Atorvastatin Prevents Angiotensin II–Induced Vascular Remodeling and Oxidative Stress

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Abstract—Angiotensin II (Ang II) modulates vasomotor tone, cell growth, and extracellular matrix deposition. This study analyzed the effect of atorvastatin in the possible alterations induced by Ang II on structure and mechanics of mesenteric resistance arteries and the signaling mechanisms involved. Wistar rats were infused with Ang II (100 ng/kg per day, SC minipumps, 2 weeks) with or without atorvastatin (5 mg/kg per day). Ang II increased blood pressure and plasmatic malondialdehyde levels. Compared with controls, mesenteric resistance arteries from Ang II–treated rats showed the following: (1) decreased lumen diameter; (2) increased wall/lumen; (3) decreased number of adventitial, smooth muscle, and endothelial cells; (4) increased stiffness; (5) increased collagen deposition; and (6) diminished fenestrae area and number in the internal elastic lamina. Atorvastatin did not alter blood pressure but reversed all of the structural and mechanical alterations of mesenteric arteries, including collagen and elastin alterations. In mesenteric resistance arteries, Ang II increased vascular O2− production and diminished endothelial NO synthase and CuZn/superoxide dismutase but did not modify extracellular-superoxide dismutase expression. Atorvastatin improved plasmatic and vascular oxidative stress, normalized endothelial NO synthase and CuZn/superoxide dismutase expression, and increased extracellular-superoxide dismutase expression, showing antioxidant properties. Atorvastatin also diminished extracellular signal–regulated kinase 1/2 activation caused by Ang II in these vessels, indicating an interaction with Ang II–induced intracellular responses. In vascular smooth muscle cells, collagen type I release mediated by Ang II was reduced by different antioxidants and statins. Moreover, atorvastatin downregulated the Ang II–induced NADPH oxidase subunit, Nox1, expression. Our results suggest that statins might exert beneficial effects on hypertension-induced vascular remodeling by improving vascular structure, extracellular matrix alterations, and vascular stiffness. These effects might be mediated by their antioxidant properties. *(Hypertension. 2009;54:00-00.)*

Key Words: angiotensin II ■ atorvastatin ■ extracellular matrix ■ remodeling ■ oxidative stress

Hypertension is associated with structural changes (vascular remodeling) of resistance arteries like media thickening, reduced lumen diameter, and increased media lumen ratio.1–3 Among the cellular processes underlying these events, alterations in cell growth, migration, differentiation, and increased extracellular matrix (ECM) deposition have been described.1,3 Angiotensin II (Ang II) influences the architecture and integrity of the vascular wall by modulating cell growth and regulating ECM composition.1,2,4 Ang II mediates many of its cellular actions by stimulating the formation of reactive oxygen species (ROS), which play an important role in modulating inflammatory reactions.1 Evidence from the last few years has suggested that increased oxidative stress plays a pathophysiological role in cardiovascular disease, including atherosclerosis, hypertension, and heart failure.5–7 In fact, ROS have been shown to play a critical role in hypertrophy, fibrosis, and remodeling in the heart and vasculature.6,8,9 Statins are inhibitors of the 3-hydroxy-3-methylglutaryl coenzyme A reductase, a ubiquitous enzyme critical for the biosynthesis of cholesterol. Several clinical trials have demonstrated that statins exert beneficial effects in patients at high cardiovascular risk.10 Moreover, several studies have shown that statins decrease blood pressure in variable degrees both in humans11–14 and in experimental models,15,16 although a lack of effect of statins on blood pressure levels has also been described.17–19 Most of the benefits of statin therapy are attributable to the lowering of serum cholesterol levels. However, by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A reductase, statins can also inhibit the synthesis of isoprenoids, which are important lipid attachments for intracellular signaling molecules, eg, Rho and Rac. Thus, statins exert many effects beyond cholesterol lowering, including improvement of endothelial function, decreasing of vascular inflammation, inhibition of smooth muscle proliferation, and
immunomodulation. In addition, many of the actions of statins are thought to be mediated by decreasing ROS formation.

We have demonstrated recently that statins inhibit several intracellular signaling systems activated by Ang II (RhoA/Rho kinase and mitogen activated protein kinase [MAPK] pathways) involved in the regulation of profibrotic factors, e.g., connective tissue growth factor, and in vascular fibrosis. The aim of the present study was to evaluate whether statin treatment modulates the effects of Ang II on vascular remodeling, ECM deposition, and mechanical properties of resistance arteries and the possible role of oxidative stress in this modulation.

**Methods**

The material and methods used are described in the online data supplement (please see http://hyper.ahajournals.org).

**Results**

Atorvastatin Does not Affect Ang II–Induced Blood Pressure Increase

Systemic infusion of Ang II increased systolic blood pressure, whereas atorvastatin treatment did not affect this increase (control: 115 ± 110 mm Hg, Ang II: 152 ± 3 mm Hg, P < 0.05 versus control; Ang II + atorvastatin: 150 ± 2 mm Hg, P > 0.05 versus Ang II; n = 10 to 15 animals per group), as described.

![Figure 1](Image)

**Figure 1.** External and internal diameter-intraluminal pressure (A and B), wall thickness-intraluminal pressure (C), wall:lumen-intraluminal pressure (D), and cross-sectional area-intraluminal pressure (CSA; E) in MRAs from control, Ang II–, and Ang II plus atorvastatin (Atorv)–treated rats incubated in Ca²⁺–Krebs Henseleit solution. Data are expressed as mean ± SE; n = 9 to 10.

![Figure 2](Image)

**Figure 2.** A, Total number of adventitial cells (AC), SMCs, and endothelial cells (ECs) of MRAs from control, Ang II–, and Ang II plus atorvastatin–treated rats. Images from Hoechst 33342 stained arteries were taken from slide-mounted vessels (×63 oil objective, zoom×1) with a confocal microscope. B, Densitometric analysis and representative blots of endothelial NO synthase (eNOS) protein expression in MRAs from control, Ang II–, and Ang II plus atorvastatin–treated rats. α-Actin is also shown. Data expressed as mean ± SE; *P < 0.05 vs control, +P < 0.05 vs Ang II; n = 7 to 8.
Atorvastatin Improves Ang II–Induced Vascular Remodeling and Endothelial NO Synthase Expression

External (Figure 1A) and internal (Figure 1B) diameters were diminished in mesenteric resistance arteries (MRAs) from Ang II–treated rats compared with controls. Wall thickness and wall:lumen ratio were increased (Figure 1C and 1D), and cross-sectional area was unaffected by Ang II administration (Figure 1E). Atorvastatin treatment abolished the effect of Ang II in vessel and lumen diameters and the wall:lumen ratio (Figure 1).

The total number of adventitial, smooth muscle, and endothelial cells was diminished in Ang II–infused compared with control rats (Figure 2A) determined in whole vessels by confocal microscopy. Atorvastatin treatment improved the alterations induced by Ang II in the number of endothelial and smooth muscle cells (SMCs; Figure 2A). There was also a tendency to normalize the number of adventitial cells, but it did not reach statistical significance (Figure 2A). Ang II induced a significant decrease in endothelial NO synthase expression that was nearly reverted by atorvastatin treatment (Figure 2B).

Atorvastatin Ameliorates Ang II–Induced Vascular Stiffness

Vessels from Ang II–treated rats showed decreased elasticity, as shown by the larger value of $\beta$ (control: $4.5\pm0.1, n=10$; Ang II: $5.6\pm0.4, n=9$; $P<0.05$) and a leftward shift of the stress-strain relationship (Figure 4A). Incremental distensibility was also smaller in MRAs from Ang II–treated rats (Figure 4B). Atorvastatin abolished the effect of Ang II in all of the parameters studied (Figure 4). Thus, $\beta$ value was smaller in arteries from Ang II plus atorvastatin ($4.6\pm0.2$; $n=10$; $P<0.05$) than vessels from Ang II–treated rats.

![Figure 3. A. Representative images and quantitative analysis of collagen content in the media layer from transversal sections of MRAs from control, Ang II- and Ang II plus atorvastatin–treated rats stained with Picrosirius red. Images were captured with a light microscope (×40 objective, zoom ×1); image size: 325×325 µm. B. Representative confocal projections of the internal elastic lamina of MRAs from control, Ang II- and Ang II plus atorvastatin–treated rats. Vessels were pressure fixed at 70 mm Hg and mounted intact on a slide. Projections were obtained from serial optical sections captured with a fluorescence confocal microscope (×63 oil immersion objective, zoom ×2); image size: 119×119 µm. Low panels show quantification of fenestra area and total number of fenestra. Data are expressed as mean±SE; *P<0.05 vs control, +P<0.05 vs Ang II; n=7 to 9.](http://hyper.ahajournals.org/)}
Atorvastatin Reduces Ang II–Induced Oxidative Stress
Basal $O_2^{-•}$ production was greater in the adventitia and media layers of MRAs from Ang II–treated rats than from control rats (Figure 5A). Plasma malondialdehyde levels were also greater in Ang II–treated rats than in control rats (Figure 5B). Atorvastatin reduced the differences found in vascular $O_2^{-•}$ and malondialdehyde levels induced by Ang II (Figure 5).

Ang II increased Mn-superoxide dismutase (SOD) expression, diminished Cu/Zn-SOD expression, and did not affect MnSOD expression but increased both Cu/Zn-SOD and EC-SOD expression (Figure 6A). Atorvastatin did not affect MnSOD expression but increased both Cu/Zn-SOD and EC-SOD expression (Figure 6A). In cultured vascular SMCs (VSMCs), Ang II increased Nox1 expression that was diminished by atorvastatin preincubation (Figure 6B).

Antioxidants and Statins Inhibit Ang II–Induced Collagen Secretion in VSMCs
Ang II increased collagen type I secretion after 48 hours of incubation (Figure 7A). The superoxide dismutase analogue tempol, the antioxidant tempol, the $O_2^{-•}$ scavenger tiron, the xanthine oxidase inhibitor allopurinol, the antioxidant N-acetylcysteine, the NADPH oxidase inhibitor apocynin, and diphenylxylenediamine, an inhibitor of flavoprotein-containing enzymes, eg, reduced nicotinamide-adenine dinucleotide/NADPH oxidase, diminished Ang II–induced collagen accumulation (Figure 7A). Pretreatment with 2 different statins, atorvastatin and simvastatin (Figure 7B), also diminished Ang II–induced collagen production.

Atorvastatin Reduces Ang II–Induced MAPK Activation
To evaluate whether atorvastatin modifies the signaling pathway stimulated by Ang II, we evaluated extracellular signal–regulated kinase (ERK)1/2 and p38 MAPK activation. Ang II infusion induced ERK1/2 but not p38 activation in resistance arteries. These effects on ERK1/2 were partially prevented by atorvastatin treatment (Figure 8).

Discussion
The present study demonstrates that atorvastatin treatment reverses the structural and mechanical alterations induced by Ang II in resistance arteries even without any change in blood pressure. These effects might be mediated by their antioxidant properties.

It is accepted that Ang II plays a central role in the pathophysiology of vascular remodeling in hypertension. MRA from Ang II–treated animals showed eutrophic remodeling with increased wall thickness and an unchanged cross-sectional area, as reported previously, although hypertrophic remodeling has also been observed. Mechanisms contributing to eutrophic remodeling in Ang II–treated rats might be related, at least partially, to increased apoptosis in the vascular wall, which might counterbalance cell proliferation, thereby explaining the maintenance of wall volume. Thus, the effect of Ang II by inducing in vitro cell apoptosis has been reported, among others, for SMCs and endothelial cells. Moreover, Diep et al. in a rat model of rats infused for 7 days with Ang II, showed increased apoptosis in the media layer of the aorta. Our results point in this direction.

Table. Characteristics of Internal Elastic Lamina in Pressurized MRAs From Control, Ang II–, and Ang II Plus Atorvastatin–Treated Rats

<table>
<thead>
<tr>
<th>MRA Parameter</th>
<th>Control (n=9)</th>
<th>Ang II (n=7)</th>
<th>Ang II+Atorvastatin (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IEL thickness, μm</td>
<td>5.4±0.4</td>
<td>5.1±0.4</td>
<td>5.0±0.7</td>
</tr>
<tr>
<td>Relative area of elastin/image</td>
<td>0.813±0.01</td>
<td>0.887±0.01*</td>
<td>0.797±0.02‡</td>
</tr>
<tr>
<td>Luminal surface, mm²</td>
<td>0.86±0.02</td>
<td>0.68±0.03†</td>
<td>0.78±0.03‡</td>
</tr>
<tr>
<td>Volume elastin/artery, mm³</td>
<td>0.0038±0.0003</td>
<td>0.0031±0.0003</td>
<td>0.0032±0.0005</td>
</tr>
</tbody>
</table>

IEL indicates internal elastic lamina. Data are expressed as mean±SE.
*P<0.05 vs control.
†P<0.01 vs control.
‡P<0.05 vs Ang II.
because Ang II infusion decreased the total number of adventitial, SMCs, and endothelial cells in MRAs.

Changes in arterial wall morphology might be also influenced by increased ECM deposition. Ang II induces collagen deposition in different vascular beds. Collagen might be located in the intracellular gaps between SMCs, thus explaining the increase in wall thickness. Accordingly, we observed greater collagen content in the media layer of MRA from Ang II–infused rats. Moreover, Ang II increased collagen type I synthesis in cultured VSMCs. In MRAs from hypertensive rats, we have demonstrated previously the important role of alterations in elastin structure in determining vascular dimensions and mechanical properties.36 Herein, we have demonstrated that Ang II treatment alters elastin structure but not elastin content of small vessels. These alterations in ECM proteins might influence vascular mechanical properties, which might affect lumen diameter and, consequently, peripheral resistance to blood flow.30 Thus, we observed increased vascular stiffness and decreased wall distensibility after Ang II treatment, as reported.27,34,35 Because vascular remodeling is associated with altered mechanical properties30 and we, in fact, observed diminished cell

Figure 5. A, Vascular superoxide anion production in transversal sections of MRAs from control, Ang II–, and Ang II plus atorvastatin–treated rats. Left, Representative fluorescent photomicrographs of confocal microscopy sections labeled with the oxidative dye hydroethidine; image size: 188×188 μm. Right, Vascular superoxide anion quantification. B, Plasma malondialdehyde (MDA) levels in control, Ang II–, and Ang II plus atorvastatin–treated rats. Data are expressed as mean±SE. *P<0.05 vs control, +P<0.05 vs Ang II; n=4 to 9.

Figure 6. A, Densitometric analysis and representative blots of CuZn-, Mn-, and EC-SOD protein expression in MRAs from control, Ang II–, and Ang II plus atorvastatin–treated rats. α-Actin is also shown. B, Ang II increases Nox1 mRNA expression. VSMCs were pretreated for 18 hours with atorvastatin (10⁻⁵mol/L) and then stimulated with Ang II (10⁻⁷mol/L) for 3 hours. Similar results were observed with simvastatin (data not shown). Results of Nox-1 gene expression were obtained by real-time PCR. Data are expressed as mean±SE. *P<0.05 vs control, +P<0.05 vs Ang II; n=3 to 8.
number, we might suggest that the increased wall thickness and wall:lumen ratio after Ang II treatment are attributed to altered ECM deposition/structure rather than to changes in cell number.

The beneficial effect of statins on cardiac remodeling is well established. However, less information is available about the effect of statins on the structural alterations of resistance arteries. Pitavastatin prevented the increase in media thickening of transversal sections from mouse coronary arteries after chronic Ang II infusion. In rings of MRAs from the genetically hypertensive rat strain, fluvastatin or simvastatin treatments decreased media thickness and media:lumen ratio. We demonstrated that atorvastatin treatment improved the alterations in the structural parameters observed after Ang II infusion and normalized the alterations in cell number. Importantly, atorvastatin normal-

**Figure 7.** Ang II increases collagen release through redox-dependent mechanisms. A, VSMCs were pretreated (1 hour) with the following antioxidants: Tempol (10⁻⁵ mol/L), tiron (5×10⁻³ mol/L), apocynin (Apo; 10⁻⁶ mol/L), allopurinol (Allo; 10⁻⁶ mol/L), diphenyltetraiodoborodion (DPI; 5×10⁻⁶ mol/L), and N-acetylcysteine (NAC; 10⁻⁴ mol/L), and then incubated with Ang II (10⁻⁷ mol/L) for 48 hours (added each 24 hours). B, VSMCs were pretreated with simvastatin (10⁻⁷ to 10⁻⁵ mol/L) or atorvastatin (10⁻⁷ to 10⁻⁵ mol/L). Transforming growth factor (TGF)-β stimulated cells were used as a positive control. Type I collagen release was evaluated in supernatants. Results of type I collagen production were obtained from densitometric analysis and expressed as n-fold over control. The figure shows a representative Western blot experiment and a bar graph of data as mean±SEM of 3 experiments. *P<0.05 vs control, +P<0.05 vs Ang II.

**Figure 8.** Densitometric analysis of phospho-ERK1/2 and p38 MAPK protein expression in MRAs from control, Ang II-, and Ang II plus atorvastatin-treated rats. Representative blots for total and phospho-ERK1/2 and p38 MAPK are also shown. Results are expressed as the ratio between signals on the immunoblot corresponding with phospho-ERK1/2 and p38 and total ERK and p38, respectively. Data are expressed as mean±SE. *P<0.05 vs control, +P<0.05 vs Ang II; n=6.
ized the number of endothelial cells and the impaired endothelial NO synthase expression induced by Ang II. These mechanisms might participate in the improvement of endothelial dysfunction observed with statins in different cardiovascular pathologies.22,39,40

Atorvastatin downregulated the increase in collagen content and improved the alterations of elastin structure induced by Ang II in MRA. More importantly, these effects had a physiological consequence. Thus, atorvastatin restored the increased vascular stiffness and the altered distensibility induced by Ang II infusion. In large arteries, statin treatment reduced artery stiffness in hypertensive patients,12 and statin treatment decreased perivascular coronary artery fibrosis19 and ECM deposition in the heart17–19 and rat aorta.23

Inhibition of isoprenylation and activation of small G proteins, eg, the component of NADPH oxidase Rac110,21 and diminished expression of NADPH oxidase subunits,21 may mediate antioxidant actions of statins. Ang II increased plasma oxidative stress and local vascular O$_2^-$ production, as reported.24,25,28 Moreover, Ang II increased Mn-SOD, diminished CuZn-SOD, and did not alter EC-SOD expression. Atorvastatin treatment reversed the alterations observed in plasma oxidative stress and vascular O$_2^-$ probably because of an increase in antioxidant defenses and diminished NADPH oxidase expression. Thus, we observed increased CuZn-SOD and EC-SOD expression and diminished Nox1 expression after atorvastatin treatment. In agreement, statins reduced oxidative stress in hypertension.16,17,20–22

It is established that vascular remodeling is influenced by oxidative stress in hypertension through its effects, among others, on ECM.6–7 Recently, we have demonstrated that atorvastatin inhibits several intracellular signaling systems (Rho/Rhoa and MAPK pathways) activated by Ang II.23 This is now confirmed in our experimental model, because Ang II induced ERK1/2 but not p38 MAPK activation in resistance vessels that was prevented by atorvastatin treatment. These intracellular signaling pathways are involved in the regulation of the profibrotic factor connective tissue growth factor and, hence, in ECM deposition and are modulated by redox processes.23 Ang II increased collagen synthesis from VSMCs and antioxidant agents, and both atorvastatin and simvastatin diminished this increase. These results suggest that ROS might mediate the altered ECM deposition induced by Ang II treatment and that atorvastatin, through its antioxidant capacity, modulates Ang II–induced fibrosis in resistance vessels.

Atorvastatin treatment did not alter the increase in blood pressure induced by Ang II, thereby excluding a blood pressure–dependent mechanism in the beneficial actions of statins. The effect of statins on blood pressure levels seems to be a dose-dependent effect in animal models. Thus, our results are in agreement with other studies performed in spontaneously hypertensive rats16,39 or in Ang II–dependent hypertensive models,17,19 where low doses of statins similar to that used in the present study did not affect systolic blood pressure. However, high and moderate doses of statins variably decreased blood pressure levels in different models of hypertension.18,20,22

In conclusion, the present results demonstrate that statins exert a beneficial effect on resistance arteries through improvement of vascular remodeling and reduction of oxidative stress.

**Perspectives**

Impaired tissue perfusion because of abnormality of the microvascular system is common among the conventional cardiovascular risk factors, including hypertension, diabetes mellitus, obesity, and dyslipidemia.3 This study demonstrates that statins are therapeutic strategies that might help to restore or at least to attenuate the impaired tissue perfusion associated with cardiovascular pathologies through improvement of vascular structure and mechanical properties. We suggest that these beneficial effects of statins on resistance arteries might precede the effects of these drugs on blood pressure and, therefore, might help to explain the blood pressure–lowering effects reported on statins. The reduction of artery stiffness may also contribute to the long-term benefits of statins by contributing to the reduced risk of stroke and cardiovascular events reported on statins. ROS are central to many of the adverse changes observed in the microcirculation and may offer potential targets for therapeutic intervention.6 The attenuation by statins of ROS production in the vasculature seems to play a role in the abrogation of oxidative stress–induced vascular fibrosis.

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**Disclosures**

None.

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Atorvastatin prevents Angiotensin II-induced vascular remodelling and oxidative stress

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MATERIALS AND METHODS

Animal model

Studies were done using the model of systemic infusion of AngII (dissolved in saline) into female Wistar rats (subcutaneously by osmotic minipumps, Alza Corp., CA), at the dose of 100 ng/kg/day. To determine the effect of statins, a group of rats was daily treated with the HMG-CoA reductase inhibitor atorvastatin (5 mg/Kg/day, dissolved in 0.1% methanol in the drinking water), starting 48 h before AngII-infusion and studied after 14 days. Control groups of animals without treatments were also studied. Blood pressure was measured by tail-cuff plethysmography. All experimental procedures were approved by the Animal Care and Use Committee of our Institution, according to the guidelines for ethical care of experimental animals of the European Community. The study was conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and the current Spanish and European laws (RD 223/88 MAPA and 609/86).

The rats were decapitated and the mesenteric arcade was removed and placed in Krebs Henseleit solution (KHS) of the following composition (in mmol/L): NaCl 115.0; KCl 4.7; CaCl2 2.5; KH2PO4 1.2; MgSO4.7H2O 1.2; NaHCO3 25.0; glucose 11.1 and Na2EDTA 0.01 maintained at 4°C and continuously gassed with 95% O2 and 5% CO2. Third order branches of mesenteric artery were isolated from the mesenteric bed and carefully cleaned of surrounding tissue under a dissecting microscope.

Pressure myography

The structural and mechanical properties of mesenteric resistance arteries (MRA) were studied with a pressure myograph (Danish Myo Tech, Model P100, J.P. Trading I/S, Aarhus, Denmark), as previously described. Briefly, the vessel was placed on two glass microcannulae and secured with surgical nylon suture. After any small branches were tied off, vessel length was adjusted so that the vessel walls were parallel without stretch. Intraluminal pressure was then raised to 140 mm Hg and the artery was unbuckled by adjusting the cannulae. The segment was then set to a pressure of 70 mm Hg and allowed to equilibrate for 60 min at 37°C in calcium-free KHS (0Ca2+; omitting calcium and adding 10⁻³ mol/L EGTA) intra and extra-vascular perfused gassed with a mixture of 95% O₂ and 5% CO₂. Intraluminal pressure was reduced to 3 mm Hg. A pressure-diameter curve was obtained by increasing intraluminal pressure in 20 mm Hg steps between 3 and 140 mm Hg. Internal and external diameters were continuously measured under passive conditions (D_{i0Ca}, D_{e0Ca}) for 3 min at each intraluminal pressure. The final value used was the mean of the measurements taken during the last 30 seconds when the measurements reached a steady state. Finally, the artery was set to 70 mm Hg in 0Ca²⁺-KHS, pressure-fixed with 4% paraformaldehyde (PFA, in 0.2 mol/L phosphate buffer, pH 7.2-7.4) at 37 °C for 60 min and kept in 4% PFA at 4°C for confocal microscopy studies.

Calculation of passive structural and mechanical parameters. From internal and external diameter measurements in passive conditions the following structural and mechanical parameters were calculated:

Wall thickness, WT = (D_{e0Ca} - D_{i0Ca})/2
Cross sectional area, CSA = \((\pi/4) \times (D_{\text{e0Ca}}^2-D_{\text{i0Ca}}^2)\)

Wall:lumen =\( (D_{\text{e0Ca}}-D_{\text{i0Ca}})/2D_{\text{i0Ca}}\)

Luminal surface area (LSA) was calculated at 70 mm Hg intraluminal pressure according to the formula:

\[ \text{LSA} = 2\pi L \left( \frac{D_{\text{i0Ca}}}{2} \right) \]

where \(L\) is 1 mm length of the vessel.

Incremental distensibility represents the percentage of change of the arterial internal diameter for each mm Hg change in intraluminal pressure and was calculated according to the formula:

\[ \text{Incremental distensibility} = \frac{\Delta D_{\text{i0Ca}}}{D_{\text{i0Ca}} \times \Delta P} \times 100. \]

Circumferential wall strain \((\varepsilon)\)= \(\frac{(D_{\text{i0Ca}} - D_{\text{00Ca}})}{D_{\text{00Ca}}}\)

where \(D_{\text{00Ca}}\) is the internal diameter at 3 mm Hg and \(D_{\text{i0Ca}}\) is the observed internal diameter for a given intravascular pressure both measured in 0Ca\(^2+\) medium.

Circumferential wall stress \((\sigma)\)= \(\frac{(P \times D_{\text{i0Ca}})}{2WT}\)

where \(P\) is the intraluminal pressure (1 mm Hg = 1.334 x 10\(^3\) dynes/cm\(^2\)) and \(WT\) is wall thickness at each intraluminal pressure in 0Ca\(^2+\)-KHS.

Arterial stiffness independent of geometry is determined by the Young’s elastic modulus \((E=\text{stress/strain})\). The stress-strain relationship is non-linear; therefore, it is more appropriate to obtain a tangential or incremental elastic modulus \((E_{\text{inc}})\) by determining the slope of the stress-strain curve \((E_{\text{inc}}=\delta \sigma/\delta \varepsilon)\). \(E_{\text{inc}}\) was obtained by fitting the stress-strain data from each animal to an exponential curve using the equation:

\[ \sigma = \sigma_{\text{orig}} e^{\beta \varepsilon} \]

where \(\sigma_{\text{orig}}\) is the stress at the original diameter (diameter at 3 mmHg). Taking derivatives on the above equation we see that \(E_{\text{inc}}=\beta \sigma\). For a given \(\sigma\) value, \(E_{\text{inc}}\) is directly proportional to \(\beta\). An increase in \(\beta\) implies an increase in \(E_{\text{inc}}\) which means an increase in stiffness.

**Confocal microscopy study of nuclei distribution.**

Pressure fixed intact arteries were incubated with the nuclear dye Hoechst 33342 (0.01 mg/ml) for 15 min. After washing, the arteries were mounted on slides with a well made of silicon spacers to avoid artery deformation. They were viewed using a Leica TCS SP5 confocal system fitted with an inverted microscope and Argon and Helium-Neon laser sources with oil immersion lens (x63) (Ex 351-364 nm and Em 400-500 nm). At least two serial optical sections (stacks of images) of 0.5 \(\mu\)m thick serial optical slices were taken from the adventitia to the lumen in different regions along the artery length. Individual images of the endothelial layer were also captured. Metamorph image analysis software (Molecular Devices Corp. Downingtown, PA, USA) was used for quantification. The nuclei numbers were measured in Z axis as previously described\(^2\) with minor modifications.

To allow comparison of control and AngII-treated rats, the following calculations were performed on the basis of 1-mm-long segments: artery volume (in mm\(^3\)) (volume = wall CSA (mm\(^2\)) x 1 mm); total number of adventitial and smooth muscle cells (cell \(n = n\) of nuclei per stack x \(n\) of stacks per artery volume); total number of endothelial cells (EC) was calculated per luminal surface of 1-mm-long artery; luminal surface area = \(2\pi\)diameter/2.
Elastin content and organization by confocal microscopy

The content and organization of elastin in MRA was studied in maximally relaxed intact segments pressure-fixed at 70 mm Hg using a confocal microscope as previously described. Briefly, stacks of images from the adventitia to the lumen (z step= 0.5 µm) were captured with a x63 objective (NA 1.3) using the 488-nm line of the confocal microscope. A minimum of 2 stacks of images of different regions was captured in each arterial segment. All the images were taken under identical conditions of laser intensity, brightness and contrast.

Quantitative analysis was performed with MetaMorph image analysis software, as described. From each stack of serial images internal elastic lamina (IEL) thickness was measured. Thereafter, individual projections of the IEL were reconstructed and total fenestrae number, fenestra area and relative area occupied by elastin, were measured.

Collagen determination by picrosirius red

Segments of MRA were removed from the mesentery and immediately fixed in 4% PFA in phosphate buffer for 1 h, transferred to a cryomold containing OCT embedding medium (Tissue Tek, Sakura) and frozen in liquid nitrogen. Frozen transverse sections (10 µm) were incubated with picrosirius red [0.1% (wt/vol) Sirus red 3FB in saturated aqueous picric acid] for 30 min with gentle agitation for collagen staining. Colour images were captured with a microscope (Nikon Eclipse TE 2000-S, x40 objective) using a digital camera (Nikon DXM 1200F). Sections from control, AngII and AngII plus atorvastatin MRA were imaged every day in parallel. Quantitative analysis of collagen content both in the adventitia and media layers was performed with Metamorph image analysis software. Collagen content was estimated by subtracting the background from the intensity values obtained in each cross section.

In situ detection of vascular O$_2^-$ production

The oxidative fluorescent dye dihydroethidium (DHE) was used to evaluate O$_2^-$ production in situ, as previously described. Hydroethidine freely permeates cells and is oxidized in the presence of O$_2^-$ to ethidium bromide, which is trapped by intercalation with DNA. Ethidium bromide is excited at 546 nm and has an emission spectrum of 610 nm. Frozen tissue segments were cut into 10 µm thick sections and placed on a glass slide. Serial sections were equilibrated under identical conditions for 30 min at 37°C in Krebs-HEPES buffer (in mmol/L: 130 NaCl, 5.6 KCl, 2 CaCl$_2$, 0.24 MgCl$_2$, 8.3 HEPES, 11 glucose, pH=7.4). Fresh buffer containing DHE (2x10$^{-6}$ mol/L) was applied topically onto each tissue section, cover-slipped, incubated for 30 min in a light-protected humidified chamber at 37°C and then viewed with a fluorescent laser scanning confocal microscope (Leica TCS SP2 equipped with a krypton/argon laser, x20 objective, zoom 4x), using the same imaging settings in control, AngII and AngII plus atorvastatin treated MRA. Fluorescence was detected with a 568 nm long-pass filter. For quantification, three rings per animal were sampled for each experimental condition and averaged. The mean fluorescence densities in the target region were calculated.

Measurement of MDA production

Plasmatic MDA levels were measured by a modified thiobarbituric acid (TBA) assay. Plasma was mixed with 20% trichloracetic acid in 0.6 mol/L HCl (1:1, v/v),
and tubes were kept in ice for 20 min to precipitate plasma components and so avoid possible interferences. Samples were centrifuged at 1500xg for 15 min before adding TBA (120 mmol/L in Tris 260 mmol/L, pH=7) to the supernatant in a proportion of 1:5 (v/v); then, the mixture was boiled at 97°C for 30 min. Spectrophotometric measurements at 535 nm were made at 20°C.

Cell cultures

Vascular smooth muscle cells (VSMC) were obtained from thoracic aorta of Sprague Dawley rats by collagenase method. VSMC from passages 2 to 7 were used showing >99% positive immunostaining against smooth muscle α-actin (not shown). For experiments, cells at 80% confluence were arrested by serum-starvation for 48h. Thereafter, cells were stimulated with AngII (10^-7 mol/L) in the presence or in the absence of different drugs. The participation of reactive oxygen species on the observed effects was studied by preincubation of the cells for 1 h with tempol, tiron, apocynin, allopurinol, N-acetylcysteine and dyphenyleneiodonium before AngII treatment. In some experiments, atorvastatin and simvastatin were used as HMG-CoA reductase inhibitors. Cell culture reagents were from Life Technologies, Inc.

Protein studies by Western Blot

Collagen content was determined in the supernatants from treated cells by Western blot. 50 µg of proteins and markers of molecular weight sizes were resolved in 10% SDS-PAGE gels, transferred, blocked and incubated with specific rabbit anti-mouse collagen type I polyclonal antibody (Chemicon, 1:10000 dilution) overnight at 4°C. Detection was made with an anti-rabbit peroxidase-conjugated secondary antibody and developed using an enhanced horseradish peroxidase/luminol chemiluminescence system (ECL Plus, GE Healthcare, Little Chalfont, UK) and subjected to autoradiography. Protein content was determined by BCA method (Pierce) and the same amount of protein was loaded for each experimental condition. The efficacy of protein loading and transfer to membranes was also assessed by Ponceau S staining.

In another group of experiments, proteins from homogenized mesenteric arteries (5 µg for Cu/Zn- and Mn-SOD and, 40 µg for eNOS, EC-SOD, p38 and ERK1/2) and markers of molecular weight sizes were separated by 7.5% (eNOS), 10% (EC-SOD, p38 and ERK) or 12% (Cu/Zn- and Mn-SOD) SDS-PAGE. Proteins were transferred to polyvinyl difluoride membranes and, after blockade, the membranes were incubated overnight at 4°C with mouse monoclonal antibody for eNOS (1:1000, BD Transduction Laboratories, Lexington, UK) or with rabbit monoclonal phospho-ERK1/2 (1:1000, Upstate), or polyclonal antibodies for Cu/Zn-SOD (0.05 µg/ml, StressGen, Victoria, Canada), Mn-SOD (0.05 µg/ml, StressGen), EC-SOD (10 µg/ml, StressGen), total-ERK1/2 (1:2000, upstate), phospho-p38 (1:500, Cell Signaling Technology, Inc) or total-p38 (1:250, Cell Signaling Technology, Inc). After washing, membranes were incubated with anti-mouse (1:5000, StressGen) or anti-rabbit (1:2000, Bio-Rad, Hercules, CA) IgG antibody conjugated to horseradish peroxidase. The membrane was thoroughly washed and the immunocomplexes were detected as described above. The same membrane was used to determine α-actin expression using a mouse monoclonal antibody (1:800,000, Sigma Chemical Co, St Louis, MO, USA).
For protein expression in mesenteric resistance arteries, data are expressed as the ratio between signals on the immunoblot corresponding to the studied protein and α-actin. To compare the results for protein expression within the same experiment and with others, we assigned a value of 1 to the ratio in arteries from AngII animals, and used that value to calculate the relative density of other bands from the same gel.

**Gene studies by RT-PCR**

RNA was isolated by Trizol (Invitrogen). Real-time PCR reactions were performed on ABI Prism 7500 sequence-detection PCR system (Applied Biosystems, Foster City, CA), and cDNA was synthesized with 2 µg of total RNA, according to manufacturer’s protocol. Assay identification for Nox-1 was Rn00586652_m1 (Applied Biosystems). Data were normalized with GAPDH and 18S ribosomal RNA expression (assay identifications: Rn99999916_s1 and Hs99999901_m1, respectively).

**Statistical analysis and drugs**

Results are expressed as means ± SE and “n” denotes the number of animals used in each experiment. Results of protein levels are expressed as n-fold increase over control or AngII in densitometric arbitrary units, expressed as mean±SE of the experiments made. The dependency of either vascular structure or mechanics on rat treatment group and intraluminal pressure was studied by two-way analysis of variance (ANOVA) followed by a Bonferroni’s post hoc test. For specific two means comparison, unpaired Student’s t-test was used. A value of \( P < 0.05 \) was considered significant.

Hoechst 33342, Sirius Red, tempol, tiron, apocynin, allopurinol, N-acetylcysteine and dyphenyleneiodonium were obtained from Sigma Chemical (St Louis, MO, USA). DHE was obtained from Molecular Probes™ (Invitrogen, Carlsbad, CA, USA). AngII was obtained from Bachem (Germany). Atorvastatin was kindly donated by Pfizer (Madrid, Spain). Simvastatin was obtained from Calbiochem (Germany). All other chemicals were of reagent grade or better and obtained from Sigma Chemical or other commercial suppliers.

**REFERENCES**


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