Simvastatin Prevents Angiotensin II–Induced Cardiac Alteration and Oxidative Stress

Sandrine Delbosc, Jean-Paul Cristol, Bernard Descomps, Albert Mimran, Bernard Jover

Abstract—The influence of the HMG-CoA reductase inhibitor simvastatin was assessed on the cardiovascular alterations and production of free radicals associated with chronic angiotensin II (Ang II) infusion. Simvastatin (60 mg/kg per day PO) or placebo were given concomitantly for 10 days in Sprague-Dawley rats infused with Ang II (200 ng/kg per minute SC, osmotic pump). In addition, simvastatin or placebo was also given in vehicle-infused rats. Tail-cuff pressure and albuminuria were measured before and at the end of the treatment period. Cardiac weight, carotid structure, production of reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide, and hydroxyl radicals. Together, these events may lead to phenotype transformation of the arterial wall and vascular hypertrophy.

Experimental evidence and clinical studies strongly suggest that 3-hydroxy-3-methylglutaryl-CoA (HMG CoA) reductase inhibitors (statins) might have antiatherosclerotic effects independent of low-density lipoprotein (LDL) cholesterol reduction. Among the pleiotropic effects of statins are inhibition of smooth muscle cell proliferation (except for pravastatin), reduction of matrix metalloproteinase expression, and stimulation of the antithrombotic system. In addition, it has been shown that an inhibitory effect of statins on vascular superoxide generation is evoked because lovastatin increased circulating LDL resistance to oxidation in humans and decreased the vascular content of LDL lipid peroxides and conjugated dienes in cholesterol-fed rabbits. Alternatively, simvastatin increased human paraoxonase activity, a protective enzyme against LDL oxidation. In addition, various statins (atorvastatin, cerivastatin, and pravastatin) inhibited the NADPH oxidase–dependent superoxide anion formation by endothelium-intact segments of aorta obtained from normal rats.

In this study, we assessed the influence of long-term administration of simvastatin on the Ang II–stimulated generation of ROS by polymorphonuclear leukocytes (PMNL) and aortic tissue and the development of hypertension and cardiovascular hypertrophy associated with a 10-day infusion of Ang II in rats.

Methods

Animals and Protocol
Male Sprague-Dawley rats (Ifra-Credo, L’Arbresle, France) weighing 310 to 400 g were placed in metabolic cages and maintained on...
a regular rat chow with free access to tap water at least for 1 week before studies. After a 3-day baseline period, rats were randomly assigned to 4 experimental groups of 8 rats. Ang II or its vehicle (distilled water) was infused alone (Ang II and control groups, respectively) or in association with simvastatin (Ang II–Simva and Simva groups, respectively). Ang II (Sigma Chemical Co) was infused subcutaneously by osmotic pumps (model 2002, Alza Corporation) at a dose of 200 ng/kg per minute for 10 days. Simvastatin (a generous gift from Merck, Sharpe and Dohme Laboratories, Rahway, NJ) was administered orally once daily (between 8 and 10 AM) at a dose of 60 mg/kg (in 1 mL/kg of a suspension of gum arabic) 24 hours before and during the 10-day period of Ang II infusion. The dose of simvastatin used in the current study was derived from preliminary experiments in which the production of hydrogen peroxide by PMNL and aorta and plasma concentrations of creatine phosphate kinase (CPK) were measured in Ang II–infused rats receiving simvastatin at doses ranging from 1 to 120 mg/kg (n=3 for each dose).

Metabolic Parameters and Cardiovascular Morphology

Body weight, food and water consumption, and urinary volume and sodium and potassium were measured daily. Plasma concentration and urinary excretion of creatinine (colorimetric method) were measured at the end of experiments for estimation of creatinine clearance as an index of renal function. Tail-cuff pressure (TCP, Narco Biosystems) and urinary excretion of albumin (immunonephelometry) were determined before and at the end of the treatment period.

At the end of the treatment period, rats were anesthetized with sodium pentobarbital (60 mg/kg body wt IP) and a catheter was implanted in the right carotid artery, allowing the measurement of arterial pressure. Blood (15 mL) was then collected on heparin and EDTA–coated tubes, and the thoracic aorta was immediately removed, cleaned of adherent fat, washed in an ice-cold bicarbonate buffer, and kept at 4°C until measurement of hydrogen peroxide production. Plasma and buffy coat were obtained by low-speed centrifugation. Buffy coat was immediately used to obtain PMNL, and plasma samples were stored at −20°C until analysis. The heart was removed and weighed for the calculation of heart-to-body weight ratio. The carotid artery was fixed in formalin (10%) at a constant pressure of 120 mm Hg, and cross-sectional area was measured on hematoxylin-colored slices (20-μm thickness). All procedures were designed in accordance with French law and institutional guidelines for the care and use of laboratory animals.

Biochemical Analysis, Lipoprotein Isolation, and Oxidation

Cholesterol, triglycerides, high-density lipoprotein cholesterol, and phospholipids were measured by an enzymatic method. Plasma concentration of thiobarbituric acid reactive substances (TBARS, mol/L), taken as an index of lipid peroxidation, was estimated by fluorimetry.13 Plasma concentration of advanced oxidation protein products (AOPP), considered an index of protein oxidation, was measured by spectrophotometry (340 nm) with chloramine T and expressed as micromoles per liter of chloramine T equivalents.14,15

Estimation of Generation of ROS

PMNL were isolated by centrifugation of the buffy coat on a Percoll gradient (1.07 g/mL) and resuspended in RPMI medium to obtain a final concentration of 500,000 cells/mL. An aliquot of 500 μL of the suspension was incubated for 30 minutes with 100 μmol/L of lucigenin, a dose that does not interfere with measurements (data not shown). Superoxide anion production by PMNL was measured after addition of phorbol 12-myristate 13-acetate (PMA 10−7 mol/L) as previously described.16–18 Hydrogen peroxide (H2O2) production was determined on PMNL and aorta segments by use of a specific bioluminescence probe consisting of a mixture of luminol and horse radish peroxidase (HRP). The probe contained 100 μmol/L of luminol and 0.05 g/L of HRP for PMNL or 200 μmol/L of luminol and 0.098 g/L of HRP for aorta segments. After a 30-minute period of incubation with the bioluminescence probes, PMA (10−6 and 10−7 mol/L in PMNL and aortic tissue, respectively) was added and the chemiluminescence was measured in a luminometer (Wallac LKB 1251).

Statistical Methods

Results are expressed as mean±SEM and analyzed by 1- or 2-factor ANOVA for repeated measures as appropriate. Between-group comparisons were made with the Bonferroni test and within-group differences were determined with the Student’s t test for paired values with a significance level set for P<0.05.

Results

Dose Response to Simvastatin

The production of H2O2 by PMNL and aorta segments was similar in untreated Ang II–infused rats and rats treated with low doses (1 and 3 mg/kg), and it was maximally reduced by doses of 60 and 120 mg/kg of simvastatin. Plasma CPK was similar in untreated rats and rats receiving 60 mg/kg of simvastatin (143±4 versus 141±4 IU/L). Unfortunately,
CPK markedly increased (347±36 UI/L) in rats treated with the dose of 120 mg/kg per day of simvastatin (Figure 1).

Cardiovascular Parameters
As depicted in Figure 2, simvastatin had no effect on TCP in control rats. The increase in TCP induced by Ang II (58±8 mm Hg at day 10) was blunted by 67% in rats receiving Ang II and simvastatin (19±5 mm Hg).

As shown in Figure 3, the development of cardiac hypertrophy and carotid remodeling were prevented by simvastatin.

Metabolic and Plasma Lipid Parameters
As summarized in Table 1, no effect on food intake and growth of rats was found. The dipsogenic effect of Ang II was not influenced by simvastatin. Interestingly, urinary sodium excretion was similar in Ang II and Ang II+simvastatin groups, thus suggesting that sodium balance was not affected by simvastatin.

At the end of the study, serum creatinine as well as calculated creatinine clearance were similar in each group. The increase in urinary albumin excretion associated with Ang II infusion was markedly blunted (by 78%) but not prevented by simvastatin.

In addition, the dose of simvastatin used in this study had no detectable effect on serum cholesterol levels (Table 2).

Production of ROS and Markers of Oxidative Stress
In PMA-stimulated PMNL, simvastatin prevented the 50% increase of superoxide anion and the 3-fold increase in hydrogen peroxide production associated with Ang II infusion (Figure 4). As depicted in Figure 5, the 50% increase in hydrogen peroxide production by aorta segments induced by Ang II was also prevented by simvastatin. Simvastatin alone had no effect on the production of reactive oxygen species by leukocytes and aorta segments.

As shown in Table 2, plasma concentrations of TBARS and AOPP were similar in control and simvastatin-treated rats. Ang II was associated with an increase in both markers of oxidation, and simvastatin treatment prevented the effect of Ang II.

Discussion
This study demonstrated that simvastatin prevented the development of hypertension, cardiac and vascular hypertrophy, and proteinuria associated with long-term infusion of Ang II. In parallel, simvastatin prevented the Ang II−induced increase in ROS production by PMNL as well as aortic segments; concomitantly, circulating markers of lipid (TBARS) and protein (AOPP) peroxidation were decreased. The dose-response studies of simvastatin indicated that the maximal effect on the production of ROS was achieved at the dose of 60 mg/kg. This dose of simvastatin did not affect the growth or the food and water intake of animals and was devoid of muscular effect, as indicated by normal levels of serum CPK.

Table 1. Influence of Angiotensin II and Simvastatin Metabolic Parameters

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>Simvastatin</th>
<th>Ang II</th>
<th>Ang II + Simvastatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight, g</td>
<td>319±10</td>
<td>309±13</td>
<td>317±22</td>
<td>307±13</td>
</tr>
<tr>
<td>Body weight gain, g/10 days</td>
<td>47±15</td>
<td>45±3</td>
<td>37±13</td>
<td>39±8</td>
</tr>
<tr>
<td>Cumulative values (g per 10 days)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food intake, g</td>
<td>239±9</td>
<td>233±7</td>
<td>237±10</td>
<td>229±6</td>
</tr>
<tr>
<td>Water intake, mL</td>
<td>294±21</td>
<td>315±18</td>
<td>371±11*</td>
<td>397±27*</td>
</tr>
<tr>
<td>Urine volume, mL</td>
<td>102±20</td>
<td>131±13</td>
<td>165±12*</td>
<td>190±17*</td>
</tr>
<tr>
<td>Urinary sodium excretion, mol</td>
<td>29.0±1.0</td>
<td>26.5±0.4</td>
<td>28.4±0.9</td>
<td>26.2±0.7</td>
</tr>
<tr>
<td>Urinary potassium excretion, mol</td>
<td>47.2±2.0</td>
<td>41.8±1.1</td>
<td>44.9±2.8</td>
<td>40.8±1.6</td>
</tr>
<tr>
<td>Serum creatinine, μmol/L</td>
<td>26.6±2.0</td>
<td>26.0±1.1</td>
<td>24.8±2.2</td>
<td>24.1±1.0</td>
</tr>
<tr>
<td>Creatinine clearance, μL/min/g KW</td>
<td>678±60</td>
<td>648±58</td>
<td>814±128</td>
<td>733±62</td>
</tr>
<tr>
<td>Final albuminuria, μg/24 h</td>
<td>176±20</td>
<td>163±28</td>
<td>986±177*</td>
<td>352±44†</td>
</tr>
</tbody>
</table>

Values are mean±SE. KW indicates kidney weight.
*P<0.05 vs control group; †P<0.05 vs Ang II group.
In this study, simvastatin markedly prevented the development of hypertension and target organ damage independent of a cholesterol-lowering effect. In young (4-week-old) spontaneously hypertensive rats (SHR), treatment with lovastatin (10 mg/kg per day for 4 weeks) attenuated the development of hypertension, as did hydralazine treatment, but remodeling of intrarenal arterioles was corrected only by lovastatin. Cerivastatin (0.5 mg/kg per day from weeks 4 to 7) reduced the progressive rise in arterial pressure observed in 4-week-old rats transgenic for human renin and angiotensinogen (dTGR), a model of Ang II–dependent and end-organ damage.

Among the mechanisms that may participate in the blood pressure–lowering effect of statins is a reduction of extracellular fluid volume. In the young SHR, lovastatin attenuated hypertension and shifted the pressure-natriuretic curve to a lower perfusion pressure. In the current study, the 10-day cumulative values of water and sodium excretion were similar in simvastatin-treated and untreated Ang II–infused rats. Although we cannot exclude an effect of simvastatin on renal function, the current findings do not favor a major involvement of extracellular fluid volume reduction in the effect of simvastatin. It is therefore likely that simvastatin prevented the rise in arterial pressure through the blunting of the pressor effect of the octapeptide.

The current observation that simvastatin obliterated the increase in superoxide anion and hydrogen peroxide production by polymorphonuclear leukocytes as well as hydrogen peroxide formation by aortic segments indicated that both the circulating and tissue production of reactive oxygen species were reduced to levels found in control animals. The mechanism by which simvastatin produces its beneficial effects beyond the single action of blocking synthesis of cholesterol was not investigated in the current experiments. A direct inhibition of NADPH oxidase could account for these effects. NADPH oxidase activation required p21 Rac translocation, which is dependent on prenylation. Prevention of superoxide production in endothelial cells, smooth muscle cells, and in phagocytic cells by statins could be linked to prenylation-dependent rac translocation. Moreover, addition of mevalonic acid and isoprenoid compounds (geranyl geraniol) restore ROS production.

In addition, the Ang II–induced increase of markers of protein and lipid oxidation (AOPP and TBARS, respectively) was prevented by simvastatin. AOPP levels are correlated with a known protein oxidation marker, di-tyrosine in humans. An increase in lipid peroxidation, equated with TBARS, was detected in the current experiments as well as in a previous study conducted in rats infused for 14 days with a subpressor dose of Ang II.

A causal association of reduction of oxidative stress and antihypertensive effects of simvastatin cannot be unequivocally evoked from the current experiments. Several reports

### Table 2. Influence of Angiotensin II and Simvastatin on Plasma Lipids

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>Simvastatin</th>
<th>Ang II</th>
<th>Ang II + Simvastatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum cholesterol, mmol/L</td>
<td>1.41±0.06</td>
<td>1.61±0.03</td>
<td>1.49±0.11</td>
<td>1.52±0.07</td>
</tr>
<tr>
<td>Serum HDL-cholesterol, mmol/L</td>
<td>0.63±0.12</td>
<td>0.55±0.08</td>
<td>0.73±0.12</td>
<td>0.80±0.08</td>
</tr>
<tr>
<td>Serum triglycerides, mmol/L</td>
<td>0.93±0.06</td>
<td>0.95±0.04</td>
<td>0.96±0.04</td>
<td>0.96±0.05</td>
</tr>
<tr>
<td>Serum phospholipids, mmol/L</td>
<td>1.33±0.06</td>
<td>1.35±0.05</td>
<td>1.4±0.11</td>
<td>1.54±0.06</td>
</tr>
</tbody>
</table>

Values are mean±SE. TBARS indicates thiobarbituric acid reactive substances; AOPP, advanced oxidation protein products.

\( P<0.05 \) vs control group; \( P<0.05 \) vs Ang II group.

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**Figure 4.** Production of superoxide anion (lucigenin) and hydrogen peroxide (luminol-HRP) by PMNL at end of treatment period. \( *P<0.05 \) compared with control normotensive rats.

**Figure 5.** Production of hydrogen peroxide by aorta at end of treatment period. \( *P<0.05 \) compared with control normotensive rats.
suggest that oxidative stress contributes to the rise in arterial pressure in various experimental models. Administration of a glutathione synthase inhibitor resulting in an impairment of antioxidant defense mechanisms was associated with severe hypertension in rats.23 Conversely, it was recently demonstrated that antioxidants such as vitamin E, superoxide dismutase, or its mimetic, tempol, inhibited the rise in arterial pressure associated with short-term24 and long-term infusion of pressor doses of Ang II.1,25 Therefore, maneuvers reducing overproduction of ROS, as did simvastatin in the current study, may have a beneficial influence in the prevention of Ang II–dependent hypertension. Ang II is a potent activator of ROS production by vascular and renal cells both in vitro26–28 and in vivo in chronically infused rats1,29 and through stimulation of type 1 Ang II receptors.26,29 Stimulation of ROS production by Ang II resulted from activation of NADH/NADPH oxidase,26,29 as demonstrated by blockade of this effect by diphenylene iodonium30 or the use of knockout mice deficient in a NAD(P)H oxidase subunit protein.31

In addition to the reduction of oxidant stress, simvastatin may have altered other mechanisms involved in the pressor effect of Ang II. Simvastatin and atorvastatin were shown to reduce the density of AT-1 receptors of platelets isolated from hypercholesterolemic patients.32 Atorvastatin was also reported to reduce AT-1 receptor expression in isolated vascular smooth muscle cells.12,33 Although a downregulation of AT-1 receptors by simvastatin remains to be demonstrated in the current model, such an effect may partly explain the prevention of Ang II hypertension. Another mechanism that may participate in the antihypertensive effect of simvastatin is the inhibition of endothelin. Blockade of type A and B endothelin receptors alleviated the development of hypertension induced by Ang II.34 and simvastatin was shown to inhibit endothelin mRNA expression and reduced immunoreactive endothelin-1 levels in bovine aortic endothelial cells.35 Similarly, the concomitant administration of tempol, a superoxide dismutase mimetic, prevented the rise in plasma endothelin-1 levels and arterial pressure associated with a 15-day infusion of a low dose (5 ng/kg per minute IV) of Ang II.25 Blunting by simvastatin of the pressor effect of Ang II may also result from attenuation of nitric oxide (NO) inhibition and/or NO degradation by ROS. Uptregulation of e–NO synthase expression by simvastatin or lovastatin in saphenous vein endothelial cells in the presence of oxidized LDL36 as well as increase in i–NO synthase mRNA and protein in rat vascular smooth muscle cells37 was demonstrated. Wagner et al11 reported that several HMG-CoA reductase inhibitors improved NO-dependent vasodilation of rat aorta segments through attenuation of endothelial superoxide anion formation in vitro. In addition to its blood pressure–lowering effect, simvastatin prevented the increase in cardiac mass and cross-sectional area of the carotid artery associated with Ang II infusion. The prevention of the cardiovascular hypertrophy by the statin may be related to the absence of rise of blood pressure. However, the trophic effect of Ang II may be independent of high pressure, as previously shown in rats infused with nonpressor doses of the octapeptide or in rats concomitantly treated by hydralazine.38 In SHR, it was shown that lovastatin but not hydralazine reduced renal vascular hypertrophy19 and normalized cross-sectional area of cerebral arterioles despite only slight reduction in arterial pressure.39 These findings support the idea that reduction of blood pressure was not the only factor involved in the antihypertrophic effect of statins. Besides its beneficial effect on the cardiovascular system, statin treatment ameliorated but did not eliminate the Ang II–induced rise in albuminuria. Similar findings were reported in rats transgenic for human renin and angiotensinogen chronically treated with cerivastatin.20 The decrease in blood pressure may have contributed to the renoprotective effect of the statin, and the residual rise was probably related to the slight and persistent increase in systemic pressure.

Taken together, these findings indicate that HMG-CoA reductase inhibitors almost totally prevented the development of hypertension and target organ alterations associated with long-term Ang II infusion in rats. It is suggested that the reduction by statins of the overproduction of free radicals and oxidation products may participate in their beneficial influence in this model. Whether HMG CoA reductase inhibition may be efficient during the established phase of Ang II hypertension remains to be demonstrated.

Perspectives
The clinical relevance of the current findings obtained with a high dose of simvastatin (60 mg/kg per day) deserves to be assessed. In patients with essential hypertension and moderate hypercholesterolemia, pravastatin (20 to 40 mg per day) treatment resulted in a slight but significant decrease in blood pressure.40 Similarly, in hypertensive type II diabetic patients, simvastatin (20 mg per day, 10 months) treatment significantly reduces diastolic blood pressure.41 In elderly diabetic patients with or without mild hypercholesterolemia, a 3-day period of treatment with cerivastatin (0.15 mg per day) resulted in significant improvement of endothelial function and reduction in the plasma concentration of 8-isoprostanate, a marker of oxidant stress despite a lack of change in lipid parameters within this short period of treatment.42 In addition, high doses of statins could be required for pleiotropic effects in acute administration by contrast with long-term treatment. Inhibition by statins of NADPH oxidase may act synergistically with others antioxidant properties including inhibition of LDL oxidation and paraoxonase expression. Beyond LDL oxidation, superoxide anion production may be involved in the regulation of the inflammatory process and vascular remodeling, since oxidative stress increases cytokine production by myocytes43,44 and vascular smooth muscle cell proliferation and impaired endothelial function.45

References


17. Delbosco et al Statin in Angiotensin II Hypertension


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