Pharmacological characterization of mechanisms involved in the vasorelaxation produced by rosuvastatin in aortic rings from rats with a cafeteria-style diet

Jorge Skiold López-Canales,† Jair Lozano-Cuenca, † Oscar Alberto López-Canales, * José Carlos Aguilar-Carrasco, † Lidia Aranda-Zepeda, † Pedro López-Sánchez, † Enrique Fernando Castillo-Henkel, † Ruth Mery López-Mayorga† and Ignacio Valencia-Hernández*  

*Section of Postgraduate Studies, Higher School of Medicine, National Polytechnic Institute, and †Department of Cellular Biology, National Institute of Perinatology, Mexico City, Mexico

SUMMARY

The present study aimed to investigate the possible influence of several inhibitors and blockers on the vascular effect produced by the acute in vitro application of rosuvastatin to phenylephrine-precontracted aortic rings from rats with a semi-solid, cafeteria-style (CAF) diet. It also aimed to examine the effects of rosuvastatin on the expression of endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase, constitutive cyclooxygenase, and inducible cyclooxygenase in aortic rings from rats with a CAF diet. From comparisons of the effect on phenylephrine-precontracted aortic rings extracted from rats with two different diets (a standard and a CAF diet), it was found that 10−9–10−5 mol/L rosuvastatin produced lower concentration-dependent vasorelaxation on rings from the CAF diet group. The vasorelaxant effect was unaffected by the vehicle, but it was significantly attenuated by 10−5 mol/L Nω-nitro-l-arginine methyl ester, 10−5 mol/L tetraethylammonium, 10−3 mol/L 4-aminopyridine, 10−2 mol/L apamin plus 10−2 mol/L charybdotoxin, 10−5 mol/L indoethamine, or 10−2 mol/L cycloheximide. Moreover, in aortic rings from rats with a CAF diet, rosuvastatin enhanced the expression of eNOS, inducible nitric oxide synthase, constitutive cyclooxygenase, and inducible cyclooxygenase. The acute in vitro application of rosuvastatin to phenylephrine-precontracted aortic rings from rats with a CAF diet had a vasorelaxant effect. Overall, the present results suggest that the stimulation of eNOS, the opening of Ca2+-activated and voltage-activated K+ channels, the stimulation of prostaglandin synthesis and enhanced protein levels of eNOS, inducible nitric oxide synthase, constitutive cyclooxygenase, and inducible cyclooxygenase are involved in this relaxant effect.

Key words: aorta, endothelium, obesity, rate, rosuvastatin.

INTRODUCTION

Statins are 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors. It is known that they have vascular-favourable, lipid-independent pleiotropic effects,1,2 at least partly mediated through a modification of endothelial nitric oxide synthase (eNOS) expression, activity, and enzymatic coupling.3–6 Studies with sub-chronic administration of statins have been conducted in animal models of hypercholesterolaemia and metabolic syndrome to explore the effect on vascular endothelial function and eNOS expression.4,5 Moreover, there have been reports describing the effect produced by an acute in vitro application of rosuvastatin on aortic segments from experimental animals with a standard diet.8 However, no study has yet analysed the effect produced by an acute in vitro application of rosuvastatin on aortic segments from experimental animals with metabolic syndrome induced by a cafeteria-style (CAF) diet, much less the possible mechanisms that may be involved.

The aim of the present study was to analyse the following: (i) the effect produced by rosuvastatin, acetylcholine, or sodium nitroprusside on phenylephrine-precontracted aortic rings from rats given either a standard diet or a CAF diet; (ii) the influence of 10−5 mol/L Nω-nitro-l-arginine methyl ester (l-NAME; a direct inhibitor of NO synthase), 10−2 mol/L tetraethylammonium (TEA; a Ca2+-activated K+ channel blocker and non-specific voltage-activated K+ channel blocker), 10−3 mol/L 4-aminopyridine (4-AP; a voltage-activated K+ channel blocker), 10−2mol/L apamin plus 10−7 mol/L charybdotoxin (blockers of small- and large-conductance Ca2+-activated K+ channels, respectively), 10−2 mol/L indoethamine (a prostaglandin synthesis inhibitor), and 10−5 mol/L cycloheximide (a general protein synthesis inhibitor) on the vasorelaxant response produced by rosuvastatin in phenylephrine-precontracted aortic rings from rats given a CAF diet.
diet; and (ii) the expression of eNOS, inducible nitric oxide synthase (iNOS), constitutive cyclooxygenase (COX-1), and inducible cyclooxygenase (COX-2) in aortic segments pre-incubated with rosuvastatin after being extracted from rats with either a standard diet or a CAF diet.

RESULTS

Table 1 shows the metabolic parameters (bodyweight, serum insulin, glucose, total cholesterol, and triglycerides) of Wistar rats after 8 weeks with either a standard diet or a CAF diet. The values of these parameters were significantly higher in rats with a CAF diet than in animals with a standard diet.

Effect of rosuvastatin on phenylephrine-precontracted rat aortic rings

Figure 1 shows the effect of a cumulative addition of rosuvastatin on endothelium-intact and -denuded phenylephrine-precontracted aortic rings from rats with either a standard diet or a CAF diet. In all animals, $10^{-9} - 10^{-5}$-mol/L rosuvastatin elicited concentration-dependent vasorelaxation in endothelium-intact and -denuded phenylephrine-precontracted aortic rings, regardless whether the animals were fed a standard diet or a CAF diet ($P < 0.001$). Vasorelaxant responses to rosuvastatin were significantly greater ($P < 0.001$) in endothelium-intact than in endothelium-denuded aortic rings, independent of diet. Vasorelaxant responses to rosuvastatin were significantly greater ($P < 0.001$) in aortic rings from rats with a standard diet than in aortic rings from rats with a CAF diet. There was no statistically significant interaction between diet, the removal of endothelium, and the concentration of rosuvastatin ($P > 0.05$).

The maximum vasorelaxant effect in phenylephrine-precontracted aortic rings from rats given a standard diet was 84.86 $\pm$ 6.42% with the endothelium intact and 18.86 $\pm$ 2.75% with the endothelium denuded. The maximum vasorelaxant effect in phenylephrine-precontracted aortic rings from rats given a CAF diet was 59.88 $\pm$ 6.85% with the endothelium intact and 19.45 $\pm$ 2.46% with the endothelium denuded. The EC$_{50}$ values in phenylephrine-precontracted aortic rings from rats given a standard diet were $10^{-5.917}$ mol/L with the endothelium intact and $10^{-5.708}$ mol/L with the endothelium denuded. The EC$_{50}$ values in phenylephrine-precontracted aortic rings from rats given a CAF diet were $10^{-5.772}$ mol/L with the endothelium intact and $10^{-5.761}$ mol/L with the endothelium denuded.

Effect of acetylcholine or sodium nitroprusside on phenylephrine-precontracted rat aortic rings

Figure 2 shows the effect of the cumulative addition of acetylcholine or sodium nitroprusside on phenylephrine-precontracted aortic rings from rats with either a standard diet or a CAF diet. A concentration-dependent relaxation of aortic rings was elicited by $10^{-9} - 10^{-5}$-mol/L acetylcholine. The maximum vasorelaxation in aortic rings from rats with a CAF diet was 51.68 $\pm$ 1.45% compared to 97.43 $\pm$ 0.61% for those with a standard diet. The EC$_{50}$ values were $10^{-8.824}$ mol/L and $10^{-6.017}$ mol/L in aortic rings from rats with a CAF diet and a standard diet, respectively. A concentration-dependent relaxation of aortic rings was elicited by $10^{-11} - 10^{-5}$-mol/L sodium nitroprusside. The maximum vasorelaxation in aortic rings from rats with a CAF diet was 117.78 $\pm$ 5.18% compared to 105.91 $\pm$ 4.47% for those with a

Table 1 Metabolic parameters of male Wistar rats after an 8-week standard diet (control) or CAF diet

| Parameter            | Standard diet (control) | CAF diet     |
|----------------------|-------------------------|--------------|
| Bodyweight (g)       | 413.0 $\pm$ 3.5         | 454.0 $\pm$ 4.0* |
| Insulin (µU/mL)      | 0.1 $\pm$ 0.0           | 0.9 $\pm$ 0.1* |
| Glucose (mg/dL)      | 80.0 $\pm$ 4.2          | 110.0 $\pm$ 1.1* |
| Total cholesterol (mg/dL) | 58.0 $\pm$ 2.6          | 115.0 $\pm$ 4.2* |
| Triglycerides (mg/dL)| 93.0 $\pm$ 2.8          | 248.0 $\pm$ 3.5* |

Data are reported as mean $\pm$ SEM for $n$ observations ($n = 4$). *$P < 0.05$ versus control (t-test). CAF, cafeteria-style.
The presence of these compounds, respectively. The EC50 values were as follows: (i) 10^{-9}–10^{-7} mol/L of acetylcholine and (b) 10^{-11}–10^{-5} sodium nitroprusside on phenylephrine (PE)-precontracted aortic rings from rats with a standard diet (control) (closed circles) and a cafeteria-style (CAF) diet (open circles). Data are expressed as mean ± SEM for n observations (n = 6). *P < 0.05 versus control (two-way ANOVA).

standard diet. The EC50 values were 10^{-7.557} mol/L and 10^{-8.136} mol/L in aortic rings from rats with a CAF diet and a standard diet, respectively.

Effect of l-NAME, TEA, 4-AP, or apamin plus charybdoctoxin on the relaxant effect produced by rosuvastatin in phenylephrine-precontracted aortic rings from rats with a CAF diet

Figure 3 shows the effect on vasorelaxation produced by 10^{-9}–10^{-7} mol/L rosuvastatin in phenylephrine-precontracted aortic rings from rats with a CAF diet. The vasorelaxant effect was induced by the vehicle, 10^{-5} mol/L l-NAME, 10^{-2} mol/L TEA, 10^{-3} mol/L 4-AP, or 10^{-7} mol/L apamin plus 10^{-7} mol/L charybdoctoxin (Fig. 3a–c). The vasorelaxant response induced by the cumulative addition of rosuvastatin was significantly attenuated (P < 0.05) by l-NAME, TEA, 4-AP, and apamin plus charybdoctoxin. The maximum vasorelaxant effect produced by rosuvastatin was unaffected by the vehicle (64.30 ± 4.70% vs 67.31 ± 3.27% in the absence or presence of distilled water, respectively. In contrast, the maximum vasorelaxant effect produced by rosuvastatin was reduced by l-NAME (65.58 ± 1.66% vs 20.78 ± 2.17% in the absence and presence of this compound, respectively), TEA (70.31 ± 2.31% vs 16.33 ± 2.29% in the absence and presence of this compound, respectively), 4-AP (75.00 ± 1.41% vs 65.68 ± 0.68% in the absence and presence of this compound, respectively), and apamin plus charybdoctoxin (80.75 ± 2.20% vs 15.14 ± 4.09% in the absence and presence of these compounds, respectively). The EC50 values were as follows: (i) 10^{-5.965} mol/L and 10^{-6.102} mol/L in the absence and presence of distilled water, respectively; (ii) 10^{-5.501} mol/L and 10^{-5.972} mol/L in the absence and presence of l-NAME, respectively; (iii) 10^{-6.074} mol/L and 10^{-6.193} mol/L in the absence and presence of TEA, respectively; (iv) 10^{-6.650} mol/L and 10^{-6.738} mol/L in the absence and presence of 4-AP, respectively; and (v) 10^{-7.060} mol/L and 10^{-7.096} mol/L in the absence and presence of apamin plus charybdoctoxin, respectively.

Expression of endothelial and inducible NO synthase in rosuvastatin-pretreated aortic rings from rats with a CAF diet

Figure 4 shows the relative expressions of inducible NOS and endothelial NOS in rosuvastatin- or phenylephrine-pretreated aortic rings from rats with a CAF diet. Rosuvastatin, but not phenylephrine, significantly enhanced (P < 0.05) the relative expression of inducible NOS and endothelial NOS.

Effect of indomethacin on the relaxant effect produced by rosuvastatin in phenylephrine-precontracted aortic rings from rats with a CAF diet

Figure 5 shows the effect of 10^{-5}-mol/L indomethacin on the vasorelaxant response produced by 10^{-9}–10^{-5} mol/L rosuvastatin in phenylephrine-precontracted aortic rings from rats with a CAF diet. The vasorelaxant response produced by the cumulative addition of rosuvastatin was significantly attenuated (P < 0.05) by indomethacin. The maximum vasorelaxant effect produced by rosuvastatin was reduced by indomethacin (65.00 ± 1.41 vs 15.46 ± 3.94% in the absence and presence of indomethacin, respectively). The EC50 values were 10^{-7.057} and 10^{-6.441} mol/L in the absence and presence of indomethacin, respectively.

Expression of COX-1 and COX-2 in rosuvastatin-pretreated aortic rings from rats with a CAF diet

Figure 6 shows the relative expressions of COX-1 and COX-2 in rosuvastatin- or phenylephrine-pretreated aortic rings from rats with a CAF diet. Rosuvastatin, but not phenylephrine, significantly enhanced (P < 0.05) the relative expression of COX-1 and COX-2.

Effect of cycloheximide on the relaxant effect produced by rosuvastatin in phenylephrine-precontracted aortic rings from rats with a CAF diet

Figure 7 shows the effect of 10^{-5}-mol/L cycloheximide on the vasorelaxant response produced by 10^{-9}–10^{-5} mol/L rosuvastatin in phenylephrine-precontracted aortic rings from rats with a CAF diet. The vasorelaxant responses produced by the cumulative addition of rosuvastatin were significantly attenuated (P < 0.05) by cycloheximide. The maximum vasorelaxant effect produced by rosuvastatin was reduced by cycloheximide (71.93 ± 1.88% and 20.14 ± 0.62% in the absence and presence of cycloheximide, respectively). The EC50 values were 10^{-5.775} mol/L and 10^{-6.984} mol/L in the absence and presence of cycloheximide, respectively.

DISCUSSION

Effect of a CAF diet on metabolic parameters

The CAF diet is an experimental model commonly used to investigate the effect of a Western dietary regime on animal health. This diet represents the food intake of people in modern Western societies. The model is characterized by meals prepared in cafeterias.9,10 In this sense, the CAF diet is a robust experimental model of metabolic syndrome that is capable of inducing obesity,
glucose intolerance, and inflammation in rats. Consistent with these observations, our results show that an 8-week CAF diet significantly enhanced (P < 0.05) bodyweight, serum insulin, glucose, total cholesterol, and triglycerides in Wistar rats (Table 1). These findings reinforce previous research in which a 4-week CAF diet enhanced the same metabolic parameters in Wistar rats. Indeed, obesity is associated with an increase in levels of insulin, glucose, total cholesterol, and triglycerides.

Effect of rosuvastatin on endothelium-intact and -denuded phenylephrine-precontracted rat aortic rings

Aortic segments were pretreated with phenylephrine 30 min before administration of rosuvastatin, because this statin elicited only moderate concentration-dependent vasorelaxation in rat aortic rings without precontraction (E_{max} 5.51 ± 2.12% of basal contraction). Phenylephrine enhanced the vasorelaxant response produced by rosuvastatin (E_{max} 59.88 ± 6.85% of the phenylephrine-induced contraction), making the vasorelaxant effect produced by rosuvastatin more evident. This result is in accordance with previous reports on in vitro studies that employed other drugs with a vasorelaxant effect. Precontraction with phenylephrine also makes the effects produced by antagonists and blockers more evident. For these reasons, we decided to perform our experimental protocol with rat aortic rings precontracted with phenylephrine.

The fact that the vasorelaxant response to rosuvastatin was significantly greater (P < 0.001) in endothelium-intact than -
denuded aortic rings, independent of diet, suggests that endothelium play an important role in the vasorelaxant effect produced by rosuvastatin. Moreover, the fact that the vasorelaxant response to rosuvastatin was significantly greater (P < 0.001) in aortic rings from rats with a standard diet than in aortic rings from rats with a CAF diet suggests that endothelial dysfunction could be involved in the inhibitory effect produced by a CAF diet. In this sense, numerous studies suggest that 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (e.g. rosuvastatin) have a vascular-favourable, lipid-independent pleiotropic effect.1,2 The vascular effects of these inhibitors have been involved in the upregulation of both endothelial NO synthase activity in the endothelial cells of the human saphenous vein and inducible NO synthase activity in cultured rat aortic vascular smooth muscle cells.16,17

Effect of acetylcholine or sodium nitroprusside on the phenylephrine-precontracted aortic rings of obese rats

Endothelial cells play an important role in the acetylcholine-induced relaxation of vascular smooth muscle.18 Acetylcholine also produces an endothelium-dependent relaxation in vascular smooth muscle preparations that are precontracted with high concentrations of potassium, noradrenaline, or other vasoactive agents.19 Therefore, in comparisons of rats fed with a CAF diet or a standard diet for 8 weeks, the reduced vasorelaxant effect produced by acetylcholine in phenylephrine-precontracted aortic rings from the CAF group suggests that endothelial function was decreased by the CAF diet. In support of this suggestion, the maximum vasorelaxation produced by sodium nitroprusside (an NO-releasing drug) in endothelium-intact aortic segments did not significantly differ (P > 0.05) between rings derived from rats with a standard diet and those from animals with a CAF diet.20,21 The proposal that endothelial function is decreased by a CAF diet is consistent with previous reports suggesting the same idea in animal models with elevated circulating free fatty acid concentration and obesity.22,23

Involvement of NO and K⁺ channels in the vasorelaxant effect produced by rosuvastatin in phenylephrine-precontracted rat aortic rings from rats with a CAF diet

The fact that vasorelaxant responses induced by 10⁻⁹–10⁻⁵-mol/L rosuvastatin in aortic rings of rats with CAF diet were unaffected by vehicle and significantly (P < 0.05) attenuated by 10⁻⁵-mol/L l-NAME (a direct inhibitor of NOS),24,25 10⁻²-mol/L TEA (a Ca²⁺-activated K⁺ channel blocker and non-specific voltage-activated K⁺ channel blocker),26,27 10⁻³-mol/L 4-AP (a voltage-activated K⁺ channel blocker),27,28 and 10⁻⁷-mol/L apamin plus 10⁻⁷-mol/L charybdotoxin (blockers of small- and large-conductance Ca²⁺-activated K⁺ channels, respectively) suggests the involvement of eNOS, Ca²⁺-activated K⁺ channels, and to a lesser extent, voltage-activated K⁺ channels.29-31 The western blot analysis showed enhanced protein levels of iNOS and eNOS in aortic rings from rats with a CAF diet that were exposed to phenylephrine plus rosuvastatin, but not to phenylephrine alone. This analysis reinforces the results of the pharmacological study with l-NAME in regard to the involvement of NO in the vasorelaxation produced by rosuvastatin. Thus, the present western blot results are consistent with evidence suggesting that rosuvastatin enhances the expression of iNOS in myocardial-infarcted rats and with reports suggesting that statins improve endothelial function through modification of the expression, activity, and enzymatic coupling of eNOS.4,16,32,33 The involvement of NO and K⁺ channels in the vasorelaxant effect produced by rosuvastatin in aortic rings from rats with a CAF diet was investigated because it was reported that stimulation of NOS and the opening of Ca²⁺-dependent K⁺ channels are involved in those vasorelaxations to rosvastatin and methyl ester of rosuvastatin on phenylephrine-precontracted aortic rings of rats with standard diet.8,14 These findings about the involvement of NO and K⁺ channels in the vasorelaxations produced by rosuvastatin on aortic rings of rat with CAF diet are in line with previous reports in which statins produced vasorelaxation and stimulation of NO and K⁺ channels; they also corroborate several
reports that suggest that NO and K+ channels play an important role in the modulation of vascular tone.\textsuperscript{34–39}

The fact that the vasorelaxant response to rosuvastatin did not differ significantly before and after pre-incubation with the vehicle (distilled water) indicates that this vasorelaxant response is highly reproducible and excludes the possibility that attenuations produced by the inhibitors/blockers are tachyphylactic. Moreover, the combination of apamin plus charybdotoxin was used because it was previously reported that a complete blockade of Ca\textsuperscript{2+}-activated K+ channels is necessary to produce a pharmacological response.\textsuperscript{8,30,31} In this respect, a pilot experiment conducted by our laboratory showed that apamin alone did not modify the vasorelaxant response to rosuvastatin (data not shown). These observations suggest, but do not prove, that rosuvastatin produces vascular hyperpolarization attributable to the release of an endothelium-dependent hyperpolarizing factor. This effect and mechanism has been reported for acetylcholine.\textsuperscript{40,41} Certainly this idea is still speculative and requires additional experiments that are beyond the scope of the present study.

Involvement of prostaglandins in the vasorelaxant effect produced by rosuvastatin in phenylephrine-precontracted aortic rings from rats with a CAF diet

The fact that 10\textsuperscript{–5}-mol/L indomethacin, a prostaglandin synthesis inhibitor,\textsuperscript{32} significantly attenuated (P < 0.05) the vasorelaxation produced by 10\textsuperscript{–9}–10\textsuperscript{–7}-mol/L rosuvastatin suggests that prostaglandins are involved in the effect of this statin on aortic rings from rats with a CAF diet. The western blot analysis adds weight to this proposal because enhanced protein levels of COX-1 and COX-2 were found when the aortic rings from rats with a CAF diet were exposed to phenylephrine plus rosuvastatin, but not to phenylephrine alone. Nevertheless, these results contrast with previous studies in which 10\textsuperscript{–5}-mol/L indomethacin did not affect the vasorelaxant response produced by 10\textsuperscript{–9}–10\textsuperscript{–7}-mol/L rosuvastatin in aortic rings extracted from rats with a standard diet.\textsuperscript{6} The current results also contrast with previous reports suggesting that prostaglandins play an important role in the endothelial dysfunction produced in several experimental models of obesity and metabolic syndrome.\textsuperscript{43–45} One possible explanation for this apparent discrepancy is that a CAF diet may produce crosstalk between the NO and prostaglandin pathways, as reported under certain conditions of inflammation.\textsuperscript{46–48}

In our model of metabolic syndrome, rosuvastatin enhanced the enzyme expression of eNOS and iNOS and, consequently, the enzyme expression of COX-1 and COX-2. The latter result might lead one to believe that rosuvastatin produced vasoconstriction through the stimulation of prostanoid synthesis. Statins have been involved in an increased expression of prostaglandin (PG)H\textsubscript{2} synthase,\textsuperscript{49} a stimulation of the PGI\textsubscript{2}-peroxisome proliferator-activated receptor \( \alpha \) pathway, and a reduction in the generation of PGE\textsubscript{2}.\textsuperscript{50} Two COX isoforms, COX-1 and COX-2, are involved in the synthesis of PGI\textsubscript{2},\textsuperscript{51} which has vasorelaxant properties.\textsuperscript{52} However, we cannot affirm that prostanoid synthesis is involved in the vasorelaxant effect produced by rosuvastatin in aortic rings from rats with a CAF diet. To explore this idea, there is a need for additional experiments beyond the scope of the present study.

Final considerations of the present experiments

The fact that 10\textsuperscript{–5}-mol/L cycloheximide, a general protein synthesis inhibitor,\textsuperscript{53} significantly attenuated (P < 0.05) the vasorelaxant effect produced by rosuvastatin in aortic rings from rats with a CAF diet suggests the involvement of protein synthesis in this vasorelaxation. This suggestion is consistent with previous findings in which 10\textsuperscript{–5}-mol/L cycloheximide inhibited the vasorelaxant response produced by the methyl ester of rosuvastatin.\textsuperscript{14} Nevertheless, it must be taken into account that cycloheximide also facilitates the induction of cellular apoptosis.\textsuperscript{54,55} Therefore, in an attempt to elucidate whether protein synthesis really participates in the vasorelaxation produced by rosuvastatin in phenylephrine-precontracted rat aortic rings from rats with a CAF diet, the relative protein expressions of inducible NOS, endothelial NOS, COX-1 and COX-2 were explored because these enzymes play an important role in the regulation of vascular tone.\textsuperscript{57,56–58} The levels of these proteins were found to be enhanced by rosuvastatin.
Summary

The acute in vitro application of rosuvastatin to phenylephrine-precontracted aortic rings from rats with a CAF diet had a vasorelaxant effect. Overall, the present results suggest that the stimulation of eNOS, the opening of Ca²⁺-activated and voltage-activated K⁺ channels, the stimulation of prostaglandin synthesis, and enhanced protein levels of eNOS, iNOS, COX-1, and COX-2 are involved in this relaxant effect.

METHODS

Animals

Male Wistar rats were randomly distributed into two groups for an 8-week protocol: (i) rats with free access to drinking water and a standard rat chow diet containing 51% carbohydrate, 4% fat, and 21% protein (n = 36); and (ii) rats with free access to drinking water and a CAF diet containing 33% ground commercial rat chow, 33% full-fat sweetened condensed milk (Nestlé, Mexico City, Mexico), 7% sucrose, and 27% water (n = 36), as used by Holemans et al. Animals (n = 72) were purchased from the Higher School of Medicine and housed in plastic cages in a special temperature-controlled room (22 ± 2°C, 50% humidity) on a 12/12-h light/dark cycle (lights on at 0700 hours). The study was approved by the Animal Care Committee of our institution (Higher School of Medicine) and is in agreement with the Animals (Scientific Procedures) Act 1986 of the UK Parliament (http://www.legislation.gov.uk/ukpga/1986/14/contents).

Preparation of aortic rings to analyse the vascular effect of rosuvastatin

Animals were killed by decapitation, and the aortas were immediately excised, placed in cold buffer, cleaned, and freed from surrounding connective tissue. The isolated arteries were cut into rings (4–5 mm long) and placed in 10-mL tissue chambers filled with Krebs–Henseleit bicarbonate buffer (1.18 × 10⁻³-mol/L NaCl; 4.7 × 10⁻³-mol/L KCl; 1.2 × 10⁻³-mol/L KH₂PO₄; 1.2 × 10⁻³-mol/L MgSO₄·7H₂O; 2.5 × 10⁻³-mol/L CaCl₂·2H₂O; 2.5 × 10⁻²-mol/L NaHCO₃; 1.17 × 10⁻²-mol/L dextrose; and 2.6 × 10⁻³-mol/L calcium disodium EDTA). In some experiments, the KCl concentration was increased to 8 × 10⁻² mol/L, and the Na⁺ concentration was decreased to maintain osmotic equilibrium.

Aortic rings were mounted on two stainless steel hooks to fix them to the bottom of the chamber and to a BIOPAC TSD125C-50g force transducer (BIOPAC Systems, Santa Barbara, CA, USA) connected to a BIOPAC MP100A-CE data acquisition system (BIOPAC Systems) in order to record the isometric tension. Optimal tension, selected from preliminary experiments, was that which gave the greatest response to phenylephrine (10⁻⁶ mol/L). The rings were given around 2.0-g initial tension (100%) and allowed to equilibrate for 2 h. Thirty minutes after setting up the organ bath, tissues were first contracted with 10⁻⁶-mol/L phenylephrine to test their contractile responses. These were then rinsed three times with Krebs solution to restore tension to precontraction levels. Endothelial integrity was pharmacologically assessed by acetylcholine-induced vasodilatation (10⁻⁶ mol/L). Segments showing no relaxation in response to acetylcholine were considered to be endothelium-denuded. After application of 10⁻⁴-mol/L phenylephrine or 10⁻⁶-mol/L acetylcholine, tissues were rinsed three times with Krebs solution to restore basal tension. Tissue baths were maintained at 37°C and pH 7.4, and bubbled with a mixture of 95% O₂ and 5% CO₂.

Preparation of samples for NOS and COX immunoblot analysis

Briefly, aortic rings from rats with a CAF diet were pre-incubated with 10⁻⁶-mol/L phenylephrine (control) and 10⁻⁶-mol/L phenylephrine plus 10⁻⁵-mol/L rosuvastatin for 180 min at 37°C and pH 7.4, and bubbled with a mixture of 95% O₂ and 5% CO₂. After pre-incubation, these segments were homogenized in Tris-HCl, pH 7.4, with a protease cocktail (MiniComplete-EDTA free; Roche, Mannheim, Germany), and total protein was analysed by Lowry’s method. Immunoblots were carried out in duplicate with 50-µg protein per lane on a 10% sodium dodecyl sulphate-polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane (Hybond-P; Amersham Biosciences, Amersham, UK). The polyvinylidene fluoride membrane was then blocked for 2 h at room temperature with Tris-buffered saline containing 5% skim milk and 0.05% Tween. The blot was incubated overnight at 4°C with a polyclonal antibody against iNOS, eNOS, COX-1, or COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), at a final dilution of 1 : 400. The membrane was then washed and incubated for 2 h at room temperature with the corresponding secondary (anti-rabbit) horseradish peroxidase-labelled antibody (Zymed, San Francisco, CA, USA). Data are expressed as normalized absorbance (A).

Drugs

All drugs except rosuvastatin were purchased from Sigma-Aldrich (St Louis, MO, USA). Rosuvastatin was a gift from AstraZeneca (Mexico City, Mexico). All compounds were dissolved in distilled water. Fresh solutions were prepared for each experiment.

Experimental protocol

The mechanisms involved in the relaxant effect produced by rosuvastatin in phenylephrine-precontracted aortic rings from rats with a CAF diet were determined by conducting four main sets of experiments.

First set of experiments

Thirty minutes after restoration of basal tension, 10⁻⁶-mol/L phenylephrine was added to the following: (i) endothelium-intact aortic rings from rats with a standard diet; (ii) endothelium-denuded aortic rings from rats with a standard diet; (iii) endothelium-intact aortic rings from rats with a CAF diet; and (iv)
endothelium-denuded aortic rings from rats with a CAF diet. Twenty minutes after the addition of $10^{-6}$ mol/L phenylephrine, the contraction plateaued. Thirty minutes after the addition of phenylephrine, rosuvastatin began to be cumulatively added ($10^{-9}$–$10^{-5}$ mol/L) in intervals of around 20 min. Tension was expressed as a percentage of the phenylephrine-induced contraction ($3.56 \pm 0.28 \text{ g} = 100\%$ for endothelium-intact aortic rings from rats with a standard diet; $3.81 \pm 0.32 \text{ g} = 100\%$ for endothelium-denuded aortic rings from rats with a standard diet; $3.92 \pm 0.34 \text{ g} = 100\%$ for endothelium-intact aortic rings from rats with a CAF diet; and $4.11 \pm 0.37 = 100\%$ for endothelium-denuded aortic rings from rats with a CAF diet).

Second set of experiments

Thirty minutes after the addition of phenylephrine, the vasorelaxant response to the cumulative addition of $10^{-9}$–$10^{-5}$ mol/L acetylcholine or $10^{-11}$–$10^{-5}$ mol/L sodium nitroprusside was assessed in rat aortic rings from rats with a standard diet or with a CAF diet. Acetylcholine or sodium nitroprusside was added at intervals of around 3 min.

Third set of experiments

Thirty minutes after the addition of phenylephrine, rat aortic rings were pre-incubated with inhibitors or blockers for 30 min. The vasorelaxant response to rosuvastatin was assessed in these rings before and after pre-incubation with the following: (i) the vehicle (distilled water); (ii) $10^{-2}$ mol/L l-NAME; (iii) $10^{-2}$ mol/L TEA; (iv) $10^{-3}$ mol/L 4-AP; (v) $10^{-2}$ mol/L apamin plus $10^{-7}$ mol/L charybdoxin; (vi) $10^{-5}$ mol/L indomethacin; or (vii) $10^{-5}$ mol/L cycloheximide. After pre-incubation, increasing concentrations of rosuvastatin were added in intervals of around 20 min.

Fourth set of experiments

After pretreatment of aortic segments with $10^{-6}$ mol/L phenylephrine or $10^{-6}$ mol/L phenylephrine plus $10^{-9}$–$10^{-5}$ mol/L rosuvastatin, the levels of NOS and COX were explored.

Data analysis and statistics

Data are presented as mean $\pm$ SEM. In all experiments, $n$ equals the number of animals from which aortic segments were obtained (six in each case). The effect produced by the concentration of rosuvastatin, the removal of endothelium, the diet, and their interactions was assessed in rat aortic rings from rats with a standard diet; $3.81 \pm 0.32 \text{ g} = 100\%$ for endothelium-denuded aortic rings from rats with a standard diet; $3.92 \pm 0.34 \text{ g} = 100\%$ for endothelium-intact aortic rings from rats with a CAF diet; and $4.11 \pm 0.37 = 100\%$ for endothelium-denuded aortic rings from rats with a CAF diet).

ACKNOWLEDGEMENTS

The authors greatly appreciate the technical assistance of Lizeth Ledesma Rodea and Oscar Martín Boche Olivan.
18. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 1980; 288: 373–6.

19. Furchgott RF. Role of endothelium in responses of vascular smooth muscle. Circ. Res. 1983; 53: 557–73.

20. Wu-Wong JR, Li X, Chen YW. Different vitamin D receptor agonists exhibit differential effects on endothelial function and aortic gene expression in 5/6 nephrectomized rats. J. Steroid Biochem. Mol. Biol. 2015; 148: 202–9.

21. Kesavan M, Sarath TS, Kannan K et al. Atorvastatin restores arsenic-induced vascular dysfunction in rats: Modulation of nitric oxide signaling and inflammatory mediators. Toxicol. Appl. Pharma- col. 2014; 260: 107–16.

22. Mathie A, Wooltorton JR, Watkins CS. Voltage-activated potassium channels in mammalian neurons and their block by novel pharmacological agents. Gen. Pharmacol. 1998; 30: 13–24.

23. Ok SH, Han JY, Sung HJ et al. Ropivacaine-induced contraction is attenuated by both endothelial nitric oxide and voltage-dependent potassium channels in isolated rat aortae. Biomed. Res. Int. 2013; 2013: 565271.

24. Shieh CC, Kirsch GE. Mutational analysis of ion conduction and drug binding sites in the inner mouth of voltage-gated K+ channels. Biophys. J. 1994; 67: 2316–25.

25. Zografos P, Li JH, Kau ST. Comparison of the in vitro effects of K+ channel modulators on detrusor and portal vein strips from guinea pigs. Pharmacology 1992; 45: 216–30.

26. Potassium channels sensitive to combination of charybdoxin and apamin regulate the tone of diabetic isolated canine coronary arteries. Acta Physiol. (Oxf.) 2008; 194: 35–43.

27. Qi Y, Quille J. Apamin/charybdoxin-sensitive endothelial K+ channels contribute to acetylcholine-induced, NO-dependent vasorelaxation of rat aorta. Med. Sci. Monit. 2001; 7: 1129–36.

28. Zaltone SA, Abo-Gresha NM. Rosuvastatin promotes angiogenesis and reverses isoproterenol-induced acute myocardial infarction in rats: Role of eNOS and VEGF. Eur. J. Pharmacol. 2012; 691: 134–42.

29. Aoki C, Nakano A, Tanaka S et al. Fluvastatin upregulates endothelial nitric oxide synthase activity via enhancement of its phosphorylation and expression and via an increase in tetrahydrobiopterin in vascular endothelial cells. Int. J. Cardiol. 2012; 156: 55–61.

30. Sonmez Udyes-Dogan B, Topal G, Takir S, Ilkay Alp F, Kaleli D, Ozdemir O. Relaxant effects of pravastatin, atorvastatin and cerivastatin on isolated rat aortic rings. Life Sci. 2005; 76: 1771–86.

31. Alzamena Biochem. Soc. Trans. 1988; 16: 484–6.

32. Vane JR, McGiff JC. Possible contributions of endogenous prostaglandins to the control of blood pressure. Circ. Res. 1975; 36: 68–75.

33. Leblanc N, Wan X, Leung PM. Physiological role of Ca(2+)-activated and voltage-dependent K+ currents in rabbit coronary myocytes. Am. J. Physiol. 1994; 266: C1523–37.

34. Knot HJ, Nelson MT. Regulation of membrane potential and diameter by voltage-dependent K+ channels in rabbit myogenic cerebral arteries. Am. J. Physiol. 1995; 269: H348–55.

35. Alvarez De Sotomayor M, Herrera MD, Marhuenda E, Andriani-shtoihana R. Characterization of endothelial factors involved in the vasodilatory effect of simvastatin in aorta and small mesenteric artery of the rat. Br. J. Pharmacol. 2000; 131: 1179–87.

36. Kuhlmann CR, Gast C, Li F et al. Cerivastatin activates endothelial calcium-activated potassium channels and thereby modulates endothelial nitric oxide production and cell proliferation. J. Am. Soc. Nephrol. 2004; 15: 868–75.

37. Moncada S, Palmer RM, Higgs EA. The discovery of nitric oxide as the endogenous nitrovasodilator. Hypertension 1988; 12: 365–72.

38. Moncada S, Higgs EA, Palmer RM. Characterization and biological significance of endothelium-derived relaxing factor. Biochem. Soc. Trans. 1988; 16: 484–6.