Angiotensin Inhibition Decreases Progression of Advanced Atherosclerosis and Stabilizes Established Atherosclerotic Plaques

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ABSTRACT

Although increased extracellular matrix (ECM) is pathogenic in a variety of chronic tissue injuries, reduced and/or disrupted ECM may be detrimental in atherosclerosis and rather destabilize existing atherosclerotic lesions. This study therefore assessed the effects of angiotensin II (AngII) antagonism on ECM components of advanced atherosclerosis. Twenty-four-week-old apolipoprotein E–deficient mice were treated with the AngII antagonist losartan for 12 wk. Controls received water or hydralazine. AngII antagonism significantly reduced progression of established atherosclerosis, whereas hydralazine showed no benefit despite similar decrease in BP. Although there was no difference in the macrophage component, AngII antagonism increased the relative collagen portion of the lesions; lessened elastin fragmentation, increased the total elastin content of the aorta; and reduced the mRNA and activity/protein of the elastolytic proteases, cathepsin S, and metalloproteinase-9. Extracellular elastin degradation by cultured smooth muscle cells (SMC) was reduced by losartan, as was SMC invasion through an elastin gel barrier. Thus, AngII antagonism lessens progression of atherosclerosis, increases collagen, and preserves elastin components of ECM within the vascular lesions, which, at least in part, is modulated by effects on SMC. These effects not only decrease further expansion of advanced lesions but also stabilize the established atherosclerotic plaques and may underlie the decreased incidence of acute cardiovascular events that are observed in patients in whom AngII antagonism is begun after atherosclerosis is already established.

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For decades, the therapeutic goal to lessen adverse cardiovascular events has focused on halting progression of atherosclerotic lesions. However, more recent angiographic data reveal that acute cardiovascular events do not necessarily correlate with the extent of obstructive coronary artery lesions.1,2 Furthermore, interventions that improve outcome may have only a minimal impact on severity of obstruction yet profoundly modulate plaque echogenicity.2,3 Such observations suggest that the atherosclerotic plaque is not merely a static repository for lipid waste but rather a dynamic lesion that can be remodeled in ways that influence the likelihood of plaque instability and acute vascular events.3,4 The vascular remodeling that is thought to stabilize atherosclerotic plaques includes decreased lipid or cellular components but especially increased extracellular matrix (ECM). Recent findings suggest that prevention of ECM degradation or even increasing ECM components such as collagen and elastin, so-called stabilization of plaque, improves outcome in atherosclerotic disease by decreasing risk for plaque rupture.5–8 Optimizing such interventions may be especially crucial in patients with chronic kidney
Observations that increased ECM is beneficial in atherosclerosis stand in stark contrast with nonatherogenic progressive arteriosclerotic and fibrotic disorders, including CKD, in which ECM accumulation is detrimental. Chief among the interventions to decrease ECM accumulation is antagonism of angiotensin II (AngII), which has become the therapeutic mainstay for a variety of progressive cardiovascular and renal scarring diseases. It is interesting that AngII antagonism is now also promoted as an effective intervention in preventing the acute clinical sequelae of atherosclerosis, although the mechanisms for such benefits remain largely unknown. In this connection, our recent study in a mouse model of atherosclerosis and reduced renal mass found that AngII antagonism with losartan between 12 and 24 wk reduced development of atherosclerosis, complementing previous results of AngII inhibition that is begun at the early stages of experimental atherogenesis in animals with intact kidneys. The losartan-linked decrease in atherosclerotic disease at this early stage was associated with reduction in macrophage migration and intimal infiltration as well as reduction in elastin disruption and vascular cathepsin S. On the basis of these results, we proposed that AngII antagonism lessens early atherogenesis by limiting macrophage infiltration and squelching macrophage synthesis of the elastolytic enzyme cathepsin S. However, the cellular and ECM composition of atherosclerotic lesions changes as disease progresses, with waning of macrophages. It is not known whether or by what mechanisms AngII antagonism may benefit the clinically relevant later stages of atherosclerotic vascular remodeling. Such considerations seem especially pertinent because AngII antagonism is usually initiated in patients, including those with CKD, well after atherosclerotic lesions are established. We therefore aimed to determine whether AngII antagonism can modify the later stages of atherosclerotic disease to elucidate mechanisms of the seemingly paradoxical effects of AngII antagonism to enhance ECM components in established atherosclerotic lesions.

### RESULTS

#### Whole-Body Parameters

Table 1 shows whole-body parameters in control mice that were killed at age 24 or 36 wk, 24-wk-old mice that were treated with losartan for 12 wk, and 24-wk-old mice that were treated with hydralazine for 12 wk. Before treatment, there were no differences in BP. Right before the mice were killed, BP was lower in losartan-treated versus control mice but similar between hydralazine- and losartan-treated groups. Body weights and serum triglycerides were not different among the groups, whereas serum cholesterol was lower in hydralazine-treated than in control or losartan-treated mice.

#### Atherosclerosis Quantification

Extent of atherosclerotic lesions in the proximal and en face aortas increased over time in untreated apolipoprotein E-deficient (apoE−/−) controls (Table 1, Figure 1). Losartan significantly decreased the extent of the lesions both in cross-sectional proximal aortas and in en face assessments. By contrast, hydralazine did not lessen the extent of atherosclerosis as assessed by either method. Whereas the number of individual lesions increased between 24 and 36 wk of age in untreated mice, this measure was unaffected by treatment with losartan or hydralazine.

#### Characteristics of Atherosclerotic Lesions

Macrophage content as assessed by monoclonal rat antibody to mouse macrophage (MOMA-2) staining revealed that, as the atherosclerotic lesion expanded, the proportion that was macrophage positive decreased. MOMA-2 staining occupied 4.0% of lesions in mice that were killed at 24 wk and 3.2% at 28 wk, whereas serum cholesterol was lower in hydralazine-treated than in control or losartan-treated mice.

#### Table 1. Whole-body and aortic lesion parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>C24w</th>
<th>C36w</th>
<th>H36w</th>
<th>L36w</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>20.8 ± 0.3</td>
<td>23.7 ± 0.4</td>
<td>21.2 ± 0.2</td>
<td>23.7 ± 0.4</td>
</tr>
<tr>
<td>BP (mmHg)</td>
<td>107 ± 2</td>
<td>108 ± 2</td>
<td>98 ± 6c</td>
<td>95 ± 3c</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>369 ± 27</td>
<td>367 ± 35</td>
<td>242 ± 18bcd</td>
<td>402 ± 31</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>120 ± 6</td>
<td>159 ± 11</td>
<td>131 ± 40</td>
<td>150 ± 10</td>
</tr>
<tr>
<td>Proximal aortic lesion (μm² × 10³)</td>
<td>227 ± 12</td>
<td>474 ± 28d</td>
<td>442 ± 36d</td>
<td>385 ± 20bcd</td>
</tr>
<tr>
<td>En face aortic lesion (%)</td>
<td>3.6 ± 0.2</td>
<td>13.0 ± 2.3d</td>
<td>9.0 ± 2.5d</td>
<td>5.1 ± 0.9c</td>
</tr>
<tr>
<td>No. of aortic lesions</td>
<td>27 ± 2</td>
<td>32 ± 3d</td>
<td>28 ± 2</td>
<td>28 ± 2</td>
</tr>
</tbody>
</table>

aC24w, data at 24 wk baseline; C36w, data in untreated controls at 36 wk; H36w, data in mice treated with hydralazine between 24 and 36 wk; and L36w, data in mice treated with losartan between 24 and 36 wk.

bP < 0.05 versus C24w.

cP < 0.005 versus C36w.

dP < 0.005 versus C24w.
(37.3 ± 3.2 versus 21.1 ± 3.8% in 36-wk-old untreated mice; \( P = 0.028 \)). By contrast, hydralazine did not affect the proportion of the wall lesion that was occupied by collagen (Masson trichrome).

By contrast, another important component of ECM, elastin, was profoundly affected by atherosclerosis and by losartan but not hydralazine treatment. Progression of atherosclerosis in untreated mice was associated with greater disruption of elastin lamellae: At 24 wk there were 38.0 ± 6.0 versus 52.7 ± 11.0 breaks/mm² at 36 wk in untreated controls (\( P < 0.008 \)). Elastin breaks were remarkably lessened by losartan but not by hydralazine (losartan: 24.7 ± 2.5 versus controls [\( P < 0.0001 \]); hydralazine: 50.1 ± 6.3 versus controls [NS]; Figure 3). Notably, the number of elastin breaks in losartan-treated mice that were killed at 36 wk was even less than in 24-wk-old controls, suggesting that losartan not only protects against destruction of elastin but also may even promote reconstitution of elastin fibers. Desmosine, a marker of mature elastin, was also significantly higher in aortas of losartan-treated mice versus untreated controls (10.9 ± 1.0 versus 5.9 ± 1.1 ng/mg, respectively; \( P = 0.034 \)).
Elastolysis and Angiotensin

Because atherosclerotic disruption of elastin of the normal vascular architecture involves matrix metalloproteinases (MMP) and cathepsins, we compared key elastolytic systems in aortas from control and losartan-treated mice. Immunostaining for cathepsin S was decreased by losartan but not hydralazine treatment. The reduction in cathepsin S protein paralleled a decrease in the mRNA expression, which was halved in aortas of losartan-treated versus control mice (P = 0.036) but not in hydralazine-treated mice (NS versus controls). Losartan-treated but not hydralazine-treated mice, showed decreased aortic MMP-9 mRNA expression versus controls (34.1 ± 11.0% of control expression levels found in losartan-treated [P = 0.03] and 88.1 ± 16.0% in hydralazine-treated mice [NS] versus controls; Figure 4). The mRNA expression of MMP-2 among the groups did not differ in the activity for MMP-2 among the groups. The elastolytic capacity of cultured vascular smooth muscle cells (VSMC) was increased in response to AngII (27.0 ± 2.4 versus 4.7 ± 1.9 cpm × 10^4/10^6 cells per 24 h in control cells; P = 0.003; Figure 5). This AngII-induced increase in elastolysis was significantly attenuated by losartan (14.6 ± 4.2 cpm × 10^4/10^6 cells per 24 h; P = 0.035 versus AngII, NS versus controls).

**Figure 5.** Elastolytic capacity of cultured vascular smooth muscle cells increased in response to exposure to angiotensin (AngII) and was abrogated in cells exposed to AngII + L.

DISCUSSION

Antagonism of AngII actions not only lessened progression of already established atherosclerotic lesions but also changed the vascular wall composition to contain relatively less lipid and more ECM, changes that predict greater stability of the atherosclerotic plaque. These benefits were not dependent on systemic hemodynamics or lipid levels, because hydralazine treatment, which achieved similarly reduced BP and even lower serum cholesterol, provided no protection. The tail-cuff determinations of BP that were used in these studies parallel intermittent brachial pressure measurements that form the basis for considering systemic pressure as a pathophysiologic parameter and target in human disease. However, neither of these methods may detect circadian or subtle changes in pressure over time. Nevertheless, reduction in pressure that was achieved by losartan and hydralazine documented under similar conditions at the same time of day were equivalent. Moreover, the aortic medial thickness was not different between losartan- and hydralazine-treated mice (data not shown), yet only losartan treatment resulted in structural benefit. These findings echo the hemodynamically independent benefits of AngII antagonism that were observed in...
other settings of experimental and clinical cardiac/renal diseases. Previous reports, including our recent study in mice with reduced renal parenchyma, showed that AngII inhibition impedes initiation and progression of early atherosclerotic disease. This study makes the additional important observation that even in the setting of established atherosclerotic disease, antagonizing AngII actions impedes further progression of the lesions and changes vessel wall composition/phenotype. These observations echo clinical studies that documented remarkable protection against acute vascular events in patients who had established atherosclerotic disease and were begun on treatment with inhibitors of AngII actions. Notably, AngII inhibition lessens cardiovascular events even in individuals with modest renal dysfunction and those who are on dialysis, who have the most aggressive cardiovascular disease and are at greater risk for plaque rupture.

The characteristics of atherosclerotic vascular remodeling change as lesions evolve. Our data reiterate that as atherosclerosis progresses, the macrophage component decreases, and at 36 wk of age, the lesional component that is macrophage positive is only approximately 12%. Contrasting previous reports of earlier stages of atherosclerosis, this study finds no difference in the macrophage-positive proportion of established atherosclerotic lesions between control and losartan-treated mice. Therefore, as lesions mature and the macrophage proportion of the lesion wanes, both the progression of disease and the impact of therapeutic interventions reflect distinct mechanisms that include contributions of nonmacrophage components of the lesion.

Previous studies focused on lipid deposition/cellular infiltration in atherosclerosis with little attention given to the other prominent component of the atherosclerotic plaque, namely the ECM. Changes in ECM, especially as affected by AngII, are now recognized as pivotal in other diseases, including cardiac remodeling, renal scarring, and hypertension-associated arteriosclerosis. In these circumstances, increased ECM, especially collagen, is linked to loss of functional parenchyma, and decreasing net ECM accumulation has become a therapeutic goal. By contrast, increasing ECM may be beneficial in atherosclerotic vasculopathy. In the atherosclerotic plaque, more ECM can stabilize the lesion and lessen the acute complications that are linked to plaque rupture. An interesting link between ECM and phenotypic characteristics of the atherosclerotic lesions in mouse models that predict stability has been reported. ApoE:cathepsin K double knockout mice had reduced atherosclerotic lesions together with increased collagen component of ECM, whereas apoE- and LDL receptor-deficient mice that were given TGF-β inhibitors had decreased vascular ECM and more plaque hemorrhages. In our study, losartan nearly doubled the relative collagen portion of the lesions. Although these results seem to be at odds with effects of angiotensin-converting enzyme inhibitors and angiotensin receptor blockers to limit ECM deposition in nonatherogenic disorders, they echo observations made in other models of atherosclerosis, including the Watanabe heritable hyperlipidemic rabbits and atherosclerotic mini-pigs, in which angiotensin-converting enzyme inhibitors/angiotensin receptor blockers increased ECM, including collagen, in the aortas. These findings complement recent observations that remodeling pathways may be tissue specific. Plasminogen activator inhibitor-1 deficiency was shown to protect against combined AngII and aldosterone-induced remodeling in the aortas but enhance AngII and aldosterone as well as senescent fibrosis in the heart. It should be noted that the Masson staining for collagen in this study was expressed as a fraction of Oil-Red-O staining for lipids, and additional biochemical measurements (i.e., HPLC-determined collagen) revealed no difference in the absolute collagen content of aortas from untreated and losartan-treated mice. Taken together, the data indicate that the relative increase in Masson staining for collagen in losartan-treated mice may reflect a greater contribution of decreased lipids induced by losartan than an absolute increase in the plaque or vessel wall collagen. Our most recent data indeed indicate that losartan has a direct effect on cellular lipid metabolism including an increase in cholesterol efflux that decreases cellular cholesterol content, which would predict increased relative collagen proportion of the vessel wall.

In addition to collagen, we examined the vascular ECM protein elastin, which was recently shown to be a crucial modulator of vascular remodeling and expansion. We found increased disruption of elastin lamellae as atherosclerosis progressed, as previously reported. Although we previously observed elastin disruption in early stages of atherogenesis, the results of this study indicate that this process not only continues into the later stages of the disease but also can be limited with AngII antagonism even in advanced disease. Losartan not only lessened the progressive increase in elastin breaks but also seems to have promoted elastin reconstitution because there were significantly fewer breaks at the end of losartan treatment than at the beginning of the therapy. Furthermore, desmosine, a marker of mature elastin, showed higher levels in aortas of losartan-treated versus control mice. The dampening of elasto-
lytic capacity of VSMC that were exposed to AngII by losartan may have contributed to this effect. It is interesting that AngII antagonism prevented an increase in serum and aortic elastolytic activity in cholesterol-fed rabbits and ameliorated arterial internal lamina ruptures in Brown Norway rats. Although elastin per se has been identified as a pivotal modulator of vascular response to injury, previous studies focused on macrophage-related elastolytic proteases. This study makes the novel observations that AngII increases elastolysis by SMC in vitro and elastin breaks in the aortic media in vivo, although a contribution from macrophages and/or endothelial cells cannot be completely excluded. Our results also show that AngII antagonism prevents disruption of elastin and may even promote reconstitution of elastin through mechanisms that, at least in part, include modulation of the elastolytic capacity of VSMC.

Elastin degradation has been most closely linked to MMP-2 and -9 and to cysteine proteases, especially cathepsin S. We found that AngII antagonism lessens cathepsin S and MMP-9 but not MMP-2 mRNA and activity. Recent observations indicate an especially relevant role for MMP-9 in extension and disruption of advanced plaques, including the pertinent finding that MMP-9 derived from resident vascular cells and not bone marrow–derived infiltrating cells was required to accelerate disease. Furthermore, MMP-9, cathepsin B, and a potential activator of both, legumin, were increased in the early stages of atherosclerosis, SMC can modulate proteolysis that promotes disruption of the elastin component of the vascular ECM and that AngII inhibition lessens the proteolytic effects in SMC. This observation may be particularly relevant to patients with CKD because even modest renal dysfunction imparts an independent risk for cardiovascular disease and death. Acute cardiovascular events typically reflect destabilization of the atherosclerotic plaques. Therefore, initiation of AngII antagonism at any stage of atherosclerosis not only may lessen progression of the atherosclerotic lesions but also may modulate the plaque characteristics toward a less vulnerable phenotype.

Our studies show that in addition to slowing progression of the later stages of atherosclerosis, inhibition of AngII decreases the elastolytic capacity of VSMC and lessens this potentially destabilizing effect on the plaque. Preservation of elastin may be a key mechanism for stabilization of atherosclerotic plaques that may underlie the decreased mortality observed in patients who have established atherosclerotic disease and are treated with AngII inhibition.

CONCISE METHODS

Mice and Experimental Groups
All experiments were done in female (apoE−/−) mice on C57BL/6 background (Jackson Laboratories, Bar Harbor, ME), maintained on normal mouse chow (RP5015; PMI Feeds, St. Louis, MO). Care and experimental procedures were in accordance with National Institutes of Health and Vanderbilt University Institutional Animal Care and Use guidelines. Angiotensin receptor antagonist losartan (n = 25; 100 mg/L drinking water) or hydralazine (n = 10; 80 mg/L drinking water) was begun at 24 wk of age and continued for 12 wk until the end of study at 36 wk of age. Dosages used in this study were based on pilot data in apoE−/− mice (n = 15), in which BP was determined two to three times per week and hydralazine and losartan dosages were titrated until similar reduction in BP was achieved. Age-matched controls received water and were killed at age 24 and 36 wk (n = 10 at each time point). Additional mice received infusions of AngII (1000 ng/kg per min for 2 wk) delivered through a subcutaneously implanted osmotic minipump; model 1002 (Durect, Cupertino, CA); n = 5) or saline (n = 4) and aortas were assessed for elastin breaks as described next.

Systemic Parameters
Systemic BP was determined by Muramachi Systems (Model MK-2000; Osaka, Japan) automated tail cuff in all mice in a conscious state after they were acclimated to the procedure, with mean values based on an average of three stable readings. BP was assessed at baseline before treatment, then at weekly intervals until the BP stabilized, and again before being killed. Serum cholesterol and triglyceride levels were determined at the end of the experiment. Mice were killed under phenobarbital anesthesia, and tissue was harvested for assessment (50 mg/kg). The heart, together with the proximal aorta, was embedded in OCT. Cryosections, 10 μm thick, were cut from the proximal aorta beginning at the end of the aortic sinus with modifications specific for computer analysis using Imaging System KS300 (Release 2.0; Kontron Elektronik GmbH, Eching, Germany) on at least 15 sections from each mouse.

Histology and Immunohistochemistry
Monoclonal rat antibody to mouse macrophages, MOMA-2 (Serotec, Raleigh, NC) was used to detect macrophage infiltration. The area stained with MOMA-2 in serial sections was measured using Imaging System KS300 (Kontron Elektronik) and calculated as the ratio of macrophage-stained to Oil-Red-O–stained areas as described previously. Five-micrometer sections of proximal aortas were stained with Masson trichrome, and the collagen-positive area was expressed as the ratio of Masson trichrome–to-Oil-Red-O–stained areas. Verhoeff-van Gieson elastin staining was performed in samples obtained from along the entire aorta as described previously.
were defined as interruptions in the elastin fiber, together with reappear-
ance of the fiber, expressed as number of elastin breaks per square
millimeter of medial area. Sections stained with the polyclonal ca-
thepsin S antibody (1:100 dilution; Calbiochem, La Jolla, CA) were
incubated overnight and subsequently incubated with secondary an-
tibodies (Vector Laboratories, Burlingame, CA) followed by incuba-
tion with ABC-AP complex.5,17 In each experiment, negative controls
without the primary antibody were included and showed no staining.

Collagen and Elastin Content by HPLC
Aortic collagen content was assessed from the concentration of hy-
droxypyroline and proline, measured as their phenylseleno thiocyanate
derivatives by reverse-phase HPLC. Collagen content is expressed as
nanograms per milligrams of aorta. Elastin content was also assessed
by reverse-phase HPLC from the concentration of desmosine and
expressed as nanograms per milligram of aorta.

Gelatin Zymography
Gelatin zymograms of aortas were performed as described previously.
49 Freshly isolated aortas were pulverized in liquid nitrogen and
transferred to lysis buffer (20 mmol/L Tris-HCl [pH 7.4], 150 mmol/L
NaCl, 10 mmol/L EDTA, 10 mmol/L benzamidine HCl, 0.02% so-
dium azide, 0.1% Triton X-100, 0.02% Tween 20, 2 mmol/L PMSF,
0.5 mmol/L leupeptin, and 5 μg/ml aprotinin), and extracts were
loaded on 10% SDS-polyacrylamide gels containing gelatin (1 mg/ml)
and electrophoresed under nonreducing conditions. Gel proteins
were renatured in 50 mmol/L Tris/0.1 mol/L NaCl/2.5% Triton X-100
at room temperature, washed, and then incubated in 50 mmol/L
Tris/10 mmol/L CaCl2/0.02% Na2VO3. Gels were stained with Coomas-
tis blue and destained in 5% acetic acid/10% methanol. The zymo-
grams were digitized, and the size-fractionated banding pattern, which
indicates MMP proteolytic activity, was determined by quan-
titative image analysis.

Messenger RNA Quantification
Total RNA extraction was performed using the RNeasy Mini kit (Qi-
gen, Valencia, CA). Quantifications of murine cathepsin S, MMP-2
and -9, and an endogenous control β-actin and 18S ribosomal RNA
levels were performed by a real-time reverse transcriptase–PCR assay
(TaqMan) using an ABI prism 7700 sequence detection system (ABI).
Probes for cathepsin S, MMP-2, MMP-9, β-actin, and 18S were ob-
tained from Applied Biosystems (Foster City, CA).

VSMC Culture and Elastase Assay
VSMC were isolated from aortas of apoE−/− mice after a procedure
that yields almost pure SMC from single mouse aortas.48 SMC identity
was confirmed with immunostaining for α-smooth muscle actin. Be-
cause of the possibility of multiply-passaged cells losing expression of
receptors for AngII, primary or secondary cell cultures were used.
Cells were exposed to medium alone or AngII (10−6 M) or AngII +
losartan (10−5 M). Elastase activity was assessed in confluent cells in
24-well plates (1 × 105 cells/well) by addition of 300 μg of [3H]elastin
(Parkin Elmer, Waltham, MA) to each well.7 Elastolysis experiments
were performed in five independently isolated cell lines with each
condition being assessed at least six times with at least three duplicates
of each experiment. Medium was collected 24 h later and centrifuged
(14,000 × g for 15 min), and the soluble radioactive elastin assayed.5,7

Transelastin SMC Invasion
SMC invasion across an elastin barrier was assessed in a modified
Boyden chamber with the membrane covered with Etna-Elastin (15
mg/ml) solution and PDGF-BB as the chemotactant as described
previously.48 SMC, isolated and cultured as described previously, were
exposed to AngII (10−6 to 10−8 M) alone or AngII (10−8 M) together
with losartan (10−5 M), or AngII with a selective cathepsin S inhibit-
or (LHVS, 5 nmol/L), a broader spectrum cysteine inhibitor (Cystatin C,
1 μg/ml), and an MMP inhibitor (GM6001; 10 μmol/L) for 24 h. The
membrane filters were fixed in methanol and stained with 1% crystal
violet. SMC adhering to the lower surface of the membrane that had
traversed the elastin barrier were counted under the microscope.
Quadruplicate wells were used for each experimental condition, and
more than four fields (×40) were counted for each well.

Statistical Analyses
Results are expressed as means ± SEM. Statistical difference was as-
essed by a single-factor variance (ANOVA) followed by unpaired t

test with corrections for multiple comparisons as appropriate. Non-
parametric data were compared by Mann-Whitney U test. P < 0.05
was considered to be significant.

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DISCLOSURES
None.

REFERENCES
EM: Extent and direction of arterial remodeling in stable versus unstable
2. Sipahi I, Tuzcu EM, Schoenhagen P, Nicholls SJ, Ozdurun V, Kapadia
S, Nissen SE: Compensatory enlargement of human coronary arteries
during progression of atherosclerosis is unrelated to atheroma bur-
den: Serial intravascular ultrasound observations from the REVERSAL
3. Chiong JR, Miller AB: Agents that stabilize atherosclerotic plaque.
Expert Opin Invest Drugs 10: 1681–1692, 2003
berger J, Wentzel J, Mizei G, Mercuri M, Badimon JJ: Lipid lowering
by simvastatin induces regression of human atherosclerotic lesions:

www.jasn.org BASIC RESEARCH

Angiotsensin in Atherosclerosis and Atherosclerotic Plaques 2317
Two years’ follow-up by high resolution noninvasive magnetic resonance imaging. Circulation 106: 2884–2887, 2002


