Flow Loading Induces Macrophage Antioxidative Gene Expression in Experimental Aneurysms

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Objective—Reactive oxygen species may act as proinflammatory mediators in abdominal aortic aneurysm (AAA) disease. Flow loading increases antioxidative enzyme expression and limits reactive oxygen species production in vascular smooth muscle cells in vitro, limits experimental AAA enlargement in rodent models, and is indirectly associated with reduced clinical AAA risk. We attempted to determine the mechanism or mechanisms by which flow loading limits AAA enlargement.

Methods and Results—Rodent AAAs were flow loaded via femoral arteriovenous fistula creation. Aortic wall shear stress and relative wall strain were significantly higher in flow-loaded rodents. Flow loading reduced AAA diameter by 26% despite evidence of flow-mediated aortic enlargement proximal to the aneurysmal segment. Messenger RNA from AAA tissue was harvested for cDNA labeling and hybridization to a 384-clone DNA microarray. Twenty-nine genes were differentially expressed (relative intensity/relative intensity of control ratio ≥1.5 and ≤0.67) in flow-loaded compared with normal flow AAA tissue, including heme oxygenase 1 (HO-1). Increased HO-1 expression was confirmed via reverse transcriptase–polymerase chain reaction. Immunohistochemistry localized HO-1 expression to infiltrative macrophages. α-Tocopherol was found to be as effective as flow loading in limiting AAA enlargement. Flow loading and α-tocopherol therapy reduced AAA reactive oxygen species production.


Key Words: abdominal aortic aneurysm ■ alpha-tocopherol ■ reactive oxygen species ■ heme oxygenase 1 ■ shear stress

Abdominal aortic aneurysm (AAA) is a common and highly lethal disease.1 Recognized clinical AAA risk factors include advanced age, male sex, cigarette smoking, and smoking-related chronic obstructive pulmonary disease and heritable predisposition.2 Inflammation-mediated proteolysis and disorganized extracellular remodeling within the aortic wall are seminal pathophysiologic events leading to progressive aortic enlargement and ultimate rupture.3 Intraluminal hemodynamic conditions (specifically flow-related wall shear stress or strain patterns) also mediate structural changes in both aneurysmal and occlusive aortic diseases.4–9

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The mechanism by which hemodynamic conditions influence aortic remodeling is incompletely understood. Elevated peripheral vascular resistance and diminished antegrade flow create oscillatory and low laminar infrafrenal aortic shear conditions.10 Recreating similar shear conditions in vitro increases proinflammatory reactive oxygen species (ROS) production in cultured vascular cells.11 Switching from oscillatory and low laminar shear to high laminar shear reduces oxidative stress.12 The ability of low and oscillatory shear forces to increase oxidative stress in vascular cells and the presence of similar prooxidative hemodynamic conditions in the human aorta during periods of lower extremity inactivity suggest that sedentary aortic hemodynamic conditions may accelerate aortic inflammation and aneurysmal enlargement in AAA disease.13

The earliest structural and mechanistic influences of AAA disease occur years or decades before surgical exposure and repair. Because surgical specimens are largely acellular and atretic, credible animal models may provide more meaningful insight into the initiating events of AAA disease. The most widely used and best-described model is the rodent porcine pancreatic elastase infusion (PPE) AAA.14 This model (created in either mice or rats) shares many important features with human AAA disease. First, elastin degradation peptides are formed early as an initiating event. These elastin degradation peptides, as well as extracts of human AAA tissue, have been shown to be chemotactic for mononuclear inflammatory cells through interactions with the 67-kD elastin binding protein.15 Second, infiltrative inflammatory cells elaborate extracellular gelatinases and proteases relevant in human AAA pathogenesis.16,17 Third, AAA formation is
absolutely dependent on elastolysis and the resulting inflammation. These critical events mimic temporal, morphological, and mechanistic features of human AAA disease. Although murine AAA models provide useful opportunities for hypothesis testing via targeted gene disruptions, the larger dimensions of the rat aorta, iliac, and femoral arteries are better suited to modification and measurement of relevant hemodynamic forces such as wall shear stress (WSS) and relative wall strain (RWS). In this experiment, we modified aortic hemodynamic conditions in the rodent PPE model to gain insight into the mechanisms by which biomechanical forces modulate aneurysm progression in experimental AAA.

Methods

All experimental procedures followed protocols approved by the Animal Care and Use Committee of Stanford University and the Guide for the Care and Use of Laboratory Animals, Health and Human Services publication No. 86-23, revised 1985. Intraportal sodium pentobarbital (25 to 50 mg) was given before all procedures for analgesia and anesthesia. Rats were recovered separately with free access to food and water.

Rat AAA Model

Porcine pancreatic elastase (PPE) (50 U type I, lot No. 31K7685, Sigma Chemical Co) was infused into the infrarenal aorta of 300- to 350-g male Sprague-Dawley rats. Aortic diameters were recorded via electronic microlapors before infusion, after infusion, and immediately before aortic harvest.

Flow Loading

Flow loading was accomplished by left femoral AVF creation. Aortic pressure and heart rate were measured intraluminally (Mikrotip catheter, Millar Instruments), and flow and wall motion were measure by direct aortic exposure (Transonics Flowmeter and Sonometrics Sonomicrometer). WSS was calculated as $4\eta Q/r^2$ ($\eta =$ viscosity, $Q =$ flow, $r =$ aortic radius) and RWS as $D_{\text{max}} - D_{\text{min}}$ ($D_{\text{max}}$ and $D_{\text{min}} =$ maximum and minimum aortic diameter, respectively).

Study Groups

Three experiments were performed. In the first 2 experiments, 4 groups were created (sham inguinal exposure/PPE infusion, AVF/PPE, sham/saline infusion, and AVF/sham). In experiment 1, PPE was infused 7 days after AVF creation. Rats were killed 7 days later. This sequence provided data on the effects of flow loading on normal aorta at 7 days and AAA diameter at 7 days. In experiment 2, PPE was infused 2 days before AVF creation. This provided earlier data on AAA response to flow loading. Aortas were harvested after molecular and histologic analysis at 3, 5, or 14 days after infusion. In experiment 3, rats received either vitamin E ($\alpha$-tocopherol, 100 mg/kg/d IM, Schering-Plough) or vehicle alone beginning 7 days before PPE infusion for pharmacologic ROS reduction. The AAA diameter response was measured 14 days after infusion. In an additional cohort, rats were killed at earlier time points for analysis of ROS production in AAA tissue.

RNA Extraction and Purification

Aortas from 4 rodents were pooled for each analysis. Total RNA was extracted using Trizol reagent (Life Technologies), treated with RNase-free DNase, and stored at –80°C.

Transcription Profiling

DNA clones were either amplified from total RNA via reverse transcriptase–polymerase chain reaction (RT-PCR)$^{21}$ or purchased from Research Genetics/Invitrogen (363) and spotted on nylon membranes (4 spots/clone, total 1536 clones). Membranes were prehybridized with Microhyb solution containing Cot-1 DNA (1 $\mu$g/mL) and poly dA (1 $\mu$g/mL) at 42°C for 2 hours. $^{33}$P-labeled cDNA probes were synthesized from 10 $\mu$g of total RNA by using ($^{33}$P)dCTP (Amersham Biosciences, Inc) and SuperScript II RT-PCR System (Life Technologies). $^{33}$P-labeled probes were purified on a size exclusion column and standardized for radioactivity before hybridization. After hybridization (42°C for 18 hours), membranes were washed (2$\times$SSC/1% SDS at 50°C for 20 minutes $\times 2$, then by 0.5$\times$SSC/1% SDS at 50°C for 20 minutes) and exposed to a phosphorimager (BioRad) for densitometric analysis (ImaGene software, Biodiscovery Inc). Local background was determined by subtracting the intensity reading immediately surrounding each spot from the intensity of the spot itself. Gene expression was defined as spot/local background intensity ratio of $>5.0$. Relative expression intensity was defined as the intensity of an individual spot/average intensity of all spots. Differential expression was defined by a relative expression intensity ratio of $>1.5$ or $<0.67$ compared with the same clone on the reference or control membrane.

RT-PCR

Complementary DNA was synthesized using the SuperScript II RT-PCR System. PCR was performed with HotStarTaq Master Mix Kit (Qiagen). Identical amounts of RNA were used to amplify cDNA of $\beta$-actin (gene bank accession No. NM 031144), matrix metalloproteinase (MMP) 9 (NM 031055), MMP-2 (NM 031054), and heme oxygenase 1 (HO-1). Primers were used as follows: $\beta$-actin (product 386 bp), forward primer (FP): GGG AAA TCG TGC GTG ACA T; reverse primer (RP): CAG GAG GAG CAT GTA TCT T; MMP-9 (product 486 bp), FP: TGG CTC TAG GCT ACA GCT TTG; RP: CGA CAC CAA ACT GGA TGA CAA; MMP-2 (product 725 bp), FP: TGC TGG AGA ACC TGA AGT GT; RP: AGA TTG ATG CCG TGT CCT AGC AG; HO-1 (product 481 bp), FP: ATT TGT CCG AGG CCT TGA A; RP: ATG CTC GGG AAG GTG AAA AA. After PCR, equal amount of cDNA products were run on 2% agarose gels containing ethidium bromide. Densitometric analysis for $\beta$-actin, MMP-9, MMP-2, and HO-1 was performed by public domain software NIH Image (version 1.61). MMP-9, MMP-2, and HO-1 expression were normalized to $\beta$-actin.

Histological Analysis

The aorta was pressure-perfused fixed in 10% neutral buffered formalin and embedded in OCP compound (Sikura Finetek USA Inc). Aortic sections (6 $\mu$m) from the PPE/sham and PPE/AVF groups harvested at the 0, 3, and 5-day postinfusion time points were stained with H&E for light microscopy. For immunohistochemistry, anti-rabbit antibody to MMP-9, MMP-2, and HO-1 (Chemicon International Inc) was applied overnight at 4°C for 18 hours, sections were incubated in 3% H$_2$O$_2$ in PBS. After biotinylated rabbit IgG (Dako USA) was applied, sections were stained with H&E for light microscopy. For immunohistochemistry, anti-rabbit antibody to MMP-9, MMP-2, and HO-1 (Chemicon International Inc) was applied overnight at 4°C for 18 hours, sections were washed (2$\times$SSC/1% SDS at 50°C for 20 minutes $\times 2$, then by 0.5$\times$SSC/1% SDS at 50°C for 20 minutes) and exposed to a phosphorimager (BioRad) for densitometric analysis (ImaGene software, Biodiscovery Inc). Local background was determined by subtracting the intensity reading immediately surrounding each spot from the intensity of the spot itself. Gene expression was defined as spot/local background intensity ratio of $>5.0$. Relative expression intensity was defined as the intensity of an individual spot/average intensity of all spots. Differential expression was defined by a relative expression intensity ratio of $>1.5$ or $<0.67$ compared with the same clone on the reference or control membrane.

Oxidative Stress

Confirmation of aneurysm ROS production was obtained from additional cohorts of flow-modified, vitamin E–treated, and vehicle-only–treated rodents at 5 days. Aneurysms were harvested and sectioned longitudinally to expose the luminal surface. Sections were immediately incubated in PBS containing lucigenin (25 $\mu$mol, Sigma). Luminescence was measured via a scintillation counter (Beckman) at 1-minute intervals for a total of 10 minutes. Counts were corrected to wet tissue weight.

Statistical Analysis

Data are expressed as mean±SD. Comparisons were made using paired and unpaired t tests, with significance assumed at the 0.05 level.

Results

We developed a novel model of AAA induction in the flow-loaded aorta to examine the hypothesis that sustained
elevations of WSS and RWS would modulate AAA progression. Femoral AVF placement successfully increased aortic flow by >300% and increased WSS and RWS by 190% and 150%, respectively, without influencing pressure or heart rate (Table). When applied to nonaneurysmal aorta before PPE infusion, increased flow, WSS, and RWS stimulated modest aortic enlargement, an effect consistent with flow-induced remodeling. Interestingly, when AAAs were induced in the flow-loaded aorta or flow loading was applied shortly after AAA induction, there was a significant and reproducible reduction in aortic dilation and AAA development (3.2 ± 0.4 mm versus 4.3 ± 0.5, *P* < 0.01, and 3.7 versus 5.3, *P* < 0.01, experiments 1 and 2, respectively, Figure 1).

Histologic analysis demonstrated marked elastin fiber degradation immediately after infusion and significant endothelial cell and medial smooth muscle cell loss by the third postinfusion day. An intense transmural inflammatory infiltrate was present throughout days 5 in both flow-loaded and normal flow AAA (Figure 2). There was some evidence of intimal and medial regeneration and cell proliferation at the 3- and 5-day time points in all specimens. Although flow did not seem to influence the intensity of the inflammatory infiltrate, intimal and medial preservation and regeneration were clearly augmented in flow-loaded AAA (data not shown).

To understand the molecular mechanisms associated with this paradoxical limitation of AAA progression in response to flow, we created a custom rat gene array enriched for genes considered relevant for aneurysm pathophysiology and vascular biology. Transcription profiling was performed on aortas obtained from sham-operated, flow-loaded nonaneurysmal aorta, AAA, and flow-loaded AAA rats. The AAA transcription profiles (normal or high flow) were determined at the 5-day time point, and AVF profiles were from the third postoperative day. Roughly 25% of clones were expressed in each of these experimental groups (saline/sham, 106; saline/AVF, 104; PPE/sham, 94; PPE/AVF, 116). Differential clone expression from these study groups is detailed in online Tables Ia, Ib, and Ic (please see http://atvb.ahajournals.org).

Taken in aggregate, the profiling data suggested that flow influenced aneurysm progression through modifications in oxidative stress within the inflamed aortic wall. The gene that generated the greatest interest in this regard was HO-1. In cultured vascular smooth muscle cells, HO-1 expression is regulated by shear and strain forces. In vascular cells, HO-1 catalyzes carbon monoxide, bilirubin, and biliverdin formation as byproducts of heme metabolism. These metabolites potentially attenuate arterial injury and responses to injury. RT-PCR confirmed that HO-1 expression was significantly higher in flow-loaded AAA compared with AAA or flow-loaded aorta alone. Concomitant with increased HO-1 expression, ROS production was significantly reduced in flow-loaded AAAs (131 ± 20 versus 200 ± 39 relative light units/10 minutes per mm/mg, *P* < 0.05.)

Increased expression of MMP-2 and MMP-9 was also noted in flow-loaded aorta and flow-loaded and normal-flow AAA tissue, respectively, consistent with their recognized roles in hemodynamic and inflammation-mediated aortic remodeling and aneurysmal degeneration (Figure 3). In flow-loaded AAA tissue, HO-1 was expressed primarily by infiltrating inflammatory cells (Figure 4), especially macrophages (confirmed via ED-1 staining, data not shown). This local-
Flow loading limits AAA progression without apparently reducing the intensity of the surrounding inflammatory cell infiltration. Endothelial cells and macrophages expressed MMP-2 and MMP-9 (Figure 4). The intensity of endothelial cell metalloproteinase expression was unexpected and may provide additional insight into the localization of proteolytic activity in early aneurysm pathogenesis.

We used a systemic antioxidant to confirm the significance of ROS production in aneurysm pathogenesis in this model and to highlight the potential impact of antioxidant gene expression induced by flow loading. Interestingly, the magnitude of AAA diameter reduction (3.6±1.2 versus 5.3±1.0 mm, *P*<0.01, Figure 5) and the reduction in AAA ROS production (274±22 versus 387±24 relative light units/10 minutes per mm²/mg, *P*<0.05, Figure 6) in vitamin E–treated rats was similar to that accomplished by flow in experiments 1 and 2 (roughly one third).

**Discussion**

AAA disease has been clinically linked to a variety of conditions that chronically alter or reduce lower-extremity blood flow, including above-knee amputation, spinal cord injury, and diminished distal arterial diameters or peripheral arterial occlusive disease. Common hemodynamic consequences of amputation, spinal cord injury, and peripheral arterial occlusive disease are increased distal arterial resistance, reduced aortic blood flow, and decreased aortic antegrade laminar WSS. Although rat aortic WSS values are significantly greater than comparable human values as a function of their reduced diameter, the percentage increase induced by AVF creation in our model was similar to that achieved after light to moderate human exercise.

Although well described and widely used in aneurysm pathogenesis research, the PPE infusion rodent model has obvious limitations, including the unlikely role of pancreatic...
elastase in human AAA disease. The high proportion of infiltrative inflammatory cells present in AAA harvested for transcription profiling also limited our insight into the early responses of constitutive aortic cells to aneurysm formation and hemodynamic modulation. Despite these limitations, however, our method proved useful and robust in its original intention (eg, defining flow-responsive gene expression in experimental AAA). Given the known similarities between human AAA disease and the PPE infusion model, we feel that the identification of increased HO-1 expression and reduced ROS in smaller flow-loaded aneurysms supports our hypothesis that AAA pathogenesis is modulated by oxidative stress.

The mechanistic relationships between ROS production and vascular diseases are well described. In addition to direct tissue injury, intracellular ROS production mediates regulatory networks and signal transduction pathways to influence both gene expression and posttranslational protein modification. As extracellular mediators, ROS activatezymogen precursors of many metalloproteinases linked to aneurysmal degeneration, as well as inactivating relevant antiproteases. ROS-stimulated cytokine release potentially perpetuates and accelerates inflammatory conditions such as AAA disease.

The relevance of HO-1 expression to aneurysm progression in our model (and ultimately in patients) will need additional confirmation via induction or inhibition experiments. Noting that length polymorphism in the HO-1 promoter is associated with variable gene upregulation in response to inflammatory stimuli, Schillinger et al recently demonstrated that AAA patients were less likely to have the antiinflammatory promoter configuration than patients with atherosclerotic occlusive disease or healthy subjects. In addition to HO-1, there are other ROS-sensitive genes potentially relevant in AAA pathogenesis, including the superoxide dismutases, catalase, NAD(P)H oxidases, glutathione peroxidases, and nitric oxide synthases, that were differentially expressed, demonstrated a trend toward differential expression, or were insufficiently expressed to be detected via microarray analysis in this model. Their significance and relevance to AAA disease remain to be determined by additional investigations.

Although the expression profile of our flow-loaded normal aorta arrays (AVF/saline group) does not correspond fully with profiles derived from sheared endothelial cells in culture or vascular smooth muscle cells exposed to varying strain conditions on a deformable membrane, these discrepancies may not limit the significance of our results. Discrepancies may result from the design of the microarrays used for transcription profiling in our experiment, the combination of shear and strain forces applied simultaneously in the flow-loaded aorta, pressure effects including apoptosis associated with the saline infusion, the time course response of the experiments in question (typically 24 hours for cultured cells versus several days for our experiments), or phenotypic differences between vascular cells in the in vitro and in vivo environments.

Vitamin E, a major lipid-soluble antioxidant, reportedly promotes vascular health by reducing ROS within the arterial wall. The fact that long-term vitamin E and β-carotene supplementation was recently found to not reduce hospital admissions for elective AAA repair or reduce the rate of AAA rupture does not necessarily contradict our observations regarding oxidative stress and experimental aneurysm progression. Our experiments are designed to define initiating mechanisms of aneurysm formation and hemodynamic modulation, events that occur presumably years or decades before clinical AAA detection in humans. The efficacy of vitamin E in our model may simply reflect timing; therapy was started 7 days before infusion in our model to ensure consistent antioxidant levels were present during and immediately after the inflammatory insult. The fact that AAA ROS production was lower in treated rats supports our conclusion that vitamin E prevents enlargement by reducing oxidative stress. Although acute administration of high-dose vitamin E therapy may increase oxidant stress under certain conditions, ROS levels were lower at all time points sampled (1, 3, and 5 days) after PPE infusion. Clinical confirmation of the potential significance of oxidative stress in AAA disease was recently provided by Miller et al, who demonstrated increased ROS production [by chemiluminescence and increased expression and activity of NAD(P)H oxidase activity] as well as evidence of oxidative injury (via lipid peroxidation and nitrotyrosine formation) in human AAA compared with nonaneurysmal aortic segments. Taken in combination with our observations regarding the ability of flow loading to reduce oxidative stress and experimental aneurysm progression, these findings support the design of additional clinical trials to test the efficacy of oxidative stress reduction in the treatment or prevention of AAA disease, perhaps including patients with smaller aneurysms or at increased risk for AAA disease, or testing alternative methods of oxidative stress reduction. Available clinical data at the present time, however, do not support the use of systemic antioxidant therapy as a means of limiting enlargement or rupture of clinically significant aneurysms.

Flow loading and systemic antioxidant therapy limit oxidative stress and early aortic enlargement in experimental aortic aneurysms. The possibility that behavioral changes, including sustained periods of lower extremity exercise in middle age patients, may reduce risk for AAA initiation and subsequent progression should be tested by an appropriately designed clinical trial.

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