Angiostatin
A Negative Regulator of Endothelial-Dependent Vasodilation

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Background—Angiostatin is known to inhibit certain aspects of endothelial function, eg, angiogenesis. Here we investigated the effects of angiostatin on another aspect of endothelial function, vasodilation, and examined mechanisms of inhibition—namely, association of heat-shock protein 90 (hsp90) with endothelial nitric oxide synthase (eNOS) and endothelial generation of nitric oxide (·NO) and superoxide anion (O₂⁻). This avenue of investigation was based on recent reports suggesting that hsp90 modulates NOS production of ·NO and O₂⁻.

Methods and Results—Effects of angiostatin on vasodilation were determined in arterioles with the use of videomicroscopy in response to endothelium- and ·NO-dependent vasodilators, acetylcholine (ACh) and vascular endothelial growth factor (VEGF), and an endothelium-independent agonist, papaverine. Association of hsp90 with eNOS was determined in rat aortas and bovine aortic endothelial cells (BAECs). Effects of angiostatin on ·NO and O₂⁻ generation by BAECs were determined by ozone chemiluminescence and superoxide dismutase (SOD)–inhibitable ferricytochrome c reduction, respectively. Angiostatin impaired vasodilation mediated by ACh and VEGF but not papaverine. Pretreating arterioles with polyethylene glycolated–SOD (PEG-SOD) improved vasodilation to ACh and VEGF. Angiostatin decreased the association of hsp90 with eNOS in aortas and BAEC cultures and increased O₂⁻ generation in stimulated BAECs by an L-·nitroargininemethylester (L-NAME)–inhibitable mechanism.

Conclusions—These data indicate angiostatin alters endothelial function by allowing eNOS to generate O₂⁻ on activation. Such changes in enzyme function begin to explain, in part, why angiostatin is antiangiogenic and impairs endothelium-dependent vasodilation. (Circulation. 2003;107:803-806.)

Key Words: angiostatin • nitric oxide synthase, endothelial • heat-shock protein 90 • nitric oxide • superoxides

The role of angiostatin as an antiangiogenic and an antineoplastic agent has recently attracted much attention.1–3 Angiostatin is a proteolytic product of plasminogen and matrix metalloproteinases (MMPs) and antagonizes the trophic effects of several growth factors, including vascular endothelial growth factor (VEGF).2 In addition to its role as an angiogenic factor, VEGF is also an endothelium-dependent vasodilator.4 Interestingly, both of these actions require ·NO.4 Evidence for ·NO in collateral growth comes from the severely compromised responses found in studies involving both endothelial nitric oxide synthase (eNOS)–knockout mice and pharmacological and naturally occurring endogenous inhibitors of eNOS.5–8 With a growing list of angiogenic and physiological mechanisms centering on ·NO, we hypothesized that angiostatin inhibits endothelial-dependent vasodilation. This hypothesis is based on observations that (1) geldanamycin and radicicol, antineoplastic agents that inhibit heat-shock protein (hsp) 90, both attenuate ·NO-dependent signaling by uncoupling eNOS activity9–12; (2) endostatin inhibits eNOS by increasing dephosphorylation at S117713,14; (3) angiostatin inhibits coronary angiogenesis to increase myocardial ischemia15,16; and (4) hsp90 enhances ·NO production from neuronal NOS.17 These reports suggest that hsp90 may modulate which radical species is generated when eNOS is activated.

Methods
Animal care and treatment were conducted in accordance with the institutional guidelines of the Medical College of Wisconsin. Male Sprague-Dawley rats (220 to 300 g, Harlan, Indianapolis, Ind) were anesthetized with sodium pentobarbital (50 mg/kg IP). The heart and aorta were removed and placed in physiological saline solution at 4°C. Aortas (3 per test group; 12 per experiment) were cleaned of fat and adventitial tissue and cut into 4 pieces (5 to 8 mm long). Angiostatin was synthesized from plasminogen5,18 and characterized by Western analysis. Digestion of plasminogen with...
MMP2+MMP9 yielded three products with molecular weights of 48, 38, and 32 kDa.18,19 This reaction mixture (∼120 nmol/L from standards on the blot) inhibited VEGF-induced (10 ng/mL) endothelial proliferation and tube formation. Purified angiostatin (No. 176700, Calbiochem) yielded similar results.

**Measurements of Vasodilation**

Arterioles (53 to 168 μm) were dissected from the interventricular septum, cannulated with glass micropipettes (tip diameter ∼30 μm), and connected to 2 reservoirs filled with physiological saline solution.20 Arterioles were stimulated with acetylcholine (ACh), VEGF, and papaverine±angiostatin, and diameters were recorded by videomicroscopy.20 To determine if O2− impaired endothelium-dependent vasodilation, arterioles were treated with polyethylene glycolated–superoxide dismutase (PEG-SOD) for 20 minutes (200 U/mL) before angiostatin.

**Immunoprecipitation of eNOS and Western Analysis**

The effects of angiostatin on hsp90–eNOS interactions were determined on native endothelial cell on rat aortas and cultured bovine aortic endothelial cells (BAECs). Aortas were treated with buffer (−) or with 120 nmol/L angiostatin (+) for 20 minutes, incubated with buffer (−) or 5 μmol/L A23187 (+) for 5 minutes, removed, flash-frozen in N2 (l), pulverized, and homogenized by hand in modified radioimmunoprecipitation buffer.11 Vascular debris was removed and aliquots (1000 μg) were preclarified. eNOS was immunoprecipitated (H32, BioMol); coprecipitated proteins separated by SDS-PAGE; and eNOS and hsp90 immunoblotted with primary antibodies 9D10 (Zymed) and H38220 (Transduction Labs) and enhanced chemiluminescence reagents (Amersham), as described.11

To determine effects of angiostatin on A23187-stimulated eNOS–hsp90 interactions and phospho-eNOS (S1177) (p-eNOS), BAEC cultures were serum-starved (0.5% FBS) in RPMI-1640 (4°C) or 5% FBS in RPMI-1640 (37°C, 15 minutes). The cultures were washed and stimulated with 5 μmol/L A23187 (+) for 10 minutes in Hank’s balanced salt solution containing L-arginine (10 μmol/L) and angiostatin. Cultures were lysed and eNOS immunoprecipitated as described.11 The proteins were separated by SDS-PAGE and immunoblotted for p-eNOS (9571, Cell Signaling), eNOS, and hsp90 as above.11 Band densities were quantified with NIH Image 1.62.11

**Effects of Angiostatin on Stimulated O2− and NO Production**

BAECs without (control [C]) and with angiostatin (120 nmol/L, 37°C, 15 minutes) were stimulated with A23187 (5 μmol/L) as described.11 L-NAME (1 mmol/L) was added 15 minutes before angiostatin and during A23187-stimulation for O2− assays. The assays were performed in triplicate and proteins in duplicate. Data are expressed as mean±SEM in nmol O2− · min−1 · mg−1 and pmol nitrite + nitrate/mg protein.

**Data Analysis**

Data were analyzed by the Student’s t test (unpaired, 2 tailed, with Welch’s correction) or by ANOVA with Fisher’s post-hoc test to determine significance among test groups, with the use of Statview 4.51 for MacIntosh computers. A probability value <0.05 was accepted as being statistically significant.

**Results**

Angiostatin induced a modest vasodilation (3% to 6%) under basal conditions. When the pressurized microvessels were stimulated, however, angiostatin impaired ACh- and VEGF- but not papaverine-induced vasodilation (Figure 1, A, B, and C, respectively). L-NAME inhibited vasodilation to ACh and VEGF to the same extent as angiostatin (data not shown). PEG-SOD improved vasodilation when angiostatin-treated arterioles were stimulated with ACh and VEGF but not papaverine (Figure 1). The inhibitory effects of angiostatin were reversible, in that washing the microvessels restored vasodilation.

Angiostatin decreased hsp90 interactions with eNOS in native endothelial cells on rat aortas by 71.8±9.0% of the levels in control aortas (Figure 2A, lane 2 versus lane 1, P<0.01, n=3). A23187 stimulation increased hsp90 association with eNOS in untreated aorta by (Figure 2A, lane 3 versus lane 1). Pretreatment of aortas with angiostatin decreased hsp90 association with eNOS in A23187-stimulated aortas, by 74.5±11.7% compared with levels in untreated A23187-stimulated aortas (Figure 2A, lane 4 versus lane 3, P<0.05, n=3).

**Figure 1.** A, ACh-induced vasodilation without angiostatin (AS) (Control), with AS, and with AS+SOD (n=7 to 8). B, VEGF-induced vasodilation without AS (Control), with AS, and with AS+SOD (n=8 to 9). C, Papaverine-induced vasodilation without AS (Control), with AS, and with AS+SOD (n=5). *P<0.01, AS vs ACh and AS vs VEGF; †P<0.01, ‡P<0.05, SOD vs AS+ACh and SOD vs AS+VEGF.
Angiostatin had little effect on the levels of p-eNOS in cultured BAECs under basal or A23187-stimulated conditions (Figure 2B). In unstimulated cultures, angiostatin increased hsp90 interactions with eNOS but decreased hsp90 association with eNOS by more than half in A23187-stimulated cultures (48.9 ± 2.4% of untreated cultures, P < 0.002, n = 3).

Next, O$_2^\cdot$ and ·NO were quantified to determine the effects of angiostatin on eNOS function. Angiostatin increased O$_2^\cdot$ from A23187-stimulated cultures (Figure 2C). L-NAME increased O$_2^\cdot$ from controls and decreased O$_2^\cdot$ from angiostatin-treated cultures. The L-NAME-induced changes in O$_2^\cdot$ generation from control and angiostatin-treated cultures confirm that angiostatin increased eNOS-dependent O$_2^\cdot$ generation. Finally, angiostatin decreased A23187-stimulated nitrite+nitrate production (3.94 ± 0.14 versus 3.37 ± 0.06 pmol/mg cell protein, P < 0.05, n = 3).

**Discussion**

These data indicate that angiostatin regulates endothelial-dependent vasodilation by decreasing hsp90 interactions with eNOS. Under these conditions, eNOS generates O$_2^\cdot$ when stimulated. Exactly how angiostatin decreases hsp90–eNOS interactions is unclear at this time. However, the impact such a change in protein interactions has on ·NO and O$_2^\cdot$ balance is clear. For every ·NO made during coupled enzyme activity, two O$_2^\cdot$ are made during uncoupled activity.

Because p-eNOS levels directly correlate with increased electron flow, and hsp90 association with eNOS enhances ·NO formation, these data indicate angiostatin alters mecha-
anisms of eNOS activation such that an increase in electron flow occurs under less than optimal conditions. Such changes in radical species generation are consistent with the idea that angiostatin decreases ·NO bioactivity in pressurized arterioles by uncoupling eNOS activity. On the basis that ·NO is required for endothelial proliferation and vasodilation, our findings provide new insight into why angiostatin is not only antiangiogenic but also able to impair endothelium-dependent vasodilation.

This mechanism for angiostatin complements the mechanism for endostatin, which increases dephosphorylation of eNOS at S1177 without inhibiting Akt activity.14 As angiostatin uncouples eNOS activity and endostatin decreases p-eNOS levels,14 it is easy to see how a shift in the balance of angiogenic and antiangiogenic factors not only inhibits collateral growth but also impairs endothelial-dependent vasodilation.

This change in association between eNOS and hsp90 may also contribute to endothelial dysfunction in diabetes, hypertension, and hyperlipidemia, in which increased MMPs were detected.22–24 Logically, an increase in vascular MMP activity could enhance plasminogen degradation to angiostatin, which, on the basis of the findings here, would uncouple eNOS activity to impair vasodilation.

In summary, angiostatin, an endogenous vasostatic molecule, alters hsp90 interactions with eNOS to impair vasodilation. Whenever the endothelium is stimulated in the presence of angiostatin, less hsp90 associates with eNOS. This shifts ·NO and O$_2^-$ generation by eNOS from ·NO toward O$_2^-$ (hsp90) in proliferating endothelial cells uncouples endothelial nitric oxide synthase from endothelial nitric oxide synthase activity. On the basis of the findings here, our findings provide new insight into why angiostatin is not only antiangiogenic but also able to impair endothelium-dependent vasodilation.

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