RESEARCH PAPER

Chronic treatment with pravastatin prevents early cardiovascular changes in spontaneously hypertensive rats

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Background and purpose: This study investigates the effect of pravastatin on blood pressure, cardiovascular remodelling and impaired endothelial function induced as early signs of cardiovascular disease in young spontaneously hypertensive rats (SHR).

Experimental approach: Eight-week-old SHR were treated for 4 weeks with pravastatin (20 mg·kg⁻¹·day⁻¹). Systolic blood pressure was measured periodically during the study using the tail-cuff method. At the end of the study, the left ventricular weight /body weight ratio was used as an index of left ventricular hypertrophy (LVH). Vascular function, superoxide (O₂⁻) production and structure were studied in aortic rings. Lipid peroxidation was measured in plasma (thiobarbituric acid reactive substances assay).

Key results: Systolic blood pressure was lower in treated SHR than in control SHR, at the end of the study (171 ± 2 mmHg, P < 0.05), and LVH was significantly reduced by pravastatin (2.7 ± 0.02 vs. 2.5 ± 0.01 mg·g⁻¹, P < 0.05). Vascular responses to sodium nitroprusside and phenylephrine were similar in both groups; nevertheless, the relaxation response to acetylcholine was higher in the treated rats (45.6 ± 2.6 vs. 58.1 ± 3.2 %, P < 0.05). Vascular O₂⁻ and plasma thiobarbituric acid reactive substances were reduced by pravastatin treatment, and urinary nitrites was elevated. Finally aortic wall became thinner after pravastatin treatment.

Conclusions and implications: Chronic treatment with pravastatin attenuated the increase of systolic blood pressure in SHR, prevented early LVH and improved vascular structure and function. These effects were accompanied by decreased measures of oxidative stress and improvements in NO production.

Keywords: pravastatin; hypertension; ventricular hypertrophy; endothelial dysfunction; oxidative stress

Abbreviations: LVH, left ventricular hypertrophy; SHR, spontaneously hypertensive rats; SBP, systolic blood pressure; TBARS, thiobarbituric acid reactive substances

Introduction

There is accumulating evidence that the statins [3-hydroxy-3methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors] exert numerous beneficial effects that are apparently independent of their action on blood lipids; these include effects on the cardiovascular system, kidneys, bones and glucose metabolism (McFarlane et al., 2002).

A number of clinical trials have shown that statins significantly reduce cardiovascular morbidity and mortality. Potential mechanisms that may mediate beneficial cardiovascular action of statins include modulation of endothelium function (Laufs et al., 1998; Alvarez de Sotomayor et al., 2000; Mital et al., 2000; Mehta et al., 2001), anti-inflammatory action (Egashira et al., 2000), antioxidant properties (Yamamoto et al., 1998; Zhou et al., 2008), plaque stabilization (Crisby et al., 2001), effects on thrombosis (Rosenson and Tangney, 1998) and vasculogenesis (Vasa et al., 2001). Moreover, although statins share a common lipid-lowering effect, there are differences within this class of drugs not only in their lipid-lowering potential but also in their non-lipid effects (Chong et al., 2001). These potential differences necessitate careful and systematic studies involving each member of the statin family.

Direct effects of statins on vascular cells could have important implications for the development of endothelial dysfunction (Wassmann et al., 2001). Disruption of the delicate balance of the NO system and especially the vascular production of reactive species (ROS) promote the development of...
endothelial dysfunction. In this context, it has been reported that, for example, the NO and endothelium system may be directly influenced by statins (Hernandez-Perera et al., 1998; Laufs et al., 1998).

In the present study we examined cardiovascular effects of pravastatin, an HMG-CoA reductase inhibitor. Experiments were developed to investigate the effects of this statin on blood pressure, endothelial dysfunction and vascular ROS in conductance arteries of young spontaneously hypertensive rats (SHR) at an early stage of cardiovascular disease. As plasma lipid levels are normally low in rats and statins usually do not modify lipid profile in rats, they provide an excellent model for studying the other cardiovascular effects of statins.

Methods

All procedures were carried out in accordance with conventional guidelines for experimentation with animals. Eight-week-old male SHR rats were used (Janvier, France). They were housed four per cage in a regulated environment with a 12 h light/dark cycle in a standard experimental laboratory of the Animal Experimentation Service of the Salamanca University. The animals had free access to food and water.

Animals were randomized to receive during 4 weeks: (i) tap water (control), and (ii) pravastatin 20 mg·kg⁻¹·day⁻¹ (P-20) dissolved in drinking water. The concentration was adjusted for the daily water intake and body weight.

Systolic blood pressure (SBP) was measured in awake rats with an automated multi-channel system, using the tail-cuff method with a photoelectric sensor (Niprem 546, Cibertec SA, Madrid, Spain) as previously described (Sevilla et al., 2004). SBP was measured before starting the study and every week during the treatment. After 4 weeks animals were placed in metabolic cages, and urine samples were collected to determine plasma concentrations of total cholesterol and the levels of lipid peroxidation.

Rats were anaesthetized (pentobarbital, 60 mg·kg⁻¹, i.p.), exsanguinated, and tissues were removed immediately. The heart and the thoracic aorta were removed from the animal and placed in chilled Krebs solution of the following composition (in mM): NaCl 118, KCl 4.7, CaCl₂ 2.5, KH₂PO₄ 1.2, and placed in chilled Krebs solution of the following composition (in mM): NaCl 118, KCl 4.7, CaCl₂ 2.5, KH₂PO₄ 1.2, and glucose 11, pH 7.4.

Histomorphology of aorta

Samples were processed as previously described (Guerrero et al., 2006). In brief, aortic samples were fixed, dehydrated and embedded in paraffin. Sections (4 µm thick) were stained with haematoxylin–eosin and examined under light microscopy with an attached video camera. Using an image software (Image J, http://rsb.info.nih.gov/ij), the internal and external perimeters of the medial layer were measured and converted into internal and external radii (Rᵢ and Rₑ respectively) according to the formula: perimeter = 2π(Rᵢ + Rₑ), where 2πRᵢ is the internal diameter (L) and Rₑ – Rᵢ is the wall thickness (W).

Medial cross-sectional area (CSAᵦᵣ) was calculated as: CSAᵦᵣ = πRₑ² – πRᵢ².

Detection of superoxide anion

Production of superoxide anion (O₂⁻) was assessed by lucigenin-enhanced chemiluminescence assay. Briefly, segments of thoracic aorta were incubated in HEPES-buffer (in mM: NaCl 119, HEPES 20, MgSO₄ 1, KCl 4.6, KH₂PO₄ 0.4, Na₂HPO₄ 0.15, NaHCO₃ 5, CaCl₂ 1.2, glucose 5.5, pH 7.4) gassed with carbogen and maintained at 37°C for 30 min. Then samples were transferred into tubes containing 1 ml of HEPES-buffer with lucigenin (5 µM). Lucigenin chemiluminescence was then recorded every 30 s for 5 min in a luminometer (LUMAT LB-9507, Berthold Technologies, Bad Wildbad, Germany). Basal and reduced nicotinamide adenine dinucleotide phosphate (NADPH, 100 µM)-stimulated production were measured and expressed as relative luminescence units (RLU)-min⁻¹·mg⁻¹ of dry tissue.

Nitrite measurements

Nitrite concentration was determined in urine by a modification of the Griess reaction as described previously (Valdivielso et al., 2001). Briefly, 500 µL of sample were mixed with 250 µL of Griess reagent (1% sulphanilamide, and 0.1% naphthyl ethylenediamine dihydrochloride, in 2.5% orthophosphoric acid) and incubated for 15 min at room temperature. Absorbance was measured at 560 nm. Calibration was carried out using sodium nitrite.

Cholesterol measurements

The total cholesterol was measured in plasma samples by using a cholesterol assay kit (Biosystems S.A., Barcelona, Spain) following the manufacturer’s indications. The enzymatic procedure involves cleavage of the cholesterol esters by cholesterol esterase and oxidation of the free cholesterol by 1 mM, and the presence of functional endothelium was assessed by the ability of acetylcholine (ACh, 1 µM) to induce relaxation. After a washout period, cumulative concentration response curves of phenylephrine (0.01–100 µM) were obtained. After precontraction with phenylephrine (1 µM) and a steady maximal contraction, cumulative concentration response curves were obtained for ACh (0.01–10 µM) or sodium nitroprusside (SNP, 0.01–10 µM). Each curve was obtained in a different ring.
cholesterol oxidase. These reactions may be quantified photometrically by use of hydrogen peroxide-dependent colour-forming reactions.

**Left ventricular hypertrophy**

The heart was removed and placed immediately in Krebs solution at 37°C to remove excess blood. The atrium was removed, and all the epicardial fat was scraped off. The right and the left ventricle were separated, regarding the interventricular septum as an integral part of the left ventricle, and this portion was weighed. Left ventricular hypertrophy index (LVH) was calculated by using left ventricular weight/body weight ratio.

**Lipid peroxidation**

Plasma levels of thiobarbituric acid reactive substances (TBARS) were determined as an index of lipid peroxidation, following the method described by Ohkawa et al. (1979) and modified by us. An aliquot of 250 µL of plasma was added to 1 mL of a reaction mixture containing thiobarbituric acid (37%), trichloroacetic acid (15%) and hydrochloric acid (11.32 N), then samples were warmed at 90°C for 30 min and centrifuged. The absorbance of the supernatants was measured at 535 nm. Data are expressed as concentration of TBARS (µM).

**Data analysis**

Data are expressed as mean ± SEM. The responses to ACh and SNP are expressed as percentages of phenylephrine contraction. EC50 values indicate the concentration of each agonist producing 50% of the maximal response and were calculated by using the GraphPad Prism 4.0 computer programme (GraphPad, San Diego, CA, USA) and expressed as negative log molar concentration (pD2). Statistical calculations for significant differences were performed by using Student’s t-test or two-way ANOVA as appropriate. Significance was accepted at P < 0.05.

**Materials**

The drugs used were: phenylephrine hydrochloride, ACh chloride, SNP, N,N-dimethyl-9,9-biacridinium nitrate (lucigenin) and NADPH tetrasodium salt, haematoxylin and eosin, all purchased from Sigma Chemical Co., USA. Pravastatin was generously supplied by Menarini S.A. (Firenze, Italy).

Stock solution of drugs were made up in ultra-pure water and stored at −20°C, and appropriate dilutions were made on the day of the experiment. Drug and molecular target nomenclature conforms to the British Journal of Pharmacology Guide to Receptors and Channels (Alexander et al., 2008).

**Results**

**Systolic blood pressure**

Before treatment, SBP was similar and pathologically elevated in both groups. Throughout the study we saw a gradual increase only in control group. Therefore a significant reduction in blood pressure was observed in the P-20 group as shown in Figure 1.

Body weights were similar in both groups at the end of the study. Pravastatin reduced significantly the left ventricular weight, and this lead to an improvement of the LVH index. Plasma total cholesterol levels were not altered by the treatment (Table 1).

**Thoracic aorta rings**

The vasorelaxation to increasing concentrations of ACh and SNP is shown in Figure 2A and B. Whereas the endothelial-independent response to SNP was not altered by the treatment with pravastatin, this statin markedly increased the ACh-induced vasodilatation. The maximum response (Emax) to ACh in the untreated (control) group was less than 50% (Table 2), which suggested the presence of endothelial dysfunction that pravastatin was able to improve.

The contractor response to phenylephrine of the aortas from control and treated rats was similar in both groups (Figure 2C). Emax and pD2 values are shown in Table 2. Responses to KCl (80 mM) were not altered by pravastatin treatment (data not shown).

**Histomorphology of aorta**

Pravastatin induced a marked reduction in wall thickness and cross-sectional area with a significantly increased lumen.
led to higher values in the wall/lumen (W/L) ratio in untreated SHR, compared with pravastatin-treated rats. (Table 3)

Detection of superoxide anion

The treatment with pravastatin in absence of NADPH caused a slight reduction of superoxide production although the difference between groups did not reach statistical differences (control, 49.4 ± 4.7 and P-20, 39.5 ± 4.3 RLU·min⁻¹·mg⁻¹, P = 0.07). When experiments were carried out in presence of NADPH, we observed a significant reduction in luminescence values in aortic rings from the pravastatin-treated group (Figure 3).

Nitrite measurements

The nitrite concentrations of treated and untreated SHR measured in urine are shown in Figure 4. Pravastatin increased significantly the levels of nitrite found in urine.

Lipid peroxidation

Plasma TBARS levels taken as an index of lipid peroxidation were lower in treated SHR than control SHR at 12 weeks of age (Figure 5).

Discussion

Our findings indicate that 20 mg·kg⁻¹·day⁻¹ of pravastatin attenuated the increase of SBP in SHR, reduced the production of ROS and improved endothelium dysfunction and wall remodelling in aorta. In addition, this treatment prevented cardiac hypertrophy, reduced circulating markers of lipid peroxidation (TBARS) and up-regulated eNOS production. Moreover these beneficial cardiovascular effects exerted by pravastatin were independent of its action on cholesterol levels.

The current observation that the chemiluminescence is smaller in resting as well as stimulated rings from treated rats indicated that tissue production of superoxide anions by NADPH oxidase were reduced by pravastatin. Several studies indicate that the prevention of superoxide production in endothelial (Wagner et al., 2000) and smooth muscle cells (Wassmann et al., 2002) by statins could be linked to Rac

Table 2  Parameters of concentration–response curves in aorta rings

<table>
<thead>
<tr>
<th></th>
<th>ACh</th>
<th>SNP</th>
<th>PE</th>
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<tr>
<td></td>
<td>pD₂</td>
<td>Eₘₐₓ (%)</td>
<td>pD₂</td>
</tr>
<tr>
<td>Control (n = 7–10)</td>
<td>7.56 ± 0.17</td>
<td>45.56 ± 2.66</td>
<td>7.33 ± 0.13</td>
</tr>
<tr>
<td>P-20 (n = 9–12)</td>
<td>7.43 ± 0.13</td>
<td>58.11 ± 3.27*</td>
<td>7.38 ± 0.12</td>
</tr>
</tbody>
</table>

Eₘₐₓ to acetylcholine (ACh) and sodium nitroprusside (SNP) are expressed as percentage relaxation of contractions evoked by phenylphrine (PE, 10⁻⁶ M), and Eₘₐₓ to PE is expressed as milligrams; pD₂ values are calculated as the negative log molar of the agonist inducing a half-maximal response.

Control, untreated spontaneously hypertensive rats; P-20, treated spontaneously hypertensive rats.

Values are means ± SEM. *P < 0.05 versus control.
Control (untreated spontaneously hypertensive rats; P-20, treated spontaneously hypertensive rats.

Values are means ± SEM. ***P < 0.001 versus control.

translocation that is required for NADPH oxidase activation. On the other hand, chronic treatment with antioxidants drugs down-regulates the expression of this enzyme (Oelze et al., 2006; Sánchez et al., 2006) and could be another possibility to explain our results as pravastatin has antioxidant properties. Further studies would be necessary to decide between these mechanisms.

While the response to SNP in aorta was unaltered, we saw that the relaxation to ACh was improved in treated SHR, compared with the control group. These results are in accord with similar studies using atorvastatin, which reported up-regulation of vascular eNOS expression and enhancement of eNOS activity (Wassmann et al., 2001). The improvement in responses to ACh could be explained by this mechanism that may result in increased production of NO that contributes to the improvement of endothelium dysfunction. The increased level of nitrites in urine observed by us in rats treated with pravastatin supports this hypothesis. On the other hand, uncoupling of eNOS results in increased formation of oxygen radicals by NOS and reduced NO production in vitro (Werner et al., 1995; Reif et al., 1999). Shinozaki et al. (2007) report an inhibition of uncoupled eNOS-dependent O$_2^-$ production by pitavastatin in insulin-resistant rats and propose that enhancement of eNOS activity in vivo is one of the initial and essential steps for the observed beneficial effects of statin treatment. Moreover, O$_2^-$ leads to the formation of hydroxyl radicals, which may be cytotoxic to endothelial cells by direct peroxidation of either lipids or proteins (Freeman and Crapo, 1982). Animals treated with pravastatin showed levels of TBARS in plasma lower than untreated SHR, indicating a reduction of lipid peroxidation. We know that this method has been frequently criticized as too unspecific and prone to artefacts during sample workup, but it still can give us some estimate of the MDA levels when a treated group is compared with a control group under the same conditions.

The SHR strain is a model of chronic essential hypertension, and their blood pressure rises from 4 weeks old. At this time, minor differences in LVH are observed, with respect to Wistar Kyoto (WKY) rats but these are increased progressively until animals are 20 weeks old (Adams et al., 1989). Our study was performed when hypertrophy is being developed and comparing untreated SHR with pravastatin-treated SHR, we observed a significant reduction in LVH. In our opinion, the mechanisms by which pravastatin prevents cardiac hypertrophy is probably not only due to its antihypertensive effect, as that was minor. It has been reported that cardiac endothelin (ET-1) levels were significantly higher at 8 weeks of age in SHR compared with WKY rats (Iyer et al., 1995), and this could be important in triggering cardiac hypertrophy (Iemitsu et al.,

**Table 3** Parameters of thoracic aorta morphometry

<table>
<thead>
<tr>
<th></th>
<th>W (μm)</th>
<th>CSA$_m$ (mm$^2$)</th>
<th>L(μm)</th>
<th>W/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 6)</td>
<td>119.8 ± 2.4</td>
<td>0.56 ± 0.01</td>
<td>1419 ± 8</td>
<td>0.084 ± 0.001</td>
</tr>
<tr>
<td>P-20 (n = 6)</td>
<td>93.4 ± 1.5***</td>
<td>0.49 ± 0.08***</td>
<td>1553 ± 8***</td>
<td>0.060 ± 0.001***</td>
</tr>
</tbody>
</table>

CSA$_m$, cross-sectional area; L, lumen; W, wall thickness; W/L, wall thickness to lumen ratio.

Control, untreated spontaneously hypertensive rats; P-20, treated spontaneously hypertensive rats.

Values are means ± SEM. ***P < 0.001 versus control.
vascular disorders in pre-hypertensive state. They found that prevention of hyper trophy by pravastatin was associated with low ET-1 levels and, furthermore, such effects were specifically prevented by mevalonic acid. Statins also prevent the synthesis of isoprenoid intermediates of the cholesterol biosynthesis pathway, such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), which serve as important lipid attachments for the post-translational modification of several proteins (Takemoto and Liao, 2001). Because Rho is a major target of GGPP, inhibition of Rho and its downstream target, Rho kinase, is a likely mechanism to be mediating some of the pleiotropic effects of statins on cardiovascular disease (Laufs et al., 2000). Recently, in a murine model of angiotensin II-induced hypertension, pravastatin attenuated the increase of Rho kinase improving hypertrophy and fibrosis of the heart (Xu et al., 2008). Simvastatin also reverses cardiac hypertrophy caused by disruption of the bradykinin-2 receptor (Osorio et al., 2008).

Studies made by our group in SHR and WKY confirm that a hypertensive state induces structural modifications in conducting arteries. Hypertension leads to thickening of arterial wall and increased lumen (Guerrero et al., 2006). In this study pravastatin treatment led to significant reduction of wall thickness and cross-sectional area in aorta indicating a beneficial effect in remodelling that could contribute to its anti-hypertensive effect.

There is an imbalance between growth and apoptosis of vascular smooth muscle cells (VSMC) during vascular remodelling development that pravastatin could reverse. The mevalonate pathway plays a role in cell growth and, particularly, this pathway yields a series of isoprenoids that are vital for the post-translational isoprenylation of proteins like Ras, involved in growth and differentiation of VSMC (Yang et al., 2001), and Rho involved in apoptosis (Gujarro et al., 1999). Short-term use of fluvastatin in rabbits inhibited proliferation of VSMC in the media and migration to the intima (Ye et al., 2000). In addition, the elevated urinary excretion of nitrates and decreased production of superoxide that we observed after pravastatin treatment improved endothelial function and could contribute to prevent remodelling.

In conclusion, our results demonstrate that pravastatin, independent of its lipid-lowering properties, could be a useful therapeutic agent to prevent the development of cardiovascular disorders in pre-hypertensive state.

Acknowledgements

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Conflict of interest

The authors state no conflict of interest.

References


Nebivolol inhibits superoxide formation by NADPH oxidase and endothelial dysfunction in angiotensin II-treated rats. *Hypertension* **48**: 677–684.


