASCULATURE instigate an interplay of vasculopathic and vasoprotective processes. Exploring the basis for such vasoprotective responses is clinically relevant because of the possibility of uncovering novel therapeutic strategies for cardiovascular diseases (35). In this regard, substantial interest centers on heme oxygenase (HO)-1, the inducible isozyme of HO, as the induction of HO-1 often confers salutary effects in models of tissue injury (1, 41). HO degrades heme, generates the gaseous product carbon monoxide (CO), produces the bile pigment biliverdin, and releases iron from the tetrapyrrole heme ring. While the induction of HO-1 is often viewed as a cytoprotective response, all products resulting from HO-based degradation of heme are potentially toxic, and, indeed, injurious effects have been ascribed to induced HO-1 (23, 27, 50). The other HO isozyme, constitutively expressed HO-2, is also of interest in tissue injury since, depending on the experimental setting, protective or injurious effects may emanate from HO-2 (30, 42, 53).

The present study examined whether the HO system is induced in the vasculature subjected to pathologic forms of shear stress. Low laminar and oscillatory shear stress are the main types of pathologic shear stress, and both are increasingly implicated in the initiation and progression of vascular disease because of their proinflammatory, proliferative, and other effects on the vessel wall (6, 7, 14). This study examined whether such alterations in shear stress upregulate HO-1 and HO-2, and the functional significance of any such induction.

To address this question, we used a model of vascular injury induced by selective ligation of the major branches of the common carotid artery, the partial carotid artery ligation (PCAL) model (32, 40). In this model, blood flow in the main trunk of the carotid artery diminishes and is accompanied by vascular remodeling and an attendant increase in intimal-media thickness (32, 40). Increased intimal-media thickness of the carotid artery in humans presages the risk for cardiovascular disease, both in general and in specific vascular beds such as the coronary artery, and has been used as an index for such risk and disease in clinical trials (46, 51).

The specific choice of the PCAL model in this study resided, however, not just in its clinical relevance but also because this model induces low laminar and oscillatory shear stress in the main trunk of the carotid artery, as definitively documented in a previous study (40). While pathological shear stress is presumed to exist in models of vascular injury, in very few studies has such shear stress been demonstrated or confirmed. Because of prior documentation of the presence of low laminar and oscillatory shear stress in the PCAL model, this clinically relevant in vivo model was thus used to examine the behavior of the HO system in response to pathological shear stress.

MATERIALS AND METHODS

Experiments using the PCAL model in mice. All experiments were approved by the Institutional Animal Care and Use Committee of Mayo Clinic and were performed in accordance with National Institutes of Health guidelines. These experiments primarily used 10- to

Address for reprint requests and other correspondence: K. A. Nath, Mayo Clinic, Guggenheim 542, 200 First St. SW, Rochester, MN 55905 (e-mail: nath.karl@mayo.edu).

0363-6135/15 Copyright © 2015 the American Physiological Society http://www.ajpheart.org
12-wk-old male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME). These experiments also used age- and sex-matched HO-1/−/− mice, HO-2/−/− mice, and their respective control mice generated from relevant colonies maintained as we have previously described (20, 30, 44). HO-1/−/− and HO-1/−/− mouse and HO-2/−/− mice were in the age range of 20 to 35 wk, and HO-2/−/+ and HO-2/−/− mice were in the age range of 10 to 50 wk.

The PCAL model was performed as previously described (32, 40). Briefly, after mice were anesthetized [pentobarbital (60 mg/kg ip)], three of the four branches (external carotid artery, internal carotid artery, and occipital artery) of the left carotid artery (LCA) were ligated using 6-0 silk suture, leaving flow through the superior thyroid artery. Sham operation for control mice included anesthesia, ventral neck incision, and exposure of the LCA. Buprenorphine (0.1 mg/kg) was administered. Sham operation for control mice included anesthesia, ventral neck incision, and exposure of the LCA. Buprenorphine (0.1 mg/kg) was administered. Briefly, after mice were anesthetized [pentobarbital (60 mg/kg ip)], three of the four branches (external carotid artery, internal carotid artery, and occipital artery) of the left carotid artery (LCA) were ligated using 6-0 silk suture, leaving flow through the superior thyroid artery. Sham operation for control mice included anesthesia, ventral neck incision, and exposure of the LCA. Buprenorphine (0.1 mg/kg) was administered. Briefly, after mice were anesthetized [pentobarbital (60 mg/kg ip)], three of the four branches (external carotid artery, internal carotid artery, and occipital artery) of the left carotid artery (LCA) were ligated using 6-0 silk suture, leaving flow through the superior thyroid artery. Sham operation for control mice included anesthesia, ventral neck incision, and exposure of the LCA. Buprenorphine (0.1 mg/kg) was administered.

In additional experiments in C57BL/6J mice, HO-1 expression was upregulated using adenovirus associated viral (AAV) vectors, whereas in other experiments, the HO product CO was delivered via a CO emitting compound [CO-releasing molecule (CORM)-3, tricarbonylchloro[glycinato]ruthenium II, Sigma-Aldrich, St. Louis, MO]. AAV9(HO-1) vector (1 × 1012 vg/mouse) or PBS was administered intravenously, and, 3 wk later, PCAL or sham surgery was performed. After 2 additional weeks, blood flow was measured. CORM-3 was administered to mice via in traperitoneal injections (40 mg/kg) (12, 60), with doses given 1 day before, the day of, and 1 day after PCAL surgery. Control mice received equivalent doses of inactivated (iCORM-3), which was prepared by allowing CO to evolve from a solution of CORM-3 at room temperature for 24 h; nitrogen gas was bubbled through the solution for 10 min to remove residual CO. One day after PCAL surgery, blood flow through the carotid arteries was assessed 45–60 min after the final dose of CORM-3 or iCORM-3 was administered.

In additional experiments in C57BL/6J mice, the effect of inhibition of HO activity using tin protoporphyrin (SnPP; catalog no. 05 PSB, Transcommit Systems, Ithaca, NY), as described in our previous study (31).

For some experiments in C57BL/6J mice, HO-1 expression was measured 45–60 min later using a perivascular flow probe (catalog no. 05 PSB, Transcommit Systems, Ithaca, NY), as described in our previous study (31).

AAV9(HO-1) vector construction and production. The self-complementary HO-1 transgene used in these experiments was made by subcloning HO-1 from pcDNA3.1/HO-1 (55) into pTRIZol method (Invitrogen, Carlsbad, CA) and further purified with an RNeasy Mini kit (Qiagen, Valencia, CA) according to each manufacturer’s protocol. Purified RNA was subsequently used in reverse transcription reactions (Transcriptor First Strand cDNA Synthesis kit, Roche Applied Science, Indianapolis, IN) using random hexamers. The resulting cDNA was used for quantitative real-time PCR analysis performed on an ABI Prism 7900HT (Applied Biosystems). FastStart Universal Probe Master mix and master mix reagent (catalog no. 04914058001, Roche Applied Science) was used for these reactions using probes and primers obtained as assay sets (TaqMan Gene Expression Assays, Applied Biosystems). Parameters for quantitative PCR were as follows: 10 min at 95°C followed by 40 cycles of amplification for 15 s at 95°C and 1 min at 60°C. Expression of 18S rRNA was used for normalization of the expression of each target gene.

Western blot analysis. Western blot analysis for HO-1 and HO-2 protein expression in carotid arteries was performed as previously described (43, 49). Two carotid arteries per sample were combined and homogenized in RIPA buffer consisting of 10 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1% Nonindet P-40 substitute, 0.5% sodium deoxycholate, 0.1% SDS, and protease/phosphatase inhibitors (catalog no. 1861281, Thermo Scientific, Waltham, MA). After quantitation by the Lowry method, 10 μg protein aliquots were separated on 12% Tris·HCl gels and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA). Membranes were block in 5% nonfat milk for 1 h and incubated overnight with rabbit polyclonal primary antibody for HO-1 or HO-2 (catalog nos. ADI-SPA-895 and ADI-SPA-897, respectively, Enzo Life Sciences, Farmingdale, NY). The next day, horseradish peroxidase-conjugated goat anti-rabbit polyclonal secondary antibody (Bio-Rad Laboratories) was used in a 1-h room temperature incubation followed by detection using chemiluminescence methods (GE Healthcare, Buckinghamshire, UK). Equivalency of protein loading was assessed by immunoblot analysis for β-actin using rabbit polyclonal antibody (catalog no. 612656, BD Biosciences, San Jose, CA). Expression of p65, phospho-p65, and IκBα was analyzed in nuclear and cytosolic protein fractions prepared as described below. Rabbit monoclonal antibody against p65 (catalog no. 8242, Cell Signaling Technology, Danvers, MA) and rabbit polyclonal antibodies against phospho-p65 and IκBα (catalog nos. 3031 and 9242, respectively, Cell Signaling Technology) were used as primary antibodies with goat polyclonal secondary antibody.

Preparation of nuclear/cytoplasmic fractions from mouse carotid arteries. Nuclear and cytoplasmic protein fractions were prepared from mouse carotid arteries for assessment of activation of NF-κB by Western blot analysis of total and phosphorylated p65 subunits as well as IκBα. Fractionation was performed using a nuclear extraction kit (catalog no. 40010, Active Motif, Carlsbad, CA). For each sample to be fractionated, three to four sham-operated or three to four PCAL carotid arteries were combined and processed according to the manufacturer’s protocol with the substitution of RIPA buffer [10 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1% Nonindet P-40 substitute, 0.5% sodium deoxycholate, 1% SDS, and protease/phosphatase inhibitors] for the kit’s complete nuclear lysis buffer. Cytosolic fractions derived with this kit method were also diluted in RIPA buffer. Nuclear and cytosolic protein aliquots were then analyzed by Western blot analysis as described above.

Immunohistochemical localization of HO-1 and HO-2 expression. Immunohistochemical analysis of HO-1 and HO-2 expression was performed as previously described (31) on 5-μm sections prepared from formalin-fixed paraffin-embedded carotid arteries from mice subjected to either sham or PCAL surgery. Briefly, immunohistochemical staining was carried out using a Bond III automated immunohistochemical staining system (Leica Microsystems, Buffalo Grove, IL) by the Mayo Pathology Research Core using the following parameters. After peroxidase quenching (catalog no. PX968M, Biocare Medical, Concord, CA) for 5 min, antigen retrieval in 10 mM EDTA (pH 9, 10 min, 95°C) was performed, and sections were blocked for 15 min with Rodent Block M (catalog no. RMB961, Biocare Medical). Sections were then incubated with primary antibodies for HO-1 and HO-2 (as described above for Western blot analysis for HO-1 and HO-2) and, after washing, incubated in peroxidase-conjugated secondary antibodies. After an additional wash, sections were incubated with peroxidase substrate. Color development was visualized using AEC substrate (Biocare Medical). Sections were then counterstained with hematoxylin. Immunohistochemical analysis of HO-1 and HO-2 expression was performed as previously described (31) on 5-μm sections prepared from formalin-fixed paraffin-embedded carotid arteries from mice subjected to either sham or PCAL surgery. Briefly, immunohistochemical staining was carried out using a Bond III automated immunohistochemical staining system (Leica Microsystems, Buffalo Grove, IL) by the Mayo Pathology Research Core using the following parameters. After peroxidase quenching (catalog no. PX968M, Biocare Medical, Concord, CA) for 5 min, antigen retrieval in 10 mM EDTA (pH 9, 10 min, 95°C) was performed, and sections were blocked for 15 min with Rodent Block M (catalog no. RMB961, Biocare Medical). Sections were then incubated with primary antibodies for HO-1 and HO-2 (as described above for Western blot analysis for HO-1 and HO-2) and, after washing, incubated in peroxidase-conjugated secondary antibodies. After an additional wash, sections were incubated with peroxidase substrate. Color development was visualized using AEC substrate (Biocare Medical). Sections were then counterstained with hematoxylin.
analysis, 1:200 for HO-1 and 1:100 for HO-2) for 60 min at room temperature followed by a 15-min incubation in anti-rabbit secondary antibody conjugated with a horseradish peroxidase-labeled polymer (EnVision+ System-HRP, catalog no. 4010, Dako, Carpinteria, CA). Staining was visualized with diaminobenzidine substrate and counterstained with hematoxylin.

**HO activity in mouse aortas.** HO activity in mouse aortas was determined as described in our previous study (45) in which the rate of generation of bilirubin from hemin was measured. Briefly, aortas were homogenized in 0.2 ml of 0.1 M potassium phosphate (pH 7.4) containing 2 mM MgCl₂, and homogenates were centrifuged at 12,000 g for 15 min. Aliquots (200 μg) of supernatant protein were incubated in 0.2-ml reaction mixtures containing 0.1 M potassium phosphate (pH 7.4), mouse liver cytosol (2 mg cytosolic protein), hemin (20 μM), glucose-6-phosphate (2 mM), glucose-6-phosphatdehydrogenase (0.2 units), and NADPH (0.8 mM) for 90 min at 37°C in the dark. A control reaction omitting NADPH, glucose-6-phosphate, and glucose-6-phosphate-dehydrogenase was also performed for each lysate. Bilirubin was extracted from each reaction with 0.2 ml chloroform and quantitated by the measurement of optical density at 464–530 nm. Using the extinction coefficient for bilirubin (40 M⁻¹·cm⁻¹), HO activity was calculated and expressed as picomoles of bilirubin formed per 60 minutes per milligram of protein.

**Serum bilirubin.** Measurement of serum bilirubin concentration was performed using a commercially available kit (catalog no. MAK126, Sigma-Aldrich), which was based on the Jendrassik-Grof method.

**Statistical analysis.** Results are expressed as means ± SE and were considered statistically significant for P < 0.05. Student’s t-test was used for parametric data, and the Mann-Whitney U-test was used for nonparametric data. Vascular patency in HO-1⁺/⁺ and HO-1⁻/⁻ mice as well as in HO-2⁺/⁺ and HO-2⁻/⁻ mice after PCAL was assessed using Fisher’s exact test.

**RESULTS**

**Characterization of the PCAL model.** Two weeks after the creation of this model, blood flow in the LCA was markedly decreased compared with the sham-operated group (Fig. 1); in contrast, blood flow in the contralateral RCA of animals subjected to partial ligation of the LCA was increased compared with the sham-operated group. Additional evidence that PCAL is a valid model for vascular injury was provided by the expression of vasculopathic candidate genes. Genes such as monocyte chemotractant protein (MCP)-1, IL-6, chemokine (C-C motif) ligand (CCL)5, and TNF-α are well recognized as vasculopathic but have not been evaluated, to date, in this model. These genes were significantly induced after PCAL (Fig. 2), along with other genes relevant to vascular injury (Table 1).

**Induction of HO-1 after PCAL.** Because induction of vasculopathic genes is often accompanied by countervailing responses, we examined one such possible response, namely, expression of HO-1. One week after PCAL, HO-1 mRNA and protein were both markedly induced, and, indeed, such induction of HO-1 protein was detectable as early as 12 h after PCAL (Fig. 3, A and B).

Immunohistochemical experiments were also performed to localize such HO-1 expression; very weak expression of HO-1 was observed in the endothelium in sham-operated arteries, whereas in the PCAL model, HO-1 was prominently expressed not shown). Two weeks after the creation of this model, blood flow in the LCA was markedly decreased compared with the sham-operated group (Fig. 1); in contrast, blood flow in the contralateral RCA of animals subjected to partial ligation of the LCA was increased compared with the sham-operated group. Additional evidence that PCAL is a valid model for vascular injury was provided by the expression of vasculopathic candidate genes. Genes such as monocyte chemotractant protein (MCP)-1, IL-6, chemokine (C-C motif) ligand (CCL)5, and TNF-α are well recognized as vasculopathic but have not been evaluated, to date, in this model. These genes were significantly induced after PCAL (Fig. 2), along with other genes relevant to vascular injury (Table 1).

**Induction of HO-1 after PCAL.** Because induction of vasculopathic genes is often accompanied by countervailing responses, we examined one such possible response, namely, expression of HO-1. One week after PCAL, HO-1 mRNA and protein were both markedly induced, and, indeed, such induction of HO-1 protein was detectable as early as 12 h after PCAL (Fig. 3, A and B).

Immunohistochemical experiments were also performed to localize such HO-1 expression; very weak expression of HO-1 was observed in the endothelium in sham-operated arteries, whereas in the PCAL model, HO-1 was prominently expressed not shown). Two weeks after the creation of this model, blood flow in the LCA was markedly decreased compared with the sham-operated group (Fig. 1); in contrast, blood flow in the contralateral RCA of animals subjected to partial ligation of the LCA was increased compared with the sham-operated group. Additional evidence that PCAL is a valid model for vascular injury was provided by the expression of vasculopathic candidate genes. Genes such as monocyte chemotractant protein (MCP)-1, IL-6, chemokine (C-C motif) ligand (CCL)5, and TNF-α are well recognized as vasculopathic but have not been evaluated, to date, in this model. These genes were significantly induced after PCAL (Fig. 2), along with other genes relevant to vascular injury (Table 1).
Sham PCAL vs. sham. Analysis for two carotid arteries with equivalency of loading assessed by immunoblot after PCAL and sham surgery. Each lane represents a sample obtained from mRNA expression in carotid arteries of sham and PCAL mice at 1 week after PCAL. Table 1.

<table>
<thead>
<tr>
<th></th>
<th>Sham Mice</th>
<th>PCAL Mice</th>
<th>Fold Change</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transforming growth factor-β₁</td>
<td>2.6 ± 0.2</td>
<td>7.7 ± 3.1</td>
<td>3.0</td>
<td>0.0021</td>
</tr>
<tr>
<td>Collagen type I</td>
<td>7.2 ± 0.8</td>
<td>18.7 ± 5.2</td>
<td>2.6</td>
<td>0.028</td>
</tr>
<tr>
<td>Collagen type III</td>
<td>2.1 ± 0.3</td>
<td>5.8 ± 1.6</td>
<td>2.8</td>
<td>0.0057</td>
</tr>
<tr>
<td>Collagen type IV</td>
<td>6.7 ± 0.7</td>
<td>9.1 ± 1.4</td>
<td>1.4</td>
<td>NS</td>
</tr>
<tr>
<td>Cyclooxygenase 1</td>
<td>9.6 ± 0.5</td>
<td>7.6 ± 1.1</td>
<td>↓ 0.8</td>
<td>0.017</td>
</tr>
<tr>
<td>Cyclooxygenase 2</td>
<td>4.0 ± 0.9</td>
<td>9.3 ± 3.3</td>
<td>2.3</td>
<td>NS</td>
</tr>
<tr>
<td>PDGF-A</td>
<td>4.6 ± 0.4</td>
<td>6.4 ± 0.5</td>
<td>1.4</td>
<td>0.016</td>
</tr>
<tr>
<td>PDGF-B</td>
<td>6.7 ± 0.5</td>
<td>11.1 ± 2.4</td>
<td>1.7</td>
<td>0.0101</td>
</tr>
<tr>
<td>Endothelial nitric oxide synthase</td>
<td>2.1 ± 0.4</td>
<td>5.0 ± 0.7</td>
<td>↓ 0.7</td>
<td>0.0152</td>
</tr>
<tr>
<td>Inducible nitric oxide synthase</td>
<td>2.7 ± 0.6</td>
<td>5.2 ± 1.4</td>
<td>1.9</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE; n = 9 and 10 for sham-operated (sham) and partial carotid artery ligation (PCAL) mice, respectively. mRNA expression was assessed by quantitative real-time RT-PCR. The above values are the result of relative quantification performed against a standard curve constructed for each mRNA target, normalized for expression of 18S rRNA, and expressed in arbitrary units. NS, not significant.

in the endothelium, adventitial cells, and, to a lesser extent, smooth muscle cells (Fig. 4, A–D).

Because HO-1 gene expression is regulated by NF-κB, we examined whether NF-κB activation occurred at an early time point in this model. In this regard, using Western blot analysis of cellular extracts of murine carotid arteries, we examined the expression of p65 and IkBα protein in cytosolic fractions and the expression of phospho-p65 protein in nuclear fractions. Because of the low yield, carotid arteries from three mice subjected to PCAL were pooled in each lane shown in Fig. 5A and four arteries were pooled in each lane shown in Fig. 5B. As shown in Fig. 5, expression of p65 and IkBα was diminished in the cytosol, and this was accompanied by increased expression of phospho-p65, the active form of p65, in the nuclear fraction 15 min after the creation of the PCAL model.

**PCAL in HO-1<sup>+/+</sup> and HO-1<sup>−/−</sup> mice.** To determine the functional significance of the induction of HO-1 in this model, we performed PCAL in HO-1<sup>+/+</sup> and HO-1<sup>−/−</sup> mice. HO-1<sup>−/−</sup> mice compared with HO-1<sup>+/+</sup> mice, subjected to this model, exhibited even more marked reduction in carotid artery blood flow and an increased number of arteries with complete loss of patency (Fig. 6, A and B). Histological examination

![Fig. 3. Heme oxygenase (HO)-1 expression in carotid arteries of sham mice and mice subjected to partial ligation of the LCA (PCAL). A: HO-1 mRNA expression 1 wk after PCAL or sham surgery as assessed by quantitative real-time RT-PCR; n = 9 and 10 for sham and PCAL groups, respectively. *P < 0.05 vs. sham. B: Western blot analysis of HO-1 protein expression 1 wk and 12 h after PCAL and sham surgery. Each lane represents a sample obtained from two carotid arteries with equivalency of loading assessed by immunoblot analysis for β-actin.](image)

![Fig. 4. Immunohistochemical analysis of HO-1 protein expression in LCAs of mice 2 wk after sham operation or partial ligation of the LCA (PCAL). A and C: low-power (A) and high-power (C) views of HO-1 expression in a carotid artery of a mouse after sham surgery. B and D: low-power (B) and high-power (D) views of HO-1 expression in a carotid artery of a mouse after PCAL surgery. E and F: isotype control staining for sham (E) and PCAL (F) groups. Scale bar = 100 μm for A and B and 50 μm for C–F.](image)
showed thickening of the arterial wall in HO-1\(^{-/-}\) mice compared with HO-1\(^{+/+}\) mice after PCAL as well as the presence of intravascular thrombus formation in some vessels (Fig. 6C).

**Effect of increased HO-1 expression mediated by AAV9 on carotid artery blood flow after PCAL.** To determine whether increased expression of HO-1 could exert functional effects in this model, we delivered HO-1 cDNA using an AAV9 vector by intravenous injection. Immunohistochemistry demonstrated upregulation of HO-1 in the endothelium and smooth muscle cells in the carotid artery when studied 5 wk after the administration of AAV9(HO-1) (Fig. 7, A–D). Such administration markedly upregulated expression of HO-1 protein in the carotid artery (Fig. 7, E and F) and increased vascular (aorta) HO activity, the aorta providing adequate amounts of the vasculature so as to undertake such measurements of HO activity (Fig. 7G). Administration of AAV9(HO-1) led to the appearance of increased amounts of full-length (32 kDa) HO-1 protein in plasma (Fig. 7H). We also measured total bilirubin levels in serum after the administration of AAV9(HO-1), as elevated serum bilirubin levels often occur in concert with, and reflect, induction of HO-1 in tissues (13, 26). After the administration of AAV9(HO-1), serum levels of bilirubin were significantly increased (3.7 ± 0.4 vs. 2.3 ± 0.2 µM, \(n = 7\) and 6 respectively, \(P < 0.05\)).

This upregulation of HO-1 by AAV9(HO-1) exerted a protective effect in the PCAL model, as demonstrated by greater blood flow in the ipsilateral, partially ligated LCA (Fig. 8). Interestingly, prior upregulation of HO-1 by AAV9 led to significantly increased carotid artery blood flow not only on the ligated side but also in the intact contralateral RCA (Fig. 8).

**Effect of CO on carotid artery blood flow after PCAL.** Because carotid artery blood flow after PCAL was decreased in the absence of HO-1 and increased after the upregulation of HO-1 by AAV9, experiments were undertaken to determine if a product of HO activity, namely, CO, could improve blood flow. As shown in Fig. 9, the administration of CORM-3, an effective delivery system for CO, increased carotid artery blood flow when measured 24 h after PCAL.

**PCAL in HO-2\(^{+/+}\) and HO-2\(^{-/-}\) mice.** Constitutively expressed HO-2 contributes to basal production of CO in the vasculature and other tissues. We thus assessed the expression and functional significance of this HO isoform in the PCAL model. After PCAL, HO-2 mRNA and protein expression were both increased in the carotid artery (Fig. 10, A–C), and immunohistochemical experiments demonstrated that such upregulation of HO-2 in the PCAL model essentially occurred in the endothelium (Fig. 10, D–G). In experiments undertaken in HO-2\(^{+/+}\) and HO-2\(^{-/-}\) mice, carotid artery blood flow was significantly decreased in HO-2\(^{-/-}\) mice compared with HO-2\(^{+/+}\) mice on the ipsilateral side after PCAL (Fig. 11A), blood flow in the contralateral carotid artery was not significantly different in HO-2\(^{+/+}\) and HO-2\(^{-/-}\) mice (Fig. 11A). There was no significant difference in patency rates of the carotid artery after PCAL in HO-2\(^{+/+}\) and HO-2\(^{-/-}\) mice (100 vs. 78%, \(P = \text{not significant}\)). Histological analyses demonstrate focal neointimal hyperplasia in three of nine carotid arteries in HO-2\(^{-/-}\) mice subjected to PCAL (Fig. 11B), whereas such lesions were not seen in any of the carotid arteries from HO-2\(^{+/+}\) mice subjected to PCAL. Thus, both HO isoforms, HO-1 and HO-2, exert vasoprotective effects in the PCAL model.

**Expression of HO-1 in HO-2\(^{+/+}\) and HO-2\(^{-/-}\) mice and expression of HO-2 in HO-1\(^{+/+}\) and HO-1\(^{-/-}\) mice after PCAL.** The mutant murine models used in this study involved global knockout of the specific HO gene. Such specific deficiency may influence the nature of the response in the expression of the other HO gene after PCAL. We thus determined whether the deficiency of one isoform influenced the degree of expression of the other isoform after PCAL. As shown in Fig. 12, the induction of HO-1 mRNA in the carotid artery was exaggerated in HO-2\(^{-/-}\) mice compared with HO-2\(^{+/+}\) mice after PCAL. Thus, the exacerbation of vascular injury when HO-2 is deficient occurred despite greater induction of HO-1 in HO-2\(^{-/-}\) mice subjected to PCAL. The results shown in Fig. 12 also demonstrate that HO-2 mRNA was comparably induced in HO-1\(^{+/+}\) and HO-1\(^{-/-}\) mice subjected to PCAL.
Thus, the exacerbation of vascular injury after PCAL in HO-1−/− mice cannot be ascribed to less induction of HO-2.

Effect of acute inhibition of HO activity on carotid blood flow after PCAL. The beneficial effects of each isoform of HO may arise from vasorelaxant effects of HO activity emanating from that isoform, effects on vascular remodeling, or a combination of both. To determine the extent to which HO activity exerts vasorelaxant effects in the PCAL model, we examined carotid blood flow after the acute inhibition of HO activity by the competitive inhibitor SnPP; such an inhibitor effectively blocks activity arising from both HO-1 and HO-2. Twenty-four hours after such inhibition (compared with vehicle-treated mice), carotid blood flow was significantly reduced on the ipsilateral side in which PCAL was performed (0.11 ± 0.01 vs. 0.07 ± 0.01 ml/min, n = 4 and 5, respectively, P < 0.05) but was not significantly altered on the contralateral, intact carotid artery (0.91 ± 0.05 vs. 0.92 ± 0.06 ml/min, n = 4 and 5, respectively, P = not significant). Thus, HO activity, arising in aggregate from HO-1 and HO-2 isoforms, exerts vasorelaxant effects since the acute inhibition of such activity leads to a reduction in carotid blood flow in the partially ligated carotid artery.

DISCUSSION

PCAL led to vascular injury, as evidenced by a prompt reduction in ipsilateral carotid artery blood flow, vascular remodeling, and the induction of vasculopathic genes. In this setting, marked induction of HO-1 mRNA and protein occurred, the significance of which was assessed in HO-1−/− mice: the imposition of the PCAL model in HO-1−/− mice caused a further reduction in ipsilateral carotid blood flow, the complete loss of patency in some arteries, and exacerbation of histological vascular injury. We pursued additional approaches in examining the countervailing, protective effects of the HO system using carotid artery blood flow as a readout of injury because this index is readily quantified and functionally significant. Such experiments demonstrated that carotid artery
blood flow in the PCAL model increased when HO-1 was upregulated by AAV9 and when the HO product CO was delivered by CORM-3; conversely, carotid artery blood flow decreased further in the absence of the constitutive HO iso-
form, HO-2.

Previous studies have clearly demonstrated that vascular injury induces HO-1 and that such an induction may exert vasoprotective effects (17, 36, 48, 52). However, the expres-
sion and significance of HO-1 have not been studied, to date, in the PCAL model. We used this specific model because, as shown in a previous study (40), low laminar and oscillatory shear stress have been documented in the main trunk of the carotid artery. We suggest that the present findings provide needed information in the field of HO-1 and shear stress because prior analyses of HO-1 expression in response to shear stress have been undertaken, almost exclusively, in vitro. An in vitro approach raises at least three issues, the first of which centers on the lack of consistency in conclusions so obtained. Shear stress includes high laminar shear stress, which is vasoprotective, and low laminar and oscillatory shear stress, both of which are vasculopathic (6, 7, 14). HO-1 is induced in endo-
thelial cells exposed in vitro to conditions intended to recapit-
ulate high (physiological) laminar shear stress (5, 8, 11, 16). However, there are very few in vitro studies that have exam-
ined HO-1 induction in response to what is regarded as a model for low (pathological) laminar shear stress (61), perhaps because of the uncertainty of the threshold for flow rates in vitro that demarcates high from low laminar shear stress. Studies of oscillatory stress in vitro have yielded quite divergent findings: compared with laminar shear stress, oscillatory shear stress-
elicited induction of HO-1 in endothelial cells has been described as exaggerated (15), equivalent (56), or nonexistent (25); induction of HO-1 in statin-exposed endothelial cells is attenuated by oscillatory shear stress but amplified by laminar shear stress (3).

In addition to this issue of consistency of findings, a second consideration is that an in vitro approach often leaves unresolved the question of the functional significance of HO-1 when shear stress is altered. The third consideration centers on the fidelity with which studies in vitro truly reflect what exists in vivo. Endothelial cells in vitro differ phenotypically from endothelial cells in vivo, and, in certain microvascular beds, >40% of the proteins expressed in endothelial cells in vivo may not be detectable in vitro (19). Additionally, gene expression profiles in in vitro systems of shear stress may not recapitulate the responses observed in vivo (6, 7, 14). For example, of the mechanosensitive genes altered in in vivo models of perturbed stress, some 50%, when examined in vitro systems, are not expressed, remain unaltered, or are altered in the opposite direction (47). Such uncertainties and vagaries observed in in vitro models of pathological shear stress systems may be circumvented by a valid, reproducible in vivo model, the latter provided by the PCAL model. Based on the present findings, we suggest that this model affords an...
Compounds that deliver products of HO activity, in particular, CO, are under active development for clinical use because of their potential to confer vasorelaxant and other vasoprotective effects (18, 39, 54). Additionally, other products of HO activity, such as biliverdin and bilirubin, possess vasoprotective properties, including the capacity to scavenge the vasoconstricting oxidant superoxide anion; epidemiologic observations (37) in humans have demonstrated that plasma levels of bilirubin in the high-normal or mildly elevated range are associated with a reduced risk for cardiovascular disease and lower mortality. Finally, a number of drugs currently used in the treatment of cardiovascular disease (statins, nitrates, aspirin, niacin, and sildenafil) and a number of salutary dietary constituents (curcumin and resveratrol) can all induce HO-1, and, for some of these, evidence is available that such induction of HO-1 contributes to their protective effects in experimental models (58). Indeed, there is substantial effort in drug discovery programs targeted to the identification of clinically approved drugs that induce HO-1 and to the screening of chem-

appealing and robust approach in analyzing the basis for the induction of HO-1 in response to pathological shear stress.

In regard to a mechanism relevant to the induction of HO-1 in PCAL, we examined activation of NF-κB, one of the main transcriptional activators of HO-1 expression (2, 34). We observed that within minutes of PCAL, activation of NF-κB occurred in the main trunk of the carotid artery. NF-κB regulates the induction of multiple vasculopathic genes, especially the genes highlighted in this study (IL-6, MCP-1, TNF-α, and CCL5), and NF-κB is increasingly regarded as an instigator of pathological shear stress, as indicated by studies conducted exclusively in vitro. We speculate that in the PCAL model, NF-κB orchestrates vasculopathic processes on the one hand (arising from IL-6, MCP-1, TNF-α, CCL5, and others shown in Table 1) and a vasoprotective response emanating from HO-1 on the other hand. The expression of vasculopathic genes in the PCAL model when HO-1 and HO-2 are deficient would be of interest.

The present findings are germane to the current interest in the HO system and its products as a therapeutic strategy.
tical libraries for compounds and chemical motifs that elicit HO-1 expression.

To the best of our knowledge, the present findings are the first to demonstrate vascular and systemic upregulation of functionally active HO-1 after the utilization of an AAV delivery system. While there is clear prior evidence that up-regulation of HO-1 by adenoviral gene therapy vectors can be vasoprotective (28, 36, 59), there are concerns using this immunostimulatory vector in humans. In contrast, AAV vectors are less immunogenic and are currently the lead vector platform for human gene therapy. Indeed, some 100 trials in humans using AAV vectors have already been conducted (4, 22). Our experiments confirmed the effective upregulation of HO-1 in the carotid artery along with a functional effect of this upregulation, namely, improvement in carotid artery blood flow. Such vascular upregulation of HO-1 by AAV9 also increased carotid artery blood flow in the contralateral, intact carotid artery, an effect likely reflecting HO-1 upregulation in that vascular segment. The administration of AAV9(HO-1) led to substantial amounts of HO-1 protein in the systemic circulation and increased vascular HO activity. The effect of AAV9(HO-1) on vasculopathic gene expression and histologic injury in the PCAL model is of interest.

The reduction in carotid artery blood flow after PCAL was alleviated by the administration of CO, with the latter administered as CORM-3. Thus, a product of HO (in this case, CO) is capable of increasing blood flow in the ligated carotid artery. It should be emphasized, however, that these experiments were performed with the objective of demonstrating that a given product of HO may increase carotid blood flow; it should not be inferred that these experiments identified CO as the basis for the vasorelaxant effects of HO. At least three additional issues may be germane to the findings from these experiments. First, CORMs may exert cardiac and systemic effects, and not just local vasorelaxant effects. Second, the findings from these experiments do not lead us to specify whether this is a vasorelaxant effect, a remodeling effect, or a combination of both; however, as the carotid blood flow experiments were performed 48 h after the administration of CO, the former may more likely underlie the increase in blood flow. Third, several mechanisms besides lesser amounts of CO may be relevant to the observed effects of HO-1 deficiency. These include 1) the loss of the antioxidant effects of the enzyme; 2) the lack of generation of bilirubin/biliverdin, products of HO that are antioxidant, anti-inflammatory, and vasoprotective; and 3) up-regulation of proliferative, proinflammatory, and procoagulant pathways in the setting of HO-1 deficiency.

Our findings also demonstrate a beneficial effect of HO-2 in the PCAL model, as assessed both by functional indexes and histologic analyses. In one-third of the HO-2−/− mice subjected to PCAL, focal neointimal hyperplasia was observed; as our analyses did not involve serial sectioning of the entire length of the trunk of the carotid artery, the true frequency of this focal histological lesion may be higher than 33%. These findings in HO-2−/− mice raise the following three considerations regarding HO-2 and vascular injury. First, the presence of neointimal hyperplasia after PCAL when HO-2 is deficient is notable, as this lesion is not a usual finding in the PCAL model when the latter is induced in otherwise unmanipulated, wild-type mice; vascular expression of HO-2 may thus represent an endogenous defense mechanism that guards against the development of neointimal hyperplasia in the injured vasculature. Second, the present findings, in conjunction with our recent study (30) demonstrating that HO-2 is required in maintaining patency and blood flow in an arteriovenous fistula, underscore the vasoprotective properties of HO-2. Finally, the HO-2 gene is readily induced by corticosteroids, with the latter commonly used in the treatment of inflammatory vasculopathies. Based on our present findings, we speculate that the beneficial effects of corticosteroids in vasculitides may involve the induction of HO-2.

The present experiments using the PCAL model underscore the usefulness of this model in the study of cardiovascular disease. In addition to demonstrating carotid intimal-media thickening and the presence of pathological shear stress (32, 40), this model exhibits endothelial dysfunction and oxidative stress (40), accelerates the progression of atherosclerosis (40), and provides important insights regarding the roles of such diverse proteins as Axl (a receptor tyrosine kinase) (33), fibronectin (10), fibroblast growth factor 2 (57), platelet/endothelial cell adhesion molecule-1 (9), and IL-17 (38) in vascular remodeling.

In summary, the present study demonstrates the induction and functional significance of the HO system in the vasculature subjected to pathological shear stress, demonstrating, in the process, protective effects conferred by the observed induction of HO-1 and the administration of CORMs, efficacy in achieving vascular upregulation of HO-1 by AAV9 along with a vasorelaxant effect, and the vasoprotective effects of HO-2. Finally, we provide evidence for the activation of NF-kB in this model. As this transcription factor is an inducer of HO-1, such NF-kB activation may not only underlie genetic responses accounting for pathological shear stress but also those that mitigate the severity of these pathological responses.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the technical expertise of Allan Ackerman and the secretarial expertise of Kara Zelinske in the preparation of this work.

GRANTS

This work was supported by National Institutes of Health Grants DK-70124, DK-47060, HL-91867, and T32-DK-007013. This work was also supported by the Mayo Clinic Center for Regenerative Medicine.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


