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
MMP2+/MMP9 yielded three products with molecular weights of 48, 38, and 32 kDa.\textsuperscript{16,17} This reaction mixture (\(\approx 120 \text{ nmol/L}\) 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 \(\mu\text{m}\)) were dissected from the interventricular septum, cannulated with glass micropipettes (tip diameter \(\approx 30 \mu\text{m}\)), and connected to 2 reservoirs filled with physiological saline solution.\textsuperscript{20} Arterioles were stimulated with acetylcholine (ACh), VEGF, and papaverine\(\pm\)angiostatin, and diameters were recorded by videomicroscopy.\textsuperscript{20} To determine if \(\text{O}_2^{-}\) 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 angiotatin \((+)\) for 20 minutes, incubated with buffer \((-\) or 5 nmol/L A23187 \((+)\) for 5 minutes, removed, flash-frozen in \text{N}_2 \text{(l)}, pulverized, and homogenized by hand in modified radioimmunoprecipitation buffer.\textsuperscript{11} Vascular debris was removed and aliquots (1000 \(\mu\text{g}\)) were precleared. eNOS was immunoprecipitated (H32, BioMol); coprecipitated proteins separated by SDS-PAGE; and eNOS and hsp90 immuno blotted with primary antibodies 9D10 (Zymed) and H38220 (Transduction Labs) and enhanced chemiluminescence reagents (Amersham), as described.\textsuperscript{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 \(\text{mol/L}\)/H11002) or 5\% FBS for 37°C, 15 minutes. The cultures were washed and stimulated with 5 nmol/L A23187 \((+)\) for 10 minutes in Hank’s balanced salt solution containing l-arginine (10 \(\mu\text{mol/L}\)) and angiotatin. Cultures were lyzed and eNOS immunoprecipitated as described.\textsuperscript{11} The proteins were separated by SDS-PAGE and immuno blotted for p-eNOS (9571, Cell Signaling), eNOS, and hsp90 as above.\textsuperscript{11} Band densities were quantified with NIH Image 1.62.11

### Effects of Angiostatin on Stimulated \(\text{O}_2^{-}\) and ·NO Production

BAECs without control ([C]) and with angiostatin (120 nmol/L, 37°C, 15 minutes) were stimulated with A23187 (5 \(\mu\text{mol/L}\)) as described.\textsuperscript{11} L-NAME (1 mmol/L) was added 15 minutes before angiotatin and during A23187-stimulation for \(\text{O}_2^{-}\) assays. The assays were performed in triplicate and proteins in duplicate. Data are expressed as mean\(\pm\)SEM in nmol \(\text{O}_2^{-} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}\) 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\% compared with levels in untreated aortas (Figure 2A, lane 2 versus lane 1). 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% \(\pm\)11.7\% compared with levels in untreated A23187-stimulated aortas (Figure 2A, lane 4 versus lane 3).
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$_2^·$ and ·NO were quantified to determine the effects of angiostatin on eNOS function. Angiostatin increased O$_2^·$ from A23187-stimulated cultures (Figure 2C). L-NAME increased O$_2^·$ from controls and decreased O$_2^·$ from angiostatin-treated cultures. The L-NAME-induced changes in O$_2^·$ generation from control and angiostatin-treated cultures confirm that angiostatin increased eNOS-dependent O$_2^·$ 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$_2^·$ 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$_2^·$ balance is clear. For every ·NO made during coupled enzyme activity, two O$_2^·$ 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 mech-
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 endothelial-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 growth15 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, (to Dr Chilian).

HL71214 (to Dr Pritchard); and NS38133, HL32788, and HL65203 (to Dr Wiederschain D, Stetler-Stevenson WG, et al. Regulation of endothelial cell survival and apoptosis during angiogenesis. Arterioscler Thromb Vasc Biol. 2002; 22: 887–893.

Acknowledgments
This work was supported in part by Marie Z. Uhlein endowed chair award (to Dr Oldham) from Children’s Hospital Foundation (Milwaukee, Wisc); National Institutes of Health grants HL61417 and HL71214 (to Dr Pritchard); and NS38133, HL32788, and HL65203 (to Dr Chilian).

References