Angiostatin Inhibits Coronary Angiogenesis During Impaired Production of Nitric Oxide

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**Background**—The in vivo mechanism by which inhibition of NO synthase impairs ischemia-induced coronary vascular growth is unknown. We hypothesized that production of the growth inhibitor angiostatin increases during decreased NO production, blunting angiogenesis and collateral growth.

**Methods and Results**—Measurements were made in myocardial tissue or interstitial fluid (MIF) from dogs undergoing repetitive coronary occlusions under control conditions or during antagonism of NO synthase (N\textsuperscript{G}-nitro-L-arginine methyl ester [L-NAME]) for 7, 14, or 21 days. A sham group was instrumented identically but received no occlusions. In controls, capillary density in the ischemic zone increased initially but returned to baseline at the later times. In the L-NAME group, capillary density was lower at 7 days compared with that of controls. MIF from control dogs induced in vitro endothelial tube formation and cell proliferation, significantly greater than that from the L-NAME group. MIF from shams did not stimulate tube formation. In controls or shams, tube formation or cell proliferation did not change after administration of antiangiostatin, but this antibody restored the responses to control levels in the L-NAME group. Angiostatin expression in MIF was increased in the L-NAME group compared with controls and shams. The activities of tissue matrix metalloproteinases (MMPs) MMP-2 and MMP-9, which generate angiostatin, were increased in the L-NAME group.

**Conclusions**—Inhibition of NO synthase increased expression of angiostatin and activities of MMP-2 and MMP-9. Our findings indicate that angiostatin inhibits coronary angiogenesis during compromised NO production and may underscore the impairment of coronary angiogenesis during endothelial dysfunction. (Circulation. 2002;105:2185-2191.)

**Key Words:** growth factors ■ collateral circulation ■ metalloproteinases ■ nitric oxide

Ischemic heart disease (IHD) is the major cause of death in the United States. Defects in coronary collateral formation and coronary angiogenesis appear to contribute to the progression of IHD.\textsuperscript{1} Clinical studies implicate impaired production or bioavailability of NO in the progression of IHD and peripheral arterial disease.\textsuperscript{,2,3} NO synthase blockade or knockout reduces angiogenesis and collateral formation in the hindlimb of rodents,\textsuperscript{4} angiogenic responses of endothelial cells,\textsuperscript{5} ischemia-induced development of coronary collaterals,\textsuperscript{6} and tumor angiogenesis.\textsuperscript{7} Some growth factors signal through NO (eg, vascular endothelial growth factor [VEGF]), but others appear to be independent of NO (eg, basic fibroblast growth factor\textsuperscript{8-9}). The mechanisms by which reductions in NO reduce vascular growth are unknown.

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Angiostatin inhibits tumor angiogenesis and metastases\textsuperscript{9,10} and is produced via degradation of plasminogen\textsuperscript{9-13} by matrix metalloproteinases (MMPs) MMP-2, -7, and -9.\textsuperscript{11,14,15} MMP-2 and -9 activities are upregulated in atherosclerotic plaques,\textsuperscript{16,17} a cellular environment under oxidant stress and with reduced NO bioavailability. Because of this inverse relationship between MMP activity and NO production, we proposed that the impairment of coronary angiogenesis and collateral growth observed during decreased NO production or bioavailability is caused by increased production of angiostatin. Accordingly, we hypothesized that blockade of NOS increases the activity of MMP-2 and -9, augments the production of angiostatin, and inhibits coronary angiogenesis. To test this hypothesis, we measured the expression of angiostatin, activities of MMP-2 and -9, and myocardial capillary density in a canine model of long-term repetitive myocardial ischemia in the presence or absence of a NO synthase inhibitor. We also assessed in vitro endothelial tube formation and proliferation after exposure to myocardial interstitial fluid from both groups of animals with and without a neutralizing antibody to angiostatin.

**Methods**

All experimental procedures and protocols used were reviewed and approved by the Animal Care and Use Committee of the Medical College of Wisconsin and conformed to the Guiding Principles in the...
Care and Use of Animals of the American Physiological Society and to the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1985.)

Repetitive Coronary Occlusion Model
Mongrel dogs (either sex; 25 to 30 kg) were anesthetized with propofol (50 mg/kg IV) and isoflurane (1.5 to 2.0%) in 100% oxygen. A left thoracotomy was performed under sterile conditions, and the following were implanted as described:15,16: a hepatic-filled catheter in the thoracic aorta; a Doppler flow transducer (20 MHz) on the left anterior descending artery (LAD); a balloon cuff occluder (in vivo metric) around the LAD for repetitive occlusions, and an intramyocardial catheter (0.8-mm OD, 0.4-mm ID) in the LAD perfusion territory for sampling of interstitial fluid. All catheters and leads were secured, tunneled subcutaneously, and exteriorized, and dogs were treated postoperatively as described.6,18 Animals recovered from surgery for 10 days before experimentation and were trained to stand quietly in a restraining sling. Systemic and coronary hemodynamics and reactive hyperemic responses were monitored.

Myocardial Interstitial Fluid
Samples of myocardial interstitial fluid (MIF) were collected from the LAD region each morning before subsequent experimentation, as described.6,19 Isotonic saline (4 mL) was flushed into the catheter as 4 mL of fluid was withdrawn. The sample was mixed with EDTA-free protease inhibitor cocktail (Roche Molecular Biochemicals), immediately placed on ice, aliquotted, frozen, and stored at −80°C until analysis.

Experimental Protocols
Repetitive episodes of myocardial ischemia were induced by occlusions (2 minutes) of the LAD once an hour, 8 times a day, for up to 21 days. In control and NOS-inhibited animals, measurements were made at 7 days (n=5 and 5, respectively), 14 days (n=3 and 3, respectively), and 21 days (n=6 and 6, respectively). Samples of MIF were collected at varying time points: days 1 to 3, 7 to 8, 12 to 14, and 18 to 21. Grouping within these ranges is justified because samples have similar properties and constituents. Sham animals (n=5), with identical instrumentation but no coronary occlusions, were used for sampling of MIF during the 3-week protocol. Inhibition of NOS was accomplished by infusion of N^ω-nitro-L-arginine methyl ester (L-NAME, Sigma; 30 mg/kg twice a day) via the atrial catheter 2 days before initiation of occlusions and throughout the 21-day protocol. Efficacy of NOS blockade was confirmed by attenuation of the hypotensive effect of intratral acetylcholine.

Capillary Density
Frozen myocardium was sectioned 10 μm thick by Cryo-Cut (Leica) and fixed in aceton for 10 minutes at −20°C. After air drying, the sections were incubated in fluorescence isothiocyanate (FITC)-conjugated lectin (Grifonia simplicifolia agglutinin I, Vector Laboratories; 1:100 dilution in PBS) for 30 minutes at room temperature.20 Sections were then washed 3 times with PBS and mounted in aqueous mounting medium (Biomedia Corp). Capillaries and myocardial muscle fibers were counted (400X) by use of fluorescent microscopy (Nikon, Japan). Vessels with a diameter <8 μm were considered capillaries. Only sections oriented perpendicular to the capillaries were counted.

MIF-Induced Tube Formation and Endothelial Proliferation
We performed MIF-induced endothelial tube formation in a fibrin gel,21 as an in vitro model of coronary angiogenesis. Plasminogen-free human fibrinogen (5 mg/mL, Calbiochem) dissolved in serum-free medium and filtered through 0.2-μm filters. Fibrin matrices were prepared by polymerizing the fibrinogen solution using thrombin (2.5 U/mL, Sigma; 2 hours at 37°C). After polymerization, gels were soaked in culture medium containing 10% fetal bovine serum (FBS) for 2 hours at 37°C to inactivate thrombin. Human aortic endothelial cells were seeded on 24-well plates containing 0.1% FBS/Dulbecco’s modified Eagle’s medium with 10% MIF from control, sham, and L-NAME–treated animals for 48 hours. We also assessed the effects of angiostatin antibody (Pharmingen; 4 μg/mL), to neutralize angiostatin, on MIF-induced tube formation. Images (200X) were digitized from a CCD camera and analyzed using NIH Image. An electronic grid (each section of the grid was 13×13 pixels) was superimposed on each image, and the number of tubes intersecting the squares was counted. Tube area was quantified as the percentage of the microscopic field covered by tubes and was derived from the following equation: Percent of field covered by tubes= [tube intersections/total grid count]×100. All samples were determined in triplicate, and the values were averaged for each point.

To evaluate the effects of MIF from L-NAME–treated animals on endothelial proliferation and to evaluate if proliferation would be affected by antiangiostatin, we ascertained proliferation of human aortic endothelial cells after 4 treatments: (1) effects of MIF from control occlusion animals, (2) effects of MIF from L-NAME–treated animals, (3) effects antiangiostatin on MIF-induced proliferation from control occlusion animals, and (4) effects of antiangiostatin on MIF-induced proliferation from L-NAME–treated animals. MIF was obtained at 4 different times of the repetitive occlusion protocol: days 1 to 3, 7 to 8, 12 to 14, and 19 to 21. Endothelial cells (n=10 000) were seeded per well of a 24-well plate, and the cells were grown for 72 hours in 0.5% FBS. Cells were maintained in growth arrest, exposed to 10% MIF from either the control occlusion or L-NAME groups or exposed to 10% MIF from both groups along with antiangiostatin (4 μg/mL). After 72 hours of these treatments, cells were trypsinized and counted using a hemocytometer. To ensure that all cells were counted, the dishes were inspected after trypsin treatment, and no remaining cells were noted. All samples were determined in triplicate, and the values of the individual experiments were averaged.

SDS-PAGE Zymogram
Transmural myocardial samples were homogenized in lysis buffer (20 mmol/L NaCl, 100 mmol/L Tris HCl at pH 7.6, and 10% SDS), which included protease inhibitor EDTA-free cocktail tablet (Boehringer Mannheim) 1 tab/50 mL, 1 mmol/L phenylmethylsulfonyl fluoride, 200 μmol/L Na orthovanadate, and 5 mmol/L Na fluoride, and supernatants were collected after centrifugation at 14 000 rpm for 15 minutes. Supernatants were used for protein determination (BCA protein assay, Bio-Rad). Samples (200 μg) were separated by dilution into zymogram sample buffer. The samples were loaded into the wells of a 10% gelatin gel and electrophoresed. The gel was removed and incubated for 1 hour at room temperature in 100 mL of renaturing buffer (2.5% Triton X-100) on a rotary shaker. The buffer was decanted and replaced with 100 mL of development buffer (50 mmol/L Tris at pH 7.5, 200 mmol/L NaCl, 5 mmol/L CaCl2, 0.02% Brij-35). The gel was incubated at 37°C for 18 to 24 hours. Each gel was stained with 100 mL of 0.5% Coomasie blue G-250 in 30% methanol and 10% acetic acid for 3 hours and then destained with 3 changes of 30% methanol and 10% acetic acid. The gels were digitized using a CCD camera-frame digitizer system and analyzed using NIH Image software (product of density and band area of both the active and pro-forms of the enzymes).

MMP-2 Activity in Endothelial Cell Culture Media
With or Without L-NAME
Human aortic endothelial cells were seeded on 100-mm dishes containing 0.1% of FBS/Dulbecco’s modified Eagle’s medium. After the culture became confluent, vehicle (saline) or L-NAME (300 μmol/L and 1 mmol/L, final concentration) were added to cell culture media, and incubated for 72 hours. One milliliter of endothelial cell-conditioned culture medium was added to 100 μL gelatin-agarose beads (Sigma Chemical Co), and the mixture was incubated for 1 hour at 4°C.22 The mixture was then centrifuged briefly and washed, and gelatinases were eluted from the agarose by adding 100 μL cold 10% DMSO. The mixture was incubated for 5
minutes and centrifuged, after which the supernatant containing the eluted gelatinases was loaded onto ultrafuge filters (30 000 NMWC) and recentrifuged (5 minutes, 3500 rpm) to remove DMSO. The filtered supernatant was subsequently used for zymogram to study activity of MMP-2.

**Generation of Angiostatin**

Human plasminogen (20 μg, Calbiochem) was incubated with human MMP-2 and -9 (50 ng each, Calbiochem) in 500 μL of development buffer for 72 hours at 37°C. Angiostatin was detected by Western blotting.

**Western Analyses of Angiostatin in MIF**

MIF was collected and diluted in SDS sample buffer. Each sample of MIF (30 μL) was separated in a 10% SDS-PAGE gel. We used mouse monoclonal angiostatin primary antibody (Upstate Biotechnology) and the secondary antibody (IGG) labeled with horseradish peroxidase. After incubation with horseradish peroxidase substrate (Pierce Supersignal), membranes were exposed on film, and signals were visualized, digitized, and analyzed by NIH Image software (density×band area) and termed optical density units.

**Data Analysis**

All data are expressed as mean±SEM. The changes in the parameters between the groups and over time were compared by 2-way ANOVA for repeated measurements. The level of significance was *P*<0.05.

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**Results**

**Capillary Density in Ischemic Myocardium**

In control animals, capillary density and capillary to myocardial fiber (C/M) ratio increased after 7 days of occlusion but returned to baseline values at 21 days (Figure 1). In contrast, in the L-NAME group, capillary density and C/M ratio only increased at the last time points. At 7 days of occlusion, capillary density and C/M ratio were significantly lower in the L-NAME group compared with controls (capillary density, 2448±70/mm² versus 2978±58/mm²; C/M ratio, 1.63±0.03 versus 2.12±0.03; *P*<0.05). In the nonischemic region, capillary density and C/M ratio did not change in either group.

**MIF-Induced Tube Formation**

MIF-induced tube formation was reduced in the L-NAME group compared with controls at 1, 7 and 14 days of repetitive occlusions (Figure 2). In controls, MIF-induced tube formation was not affected by antiangiostatin, but it was significantly augmented by antiangiostatin at all time points in the L-NAME group. MIF from the sham dogs did not induce tube formation, nor were any tubes evident after anti-angiostatin.
MIF-Induced Endothelial Cell Proliferation
MIF-induced proliferation (percentage increase in cell number) was reduced in the L-NAME group compared with controls at days 1 to 3, 7 to 8, and 12 to 14 of repetitive occlusions (Figure 3). In controls, proliferation was not affected by antiangiostatin (data not shown). In contrast, in the L-NAME group, proliferation was increased to, or above, control levels by antiangiostatin.

Angiostatin Expression in MIF
Figure 4A shows angiostatin generated by plasminogen with MMP-2 or -9. In controls or shams, angiostatin expression was lower in L-NAME group (solid bars) compared with controls (empty bars) at days 1, 7, and 14 (P<0.05). In L-NAME group, MIF-induced tube formation was augmented by angiostatin (striped bars) at all days (P<0.05). In controls, however, tube formation was not affected by angiostatin antibody (shaded bar). n=3 to 6 for each group.

Figure 2. Top, Representative images of tube formation expressed as the percentage of the field covered by tubes in response to MIF from control and L-NAME–treated groups at 7 days of occlusion. The tubes in the L-NAME group were thin with few branches compared with those of controls, but after antiangiostatin, tube formation in L-NAME group was restored. Bottom, Analyses of MIF-induced tube formation. Tube area was lower in L-NAME group (solid bars) compared with controls (empty bars) at days 1, 7, and 14 (P<0.05). In L-NAME group, MIF-induced tube formation was augmented by antiangiostatin (striped bars) at all days (P<0.05). In controls, however, tube formation was not affected by angiostatin antibody (shaded bar). n=3 to 6 for each group.

Figure 3. Endothelial cell proliferation (percentage increase in cell number) in response to positive and negative controls, 0.5 and 20% FBS, respectively, and MIF from controls and L-NAME animals. In response to MIF, proliferation was less in L-NAME group compared with controls (P<0.05) at all but days 18 to 21. Antiangiostatin (AngiostatinAb) had no effect on proliferation in response to MIF from controls (data not shown) but increased that from L-NAME group (P<0.05 vs L-NAME). n=4 to 6 for each group.
was lower than that in the L-NAME group, which was higher at all times (Figure 4B).

**MMP-2 and -9 Activity**
MMP-2 activity in myocardial tissue from the L-NAME group was significantly higher at days 7 and 21 compared with that in controls (Figure 5A). MMP-9 activity was higher in the L-NAME group than in controls at day 7. L-NAME augmented MMP-2 activity in culture medium compared with that in vehicle-treated controls (Figure 5B).

**Discussion**
The new findings of the present study are (1) inhibition of NOS synthase impaired angiogenesis in response to myocardial ischemia. We observed that L-NAME treatment reduced MIF-induced endothelial tube formation and proliferation and inhibited increases in capillary density. (2) Inhibition of NO production increased the levels of angiostatin. (3) Antiangiostatin rescued the impaired proliferative and tube-forming properties of the MIF from L-NAME–treated animals. (4) Inhibition of NO production increased the activity of MMP-2 and -9, critical enzymes for the generation of angiostatin. We conclude that the formation of angiostatin during impaired production or bioavailability of NO abrogated coronary angiogenesis. We further speculate that angiostatin production is responsible for poor coronary collateral growth during NOS inhibition.

**Impaired Coronary Angiogenesis During Inhibition of NO Synthase**
Coronary angiogenesis is important for the preservation of cardiac function during development of ventricular hypertrophy. VEGF-knockout mice developed ischemic cardiomyopathy because of limited capillary density. Patients with dilated cardiomyopathy and decreased capillary density show decreased VEGF expression. NO participates in angiogenesis by facilitating (1) endothelial proliferation and migration, (2) endothelial cell survival, and (3) integrin expression. NO is required for ischemia-induced and tumor angiogenesis and is essential for coronary collateral growth. Our results help explain the role of NO in coronary angiogenesis and collateral growth. Specifically, during NOS
inhibition, production of angiostatin was increased. The concentrations of angiostatin in myocardial interstitial fluid appeared sufficient to limit angiogenesis, because antibody neutralization in MIF augmented endothelial proliferation and tube formation. Although we did not study cell migration, we note that angiostatin inhibits endothelial cell migration.30 Capillary density in the control group increased at day 7 and then waned back to baseline/sham levels. This pattern paralleled expression of VEGF that we reported previously in our model of repetitive myocardial ischemia.6 In L-NAME animals, capillary density failed to increase during elevated VEGF expression; therefore, we believe NO is critical for coronary angiogenesis.

Angiostatin Retards Coronary Angiogenesis During Inhibition of NO Synthase

Angiostatin, a product of plasminogen proteolysis, is a potent inhibitor of tumor angiogenesis and tumor cell metastases.9–13 MMP-2, -7, and -9 can generate angiostatin from plasminogen.11,14,15 The activities of MMP-2 and -9 are increased during high oxidative stress,16,17,22 and the bioavailability of NO is impaired during oxidative stress.4,5,31–33 Consistent with these observations are our observations of the present study: L-NAME treatment augmented activities of MMP-2 and -9 in myocardium and of MMP-2 in conditioned media. We propose that increased MMP-2 and -9 activities during NOS inhibition increased production of angiostatin to a level sufficient to attenuate myocardial angiogenesis. It is important to note that a neutralizing antibody to angiostatin restored proliferation and tube formation to MIF from the L-NAME group to the level in control experiments. This observation helps eliminate the possibility that L-NAME in the MIF impaired endothelial cell responses, because antiangiostatin would not have restored proliferation and tube formation in culture if the inhibitor was the culprit.

The Western blots suggested elevated levels of plasminogen in MIF after L-NAME. Therefore, we must consider the possibility that elevated extravasation of plasminogen contributes to the production of angiostatin.

Gorrin-Rivas et al14 suggested that tumor angiogenesis is controlled by a balance between angiostatin and VEGF expression. We too have found a dissociation between VEGF angiogenesis and collateralization,6 which suggests a similar paradigm, ie, coronary collateral growth and angiogenesis are controlled by a balance between growth factors and inhibitors. At day 21 in the L-NAME group, capillary density increased despite the maintenance of angiostatin levels. We argue that the balance between growth factors and inhibitors likely involves additional factors besides just angiostatin and VEGF.

Clinical Implications

Stimulation of coronary angiogenesis and collateral growth could potentially ameliorate myocardial ischemia in patients with coronary artery disease.1 A hallmark of IHD, however, is reduced production and/or bioavailability of NO and
increased activity of MMPs. In the present context, angiotatin may suppress coronary angiogenesis and collateral growth in patients with decreased NO production or bioavailability and increased MMP activity. Our results may suggest a new strategy for therapeutic angiogenesis—neutralization of angiotatin—in certain patients who are refractory to the effects of growth factors.

In conclusion, we found that inhibition of NO synthesis impaired coronary angiogenesis. Inhibition of NO production increased activities of MMP-2 and -9, leading to the generation of angiotatin. Our results are consistent with the concept that a balance between growth factors and inhibitors controls coronary angiogenesis and collateralization.

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