Atorvastatin Prevents End-Organ Injury in Salt-Sensitive Hypertension
Role of eNOS and Oxidant Stress

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Abstract—Statins, inhibitors of cholesterol biosynthesis, are endowed with pleiotropic effects that may contribute to their favorable clinical results. Hypertensive Dahl salt-sensitive (DS) rats have endothelial dysfunction and cardiorenal injury associated with decreased NO bioavailability and increased superoxide (O$_2^-$) production linked to a functional upregulation of angiotensin II. We investigated whether atorvastatin (30 mg/kg per day; by gavage) would prevent endothelial nitric oxide (eNOS) downregulation and the increase in O$_2^-$ in DS rats, thereby reducing end-organ injury. DS rats given a high-salt diet (4% NaCl) for 10 weeks developed hypertension (systolic blood pressure [SBP]) 200±8 versus 150±2 mm Hg in DS rats fed 0.5% NaCl diet [NS]; P<0.05), impaired endothelium-dependent relaxation, functional upregulation of endothelin-1, left ventricular hypertrophy (LVH; 30%), and proteinuria (167%), accompanied by downregulation of aortic eNOS activity (0.7±0.2 versus 1.8±0.3 nmol/min per gram protein in NS; P<0.05) and increased aortic O$_2^-$ (263±316 versus 1176±112 counts/min per milligram in NS; P<0.05) and plasma 8-F$_2$α isoprostanes. Atorvastatin prevented the decrease in eNOS activity (1.5±0.3 nmol/min per gram protein) as well as the increase in O$_2^-$ (1192±243 counts/min per milligram) and plasma 8-F$_2$α isoprostanes, reduced LVH and proteinuria, and normalized endothelial function and vascular response to endothelin-1, although reduction in SBP was modest (174±8 mm Hg). Atorvastatin combined with removal of high salt normalized aortic eNOS activity, SBP, LVH, and proteinuria. These findings strongly suggest that concomitant prevention of vascular eNOS downregulation and inhibition of oxidative stress may contribute to the protection against end-organ injury afforded by this statin in salt-sensitive hypertension. (Hypertension. 2004;44:186-190.)

Key Words: nitric oxide synthase ■ oxidative stress ■ hypertension, sodium dependent ■ statins

The 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors (statins) are potent inhibitors of cholesterol biosynthesis. These agents slow the progression and foster the regression of atherosclerosis, resulting in an improvement of cardiovascular outcomes in humans with elevated and normal serum cholesterol levels.¹–⁴ Evidence is steadily increasing for the beneficial, lipid-independent or “pleiotropic” effects of statins on cardiovascular diseases, which include restoration of endothelial function, stabilization of atherosclerotic plaques, and decrease in oxidative stress and vascular inflammation.⁵–⁶ These pleiotropic effects of statins are mediated, at least in part, by their ability to block the synthesis of various isoprenoid intermediates, which serve as lipid attachments for the post-translational modification of a variety of intracellular signaling proteins.⁷

The vasculoprotective effects of statins may have important implications for the prevention of end-organ damage in hypertension.⁸⁹ Indeed, statins have been reported to upregulate endothelial nitric oxide synthase (eNOS) expression and activity, reduce endothelin-1 (ET-1) expression, downregulate angiotensin II (Ang II) receptor subtype 1 (AT₁) expression, and inhibit NAD(P)H oxidase activity.⁵⁺⁶,¹⁰,¹¹

Dahl salt-sensitive (DS) rats, which are a paradigm of low renin salt-sensitive hypertension, are characterized by a downregulation of eNOS as well as an impaired NO bioavailability.¹²–¹⁴ We have shown that the latter is linked, at least in part, to a functional upregulation of Ang II action that results in high vascular oxidative stress.¹⁵ In addition, recent studies from other laboratories reported that levels of Ang II as well as of NAD(P)H oxidase subunits p47phox and gp91phox are increased in the kidney of hypertensive DS rats.¹⁶–¹⁸

Currently, there is a consensus that supports the notion that the balance between NO, Ang II, ET-1, and vascular generation of reactive oxygen species is crucial for maintaining the homeostasis of the cardiovascular and renal systems, particularly for regulation of vascular tone, modulation of inflammation, and vascular remodeling.¹⁹–²¹ We, therefore, tested the hypothesis that statins will ameliorate endothelial dysfunction and end-

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organ injury via restoration of the balance among these vasoactive substances in salt-sensitive hypertension.

The studies reported herein demonstrate that in hypertensive DS rats fed high dietary salt, atorvastatin prevents the decrease in aortic eNOS activity and concomitantly reduces aortic superoxide anion (O$_2^-$) production; normalizes endothelial function, vascular response to ET-1, and aortic hypertrophy; and reduces systolic blood pressure (SBP), left ventricular hypertrophy (LVH), and proteinuria. Moreover, the combination of atorvastatin with removal of high dietary salt normalizes SBP and LVH. These findings support the notion that concomitant prevention of the downregulation of vascular eNOS activity and inhibition of oxidative stress contributes to the protection afforded by statins against end-organ injury in salt-sensitive hypertension.

Methods

**Animals and Experimental Protocols**

The animals were housed in facilities accredited by the American Association for Accreditation of Laboratory Animal Care. The Institutional Animal Care and Use Committee at the Miami VA Medical Center approved the studies. Six-week-old DS male rats were purchased from Harlan Sprague-Dawley (Indianapolis, Ind) and maintained under controlled conditions of light, temperature, and humidity. After 2-week accommodation to the new environment, the rats were divided into 5 groups: NS, fed 0.5% NaCl diet for 10 weeks (n=8); HS, fed 4% NaCl diet for 10 weeks (n=8); HS+AT, fed 4% NaCl diet plus atorvastatin (30 mg/kg per day by gavage, n=7) for 10 weeks; HS/NS, fed 4% NaCl diet for 6 weeks followed by 4 weeks of 0.5% NaCl diet (n=6); HS/NS+AT, fed 4% NaCl diet for 6 weeks followed by 4 weeks of 0.5% NaCl diet plus atorvastatin (n=6). SBP was measured in conscious rats by the tail-cuff method. Twenty-four-hour urinary excretion was collected in individual metabolic cages every other week. Urine protein concentration was determined with the use of a BioRad protein assay kit and expressed in milligrams per 24 hours. At the end of the study, the rats were euthanized by decapitation, and the heart, kidneys, and aorta were harvested. LV weight and aortic wet weight/cm (arch of thoracic aorta to the origin of mesenteric artery) were used as indices of LVH and aortic hypertrophy, respectively. 12

**Detection of O$_2^-$ Generation in the Aorta**

The O$_2^-$ generation in intact aortic rings was determined by chemiluminescence of lucigenin (5 μmol/L) as previously described, and the results expressed as counts/min per milligram dry tissue. Chemiluminescence of lucigenin has been validated as the method to measure O$_2^-$ in our previous studies. 13 The specificity of lucigenin to assess aortic O$_2^-$ generation was confirmed by preincubation of aortic rings with Tiron that resulted in a 90% reduction in O$_2^-$ measurement (data not shown).

**Determination of Plasma-Free 8-F$_2$α Isoprostane Levels**

Purification and enzyme immunoassay procedures for measurement of plasma-free 8-F$_2$α isoprostanes were performed by enzyme immunoassay using a commercial kit (Cayman Chemical) and following the manufacturer instructions as previously described. Concentrations were estimated from a standard curve and expressed as pg/mL.

**Organ Chamber Experiments**

Endothelial function was examined in aortic rings using an organ chamber bath, as previously described. Endothelium-dependent relaxation to acetylcholine (Ach) (10$^{-8}$ to 10$^{-4}$ mol/L) was studied in rings precontracted to 70% of maximal contraction to norepinephrine. Vascular contraction in response to ET-1 (10$^{-9}$ to 10$^{-7}$ mol/L) was studied in intact aortic rings.

**Measurement of eNOS Activity in the Aorta**

eNOS catalytic activity was measured by conversion of [14C]arginine to [14C]L-citrulline as previously described, and eNOS activity expressed as nanomoles of [14C]citrulline formed per minute per gram of protein. In our previous studies, we showed that eNOS activity measured by the citrulline assay represents maximal eNOS activity and correlates with eNOS mass as determined by Western blot.

**Data Analysis**

Relaxation of aortic rings was expressed as percent inhibition of norepinephrine-induced constriction. Vasocostriction response to ET-1 was expressed as percentage of 100 mmol/L KCl-induced constrictions. The maximal response to an agonist (Emax) and the concentration of an agonist causing a half-maximal response (ED$_{50}$) were determined from the concentration-response curves using the best fit to a logistic sigmoid function. The results were expressed as mean±SEM. Statistical analyses were performed by ANOVA with Bonferroni correction for multiple comparisons, followed by Scheffé test. Significance was assumed at P<0.05.

**Results**

**SBP and Urine Protein Excretion**

High-salt diet for 10 weeks resulted in a significant increase in SBP (200±8 versus 150±2 mm Hg; P<0.05) and in urine protein excretion in DS rats compared with that in NS controls (Figure 1). Atorvastatin in the presence of high-salt diet (HS+AT) significantly inhibited the increase in SBP (174±8 mm Hg) and proteinuria. The return to an NS diet (HS/NS) after 6 weeks of HS diet did not reduce SBP (205±7 mm Hg) or urine protein excretion (Figure 1). However, the combination of atorvastatin with removal of the HS diet (HS/NS+AT) normalized SBP (152±2 mm Hg) and urine protein excretion (Figure 1).

**Aortic and LV Hypertrophy**

Hypertensive DS rats fed a high-salt diet developed a significant increase in aortic weight (23%) and LV weight/body
weight (BW) ratio (30%). Atorvastatin in the presence of HS diet normalized the aortic weight and significantly reduced LVW/BW ratio. Return to a normal salt diet did not reduce aortic weight or LVW/BW ratio. The combination of atorvastatin with removal of HS diet normalized the aortic weight and the LVW/BW ratio (Figure 2).

**Oxidative Stress, Endothelial Function, and eNOS Activity in Aorta**

As previously shown, 15 hypertensive HS rats exhibited a significant increase in aortic $O_2^\cdot$ compared with that in NS controls ($P<0.05$) (Figure 3). Return to an NS diet alone did not reduce either aortic weight or LV weight/BW ratio. The combination of atorvastatin with removal of the HS diet normalized the aortic weight and the LV weight/BW ratio (Figure 2).

Endothelium-dependent relaxation to Ach in the aortic rings of hypertensive HS rats was significantly attenuated compared with that in NS controls (Emax: 56.6% versus 93.7% in NS rats, $P<0.05$; ED50: 6.5% versus 26.0% $-\log$ molar in NS rats, $P<0.05$) (Figure 4A). Return to an NS diet did not improve endothelium-dependent relaxation to Ach (Emax: 67.6% $\pm$ 5.5%; ED50: 6.6% $-\log$ molar). It has been reported that the maximal response to exogenous ET-1 is inversely proportional to the endogenous ET-1 concentration in the vessel wall. 24 The vascular contraction in response to ET-1 in hypertensive HS rats was significantly attenuated compared with that in NS controls (Emax: 105.2% versus 207.2% in NS rats, $P<0.05$) (Figure 4B). Return to an NS diet alone did not improve the contraction response to ET-1 (Emax: 118.2%$\pm$12.2%).

Atorvastatin normalized (1) aortic $O_2^\cdot$ (Figure 3), (2) plasma-free 8-F2α isoprostanes (HS/AT, 58.4 $\pm$ 14.2 pg/mL; NS + AT, 48.7 $\pm$ 8.5 pg/mL), (3) endothelial function (Figure 4A), and (4) vascular response to ET-1 (Figure 4B), independently of whether hypertensive DS rats were maintained on a HS diet or switched to an NS diet.

Similar to what we have shown previously, 12-13 eNOS activity in the aorta was significantly lower in hypertensive HS rats than in NS rats (0.74 $\pm$ 0.21 versus 1.82 $\pm$ 0.34 nmol/min per gram protein; $P<0.05$). Strikingly, atorvastatin also normalized eNOS activity in hypertensive DS rats independently of the dietary salt content (HS/AT, 1.52 $\pm$ 0.26 nmol/min per gram protein; HS/NS + AT, 1.56 $\pm$ 0.17 nmol/min per gram protein) (Figure 5). The return to an HS diet alone after 6 weeks of HS diet does not prevent the fall in eNOS activity (unpublished observations).

**Discussion**

In clinical trials, statins have been shown to reduce cardiovascular morbidity and mortality in patients with hypertension and normal or elevated cholesterol levels. 1-4 Our studies suggest that statins normalize endothelial function and prevent end-organ injury in salt-sensitive hypertension, at least in part by concomitantly maintaining vascular eNOS and inhibiting oxidative stress.
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and normalized the vascular response to ET-1. We surmise cardiovascular and renal injury. 26 However, whether endothelial, upregulation of Ang II, AT1 receptor, and ET-1, Indeed, in the presence of high O2

microenvironment where NO is synthesized and released.

production that is linked to a functional upregulation of Ang II and ET-1. 19,25,26 In addition, NO downregulates Ang II, AT1 receptor, and ET-1 synthesis. 25,26 NO is linked to a functional upregulation of Ang II and ET-1. 19,25,26 It has been demonstrated that vascular upregulation of NO synthesis is an adaptive response to the hemodynamic stress of increased blood pressure and that upregulation of eNOS contributes to a reduction in vascular tone and to the prevention of the pathological remodeling of the heart and vessels. 19 Mice lacking the eNOS gene manifest an exaggerated vascular hypertrophic response to increased hemodynamic stress. 27 Pharmacological inhibition of NOS results in hypertension, upregulation of Ang II, AT1 receptor, and ET-1, in addition to increased O2 production accompanied by cardiovascular and renal injury. 26 However, whether endothelial NO is a friend, as described above, or is in fact a foe is dependent on the concomitant production of O2·− in the microenvironment where NO is synthesized and released. Indeed, in the presence of high O2·−, the bioactivity of NO is not only diminished but also transformed into peroxynitrite, an oxidant molecule with proatherogenic effects. 28

Clinically, hypertensive salt-sensitive individuals are more prone than their nonsalt–sensitive counterparts to cardiovascular, renal, and endothelial injury. 29,30 DS rats are a paradigm of human salt-sensitive hypertension. We have shown that hypertensive DS rats on a high-salt diet, but not normotensive DS or Dahl salt-resistant rats given high- or normal-salt diet, manifest a decrease in NO bioactivity because of a decrease in eNOS and to inactivation of NO by increased O2·− production that is linked to a functional upregulation of Ang II. 15 Compared with similarly hypertensive rats of other strains, DS rats have more severe endothelial dysfunction, LVH, aortic hypertrophy, and renal injury. 12–14 In addition, DS rats have increased production of vascular ET-1 and peroxynitrite. Here we demonstrate that in hypertensive DS rats, atorvastatin maintained the levels of vascular eNOS and concomitantly reduced oxidant stress (O2·− and isoprostanates) and normalized the vascular response to ET-1. We surmise that these remarkable effects of the statin, which may also, at least in part, be due to downregulation of AT1 receptors, are responsible for the beneficial effects on endothelial function, LVH, aortic hypertrophy, and proteinuria observed in hypertensive DS rats. Moreover, these beneficial effects were independent of dietary salt intake as well as independent of a major reduction in blood pressure.

Recently, Rho kinases have been proposed to be part of the important signaling pathways involved in hypertensive LVH and nephropathy. 32–34 Rho kinases have also been shown to regulate eNOS expression 35 and synthesis as well as O2·− production. 34 Statins have been demonstrated to modify a variety of small GTPase protein synthesis, including Rho kinases, by inhibiting the synthesis of various isoprenoid intermediates. 35 Thus, the beneficial effects in our studies may also be related to inhibition of Rho kinase by atorvastatin.

Previous studies have shown that statins reduce blood pressure and have renal protective effects in DS rats, although the mechanisms involved have not been fully elucidated. 9 Several, but not all, human studies have demonstrated that statins contribute to lower blood pressure and proteinuria in patients with essential hypertension and primary hypercholesterolemia. 4,36,37 Consistent with those findings, the present study showed that atorvastatin significantly reduced but did not normalize blood pressure and proteinuria.

Normalization of blood pressure and proteinuria required a reduction in dietary salt to normal. 12,38 Clinically and experimentally, this would be equivalent to an addition of a diuretic. Indeed, high dietary salt has been shown to antagonize the antihypertensive and renal protective effects of angiotensin-converting enzyme inhibitors and vasopeptidase inhibitors in salt-loaded, NO-deficient rats. 39 Furthermore, clinically and experimentally, diuretics prevent renal injury and potentiate the renoprotective effects of angiotensin-converting enzyme inhibitors and AT1-receptor blockers. 32,38 Extrapolation between experimental animal studies and human studies is always speculative. Nonetheless, our studies provide evidence to support the notion that the pleiotropic effects of statins may be clinically important for arresting hypertensive cardiovascular and renal injury, particularly in high-risk patients such as those that are salt-sensitive and those with hypercholesterolemia. 4

Perspectives

We have shown that in salt-sensitive hypertension, decreased NO is linked to a functional upregulation of Ang II and increased oxidative stress. 15 The present study revealed that statins lower blood pressure, protect against hypertensive end-organ injury, and modulate the activity of at least 4 vasoactive substances generated by the endothelium, namely vascular eNOS, O2·−, ET-1, and, most likely, Ang II. Thus statins, beyond their lipid-lowering effects, strategically restore the balance among these molecules in the vasculature, thereby mitigating proatherogenic and pathological vascular remodeling responses. 5,31 Clinically and experimentally, salt-sensitive hypertension predisposes to the development of LVH, vascular dysfunction, and renal injury. 12,15,29 These studies suggest that statins may expand our armamentarium for the prevention of end-organ injury in high-risk hypertensive individuals.

Figure 5. eNOS activity in the aortas of atorvastatin-treated DS rats. High-salt diet significantly decreased aortic eNOS activity. Atorvastatin in the presence of high-salt diet or combination of atorvastatin with removal of high-salt diet prevented the fall in aortic eNOS activity. *P<0.05 vs HS.
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