Background—Recent studies have demonstrated that systemic pharmacological induction of heme oxygenase-1 (HO-1), the inducible isoform of the initial and rate-limiting enzyme for heme catabolism, attenuates neointima formation after experimental vascular injury. We have now investigated the ability of localized adenovirus-mediated HO-1 (Ad-HO-1) gene delivery to modify arterial remodeling after balloon angioplasty.

Methods and Results—Two weeks after balloon angioplasty in the rat carotid artery, elevated HO-1 protein was observed in the Ad-HO-1 arteries compared with those exposed to empty adenovirus (Ad-E) or to PBS. The arteries exposed to Ad-HO-1 exhibited significantly reduced neointimal area, medial wall area, neointimal area/medial wall area ratio, and neointimal thickness compared with arteries exposed to Ad-E. The Ad-E vessels showed subtle reductions in each morphometric parameter compared with PBS vessels. In a separate group of animals, concomitant treatment of Ad-HO-1 with the HO-1 inhibitor tin protoporphyrin completely restored each morphometric parameter to control levels. Arteries exposed to Ad-HO-1 demonstrated significantly increased TUNEL labeling of apoptotic nuclei and significantly decreased PCNA labeling of DNA synthesis in the medial wall 48 hours after injury.

Conclusions—These results indicate that HO-1 represents an important in vivo vasoprotective mediator that is capable of attenuating the pathophysiological remodeling response to endovascular injury and suggest that HO-1 may be a novel target for the treatment of vascular disease. (Circulation. 2001;104:2710-2715.)

Key Words: viruses ■ arteries ■ neointima ■ heme oxygenase

Heme oxygenase is the rate-limiting enzyme that catalyzes the degradation of heme to carbon monoxide (CO), free iron, and biliverdin. Heme oxygenase-1 (HO-1), the inducible isoform of heme oxygenase, provides cytoprotection in various in vitro and in vivo systems, and activation of the HO-1 gene has been considered to be an adaptive cellular defense mechanism. Cultured fibroblasts from HO-1–knockout mice are highly susceptible to heme- or hydrogen peroxide–mediated cytotoxicity,1 and exposure of HO-1–deficient mice to endotoxin results in increased hepatocellular necrosis and mortality from endotoxic shock.1 The first human case of HO-1 deficiency exhibited similar characteristics, including severe, persistent endothelial damage in vivo and extreme sensitivity to hemin-induced cell injury in a cell line derived from this patient.2 Several lines of evidence from our work3 and others4,5 strongly suggest that heme oxygenase plays an important physiological and pathophysiological role in the vasculature. There is increased expression of HO-1 in human atherosclerotic lesions,6 as well as in vascular endothelial and smooth muscle cells (SMCs) exposed to oxidized LDL.7 The induction of HO-1 in cardiac allografts that do not show accelerated arteriosclerosis has suggested that this enzyme may protect against the chronic rejection of transplants.8 Induction of HO-1 inhibits atherosclerotic lesion formation in LDL receptor–knockout mice.9 The adaptive response of HO-1 induction has been considered to promote the maintenance of vascular tone and patency in atherosclerotic vessels via metabolism of heme to vasodilatory CO and the antioxidant biliverdin. The removal of pro-oxidant heme (which accumulates in atherosclerotic plaques) and the concomitant induction of ferritin expression (which sequesters free iron) also contribute to the antioxidant defense of HO-1 induction in atherogenesis. Finally, HO-1 gene transfer may confer vascular protection. Transfection of the HO-1 gene into coronary endothelial cells attenuates the damaging effects of free heme and induces an angiogenic response in vitro.10,11

Recently, we3 and others4,5 have investigated the influence of pharmacologically induced HO-1 overexpression on atherosclerotic remodeling after balloon injury in the rat carotid artery (CA). Results from these studies indicate that systemic upregulation of vascular HO-1 significantly reduces neointi-
ma development after injury. CO, released as a byproduct of HO-1–catalyzed heme degradation, may be an important mediator of HO-1–induced vasoprotection.5 In support of this, recently published observations from our laboratory with a cGMP-stimulating benzyl indazole derivative, YC-1, suggest that endogenous CO-stimulated cGMP is capable of reducing postinjury neointimal development.12 Others report that free iron is important in mediating post–balloon injury neointimal development through stimulation of vascular SMC proliferation.13 Free iron rapidly upregulates and is then sequestered by ferritin, which itself contains potent antioxidant apoferroxidase activity.14 Finally, the HO-1–mediated bile pigments biliverdin and bilirubin confer vasoprotection through potent antioxidant15 and anticomplement properties.16

We report here that localized adenovirus-mediated HO-1 gene delivery to balloon-injured rat CAs significantly stimulates vascular HO-1 protein and markedly attenuates the pathophysiological neointimal and medial wall response to injury. Adenovirus-mediated transfection of the HO-1 gene significantly stimulates acute medial wall SMC apoptosis and concomitantly inhibits medial wall DNA synthesis. These results provide direct support of the hypothesis that HO-1 is an important pathophysiological determinant of postinterventional stenosis and remodeling and suggest that HO-1 gene delivery has potential therapeutic applicability.

Methods

Rat CA Balloon Angioplasty
An established rat CA model of balloon angioplasty was used to examine the in vivo response to arterial injury.17 Male Sprague-Dawley rats (463±3.8 g; Harlan, Indianapolis, Ind) were anesthetized with a combination anesthetic (ketamine, xylazine, and acepromazine; 0.5 to 0.7 mL/kg IM; VetMed Drugs). The left CA vasculature was exposed, and an isolated arterial segment was made with hemostatic clamps. A Fogarty 2F embolectomy catheter (Baxter Healthcare Corp) was introduced into an external carotid arteriotomy incision, advanced to the common CA, and inflated and withdrawn 3 times with rotation. The catheter was removed, and the injured vessel was exposed to the various treatments. After treatment, the incision was closed, and on full recovery, animals were returned to the animal care facility and provided standard rat chow and water containing acetaminophen (300 mg/kg; Sigma) ad libitum.

Adenovirus HO-1 Gene Preparation and Delivery
Recombinant adenovirus containing the HO-1 gene was prepared by use of the AdEasy System (Johns Hopkins Oncology Center). The HO-1 gene was cloned into the shuttle vector pAdTrack-CMV, and the resultant plasmid was linearized with PmeI digestion. The linearized plasmid was cotransformed into Escherichia coli B35183 cells with an adenoviral backbone plasmid, pAdEasy-1. Recombinants were selected, and recombination was determined by restriction endonuclease analysis. The linearized recombinant plasmid was transfected into 293 cells, and high-titer viral stocks were prepared.

The protocol used for treating rat CAs with adenovirus has been described previously.18 Immediately after injury, a polyethylene catheter was introduced through the external carotid arteriotomy site. The injured CA was washed with PBS, and 50 μL adenovirus (2×1010 pfu/mL) with or without the HO-1 gene, or 50 μL PBS, was infused and incubated for 30 minutes. A separate cohort of animals exposed to Ad-HO-1 was topically treated with the HO-1 inhibitor tin protoporphyrin IX (10 mg; SnPP-IX; Porphyrin Products) in gel immediately after surgery.

Western Blot for HO-1 Protein
The protocol used for Western blot analysis of HO-1 has been described previously.3 Briefly, fresh CAs were removed and immediately snap-frozen. Protein was homogenized, whole-cell lysates were boiled and sonicated, and SDS-PAGE was performed with 20 μg protein per tissue. The separated blots were transferred to nitrocellulose membranes, blocked, and incubated with an anti-HO-1 polyclonal antibody (1:500; Stressgen Biotechnologies Corp). Membranes were incubated with anti–rabbit horseradish peroxidase–conjugated secondary antibody (1:7500) followed by treatment with enhanced chemiluminescence reagents (Amersham Corp). Densitometric analysis of gel films was performed with an Ultrascan XL Enhanced Laser Densitometer (Pharmacia LKB Biotechnology).

Morphometric Analyses
Perfusion-fixed, paraffin-embedded tissues and standard tissue staining techniques were used.3 For microscopic verification of apoptotic morphology, slides were treated with hematoxylin and eosiin. For verification of viral transfection in the vessel wall, slides were analyzed under a fluorescence microscope (Olympus AX70) for the presence of green fluorescent protein (GFP). Microscopic analyses and quantification of morphological parameters were performed as previously described.3

TUNEL Assay
Perfusion-fixed, paraffin-embedded tissues were stained for apoptotic nuclei with an In Situ Cell Death Detection Kit (Roche Molecular Biochemicals). Slides were treated with proteinase K, endogenous peroxidase activity was blocked, and slides were exposed to an avidin-biotin block with antigen retrieval. Slides were permeabilized, incubated with terminal dUTP nick end-labeling (TUNEL) reaction mixture, treated with converter-peroxidase solution, and exposed to diaminobenzidine (DAB) black-nickel chromagen. Slides were counterstained with hematoxylin, coverslipped, and analyzed under light microscopy. Data are represented as a TUNEL labeling index (LI), defined as the percentage of total medial wall cells positive for TUNEL staining.

PCNA Immunohistochemistry
Standard immunohistochemical techniques were used with an anti–proliferating cell nuclear antigen (PCNA) monoclonal antibody (PC-10; 1:25; Sigma) and a biotinylated anti-mouse secondary antibody (1:100) on perfusion-fixed paraffin-embedded tissues. Slides were treated with an avidin-biotin block and exposed to DAB black chromogen with nuclear fast red counterstain. Slides were coverslipped for analysis under light microscopy. Data are represented as a PCNA LI.

Statistical Analyses
Data were stored and analyzed on personal computers with Excel 97 (Microsoft) and Sigma Plot 4.0 with Sigma Stat 2.03 (SPSS Inc). Data were grouped according to treatment and analyzed with an unpaired Student’s t test. All data are represented as mean±SEM. A value of P≤0.05 is considered statistically significant for all comparisons.

Results
Successful viral transfection to the artery wall was measured by the presence of GFP, which showed much higher levels in the Ad-HO-1 and Ad-E animals than the PBS controls 48 hours and 2 weeks after injury (data not shown). A panel of 3 separate Western blots 2 weeks after injury (Figure 1) illustrate that ipsilateral CA HO-1 protein was significantly higher in the Ad-HO-1 animals than both the empty adeno-
Ad-HO-1 animals for circumferences of the left CA and concentric neointima is evident, and the medial wall is clearly defined by the internal and external elastic laminae. Figure 2B shows a CA exposed to Ad-E. A significant but slightly reduced neointima is evident. In contrast, Figure 2C shows a CA exposed to the Ad-HO-1 gene, in which a markedly attenuated concentric neointima is noted along the luminal border. Figure 2D illustrates a CA exposed to Ad-HO-1, simultaneously treated with perivascular application of the HO-1 inhibitor SnPP-IX. The neointima was completely restored with inhibition of activity of the adenovirus-mediated induced HO-1. Histomorphometric analyses of these data are graphically illustrated in Figure 2E through 2H. Parameters for left CA neointimal area (Figure 2E), medial wall area (Figure 2F), neointimal area/medial wall area ratio (Figure 2G), and neointimal thickness (Figure 2H) were all significantly reduced in the Ad-HO-1 animals compared with the Ad-E control animals. Although a trend was evident, significant differences were not found between the Ad-E animals and the PBS controls for any parameter. Concomitant treatment of the Ad-HO-1 gene with perivascular SnPP-IX significantly restored each parameter to control levels. No significant differences were observed between the Ad-E and Ad-HO-1 animals for circumferences of the left CA external elastic laminae (3.27 ± 0.035 versus 3.06 ± 0.08 mm, respectively) or internal elastic laminae (2.95 ± 0.065 versus 2.83 ± 0.066 mm, respectively).

Figures 3 and 4 show representative photomicrographs of TUNEL- and PCNA-stained medial wall nuclei, respectively, for injured PBS (A), Ad-E (B), or Ad-HO-1 (C) CAs 48 hours after injury. Data analysis shows that treatment of the injured CAs with Ad-HO-1 immediately after injury significantly stimulated medial wall SMC apoptosis compared with both the injured Ad-E CAs (P < 0.05) and the PBS control CAs (P < 0.01; Figure 5A). Concomitantly, the Ad-HO-1 CAs also exhibited significantly diminished medial wall SMC DNA synthesis at this time, as demonstrated by reduced PCNA labeling (Figure 5B). At 48 hours after injury, these early changes in cellular apoptosis and DNA synthesis were not yet manifested as changes in cellular density, because no differences were detected between the 3 treatment groups in terms of left CA medial wall SMC counts (data not shown).

### Discussion

The results of this study support the emerging concept that inducible heme oxygenase is a vasoprotective mediator that is capable of attenuating the pathophysiological remodeling response to vascular injury. We report that targeted local HO-1 gene delivery immediately after CA balloon angioplasty significantly inhibited stenotic neointimal and medial wall remodeling 2 weeks after injury. This was associated with a concomitant increase in vascular HO-1 protein. The pathological remodeling processes were completely reversed by perivascular application of an HO-1 inhibitor. The mechanisms of these HO-1–mediated morphological effects involve stimulation of medial wall SMC apoptosis and inhibition of medial wall DNA replication.

Ipsilateral adenovirus-mediated HO-1 gene delivery was performed immediately after balloon angioplasty, and successful transfection of the medial wall was measured through GFP fluorescence and Western blot analysis for HO-1 protein. At both 48 hours and 2 weeks after injury, the Ad-HO-1 and Ad-E arteries expressed much higher levels of GFP fluorescence than the PBS control arteries. Forty-eight hours after injury, notable interanimal variation was detected in arterial HO-1 protein between the Ad-HO-1 and Ad-E groups (data not shown). Previous results from our laboratory detected systemic vascular HO-1 induction 48 hours after unilateral CA balloon injury or mechanical sham surgery. This suggests that stress proteins stimulated by surgery may still be elevated at this early time. Two weeks after balloon injury, when surgical stress was reduced, we found total vascular HO-1 protein to be significantly elevated in the
Ad-HO-1 CAs compared with both the Ad-E and PBS control vessels. These data, however, did contain a notable degree of interanimal variation. We believe that the population of medial wall SMCs transfected with the HO-1 gene will have been largely eliminated from the vessel at this time, as reflected by the significant number (19%) of apoptotic-positive medial cells in the Ad-HO-1 animals at the earlier time, 48 hours after injury. The population of HO-1–transfected apoptotic medial cells decreased to 4.6% 2 weeks after injury but was still significantly increased over the Ad-E and PBS groups at that time. This significant reduction in the apoptotic cell population in the Ad-HO-1 animals reflects the marked reduction in the number of HO-1–transfected cells at 2 weeks, which may, in turn, contribute to the variation observed in the HO-1 signal. The observed induction of vessel wall HO-1 protein ensures significant transfection of the HO-1 gene to the injured arterial wall and successful translation of the protein 2 weeks after injury. Similar temporal expression of HO-1 after adenovirus-mediated gene delivery has been reported previously in other systems.19

Histomorphometric analyses of balloon-injured CAs 2 weeks after injury demonstrate robust and highly significant diminution in stenotic neointimal formation and medial wall hypertrophy in the Ad-HO-1 animals compared with both the Ad-E and PBS groups. This HO-1–mediated attenuation of remodeling was completely reversed with simultaneous inhibition of HO-1 activity. These results extend our earlier findings that systemic vascular HO-1 induction significantly
attenuates neointimal formation and medial wall hypertrophy in balloon-injured rat CAs. Other laboratories have published pharmacological studies showing that systemic HO-1 induction mediates postinjury arterial remodeling. These HO-1-mediated effects on arterial remodeling do not involve expansion in vessel caliber and represent a reversal of inward occlusive remodeling of the vessel wall.

Arterial remodeling in response to pathological insult is a complex process that depends in part on the balance between vascular cell apoptosis and proliferation. Other mechanisms intrinsic to this process involve cellular and extracellular matrix degradation and restructuring, vascular cell migration, phenotypic modulation of resident SMCs, and regrowth of the endothelial lining. The onset and temporal regulation of medial and neointimal cell proliferation and apoptosis have been well characterized. Our study shows that adenovirus-mediated HO-1 upregulation significantly stimulates early medial wall apoptosis while concomitantly inhibiting medial wall DNA replication, thus contributing to a reduced viable cell population in the medial wall and diminished neointimal development observed in the Ad-HO-1 animals. This approach may represent a novel therapeutic strategy aimed at altering the coordinate balance between these cell cycle–dependent mechanisms in an effort to minimize vascular lesion formation.

Mechanisms for the HO-1–mediated cellular apoptosis and inhibition of DNA synthesis remain unclear. HO-1 induction at the sites of injury may serve a protective role through intrinsic cell-suicide pathways. Metalloporphyrin-induced HO-1 expression was correlated with increased apoptosis in cultured hamster and mouse fibroblasts, and HO-1 was suggested to be involved in the ischemia-induced death of pyramidal neurons. Overexpression of HO-1 in cultured human pulmonary epithelial cells resulted in cell cycle arrest, which was reversed with HO-1 inhibition. HO-1–derived CO has been shown to directly block hypoxia-induced vascular SMC growth, and exogenously supplied CO inhibits serum-stimulated SMC proliferation. Interestingly, preliminary results from our laboratory indicate that adenoviral HO-1 overexpression induces apoptosis in cultured rat vascular SMCs.

While our manuscript was under review, similar results were reported that suggest that vector-mediated HO-1 inhibits...
medial SMC proliferation and attenuates injury-induced remodeling in porcine femoral arteries. The present study supports and extends these findings by proposing that an HO-1–mediated increase in medial wall apoptosis, in addition to reduced medial wall cell proliferation, contributes to HO-1–mediated protection against injury-induced remodeling.

The results of this study provide direct new evidence that HO-1 serves a vasoprotective role in the arterial response to endovascular injury in vivo. Adenovirus-mediated HO-1 gene therapy immediately after arterial balloon angioplasty results in a highly significant attenuation of neointimal development and medial wall hypertrophy through enhanced medial wall apoptosis and inhibited DNA synthesis. These observations suggest that HO-1 may be a novel, clinically relevant therapeutic target in vascular disease.

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