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Improvement of age-related endothelial dysfunction by simvastatin: effect on NO and COX pathways

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1 The effects of oral administration of the HMG-CoA reductase inhibitor, simvastatin (SV), on age-related endothelial dysfunction were investigated in the aorta of male Wistar rats.

2 Adult (12–14 weeks) and old (60–80 weeks) rats were treated daily for 12 weeks with either vehicle or SV (1 mg kg^{-1}). In old rats, SV treatment did not significantly affect systolic blood pressure and LDL-cholesterol, but it reduced plasma cholesterol, triglycerides and oxidised LDL though it did not affect total antioxidant status.

3 SV improved endothelium-dependent relaxation to acetylcholine and A-23187 in vessels from aged, but not adult, rats. This effect was linked to a greater NO vasodilatation *via* an increased expression of endothelial NO-synthase. A mechanism sensitive to superoxide dismutase and catalase also accounts for enhanced endothelial vasodilatation.

4 Finally, SV did not affect the release of prostacyclin, but it inhibited the generation of thromboxane (TX) A_2 from COX-2 isoform. The effect of the latter was sensitive to the T_p receptor antagonist, ICI-192,605.

5 The present study provides evidence that oral administration of SV improves endothelial dysfunction in the aorta from aged rats by mechanisms associated with enhanced NO vasodilatation, reduced release of TXA_2 from cyclo-oxygenase, and increased antioxidant properties of the vessel wall. These data underscore a new therapeutic perspective for SV in age-related endothelial dysfunction.
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Abbreviations: AUC, area under curve; COX, cyclo-oxygenase; HMG-CoA, hydroxy-methyl-glutaryl coenzime A; L-NAME, N^G-nitro-L-arginine methyl ester; LDL, low-density lipoprotein; NO, nitric oxide; NOS, nitric oxide synthase; PGI_2, prostacyclin; PSS, physiological salt solution; ROS, reactive oxygen species; SBP, systolic blood pressure; SNP, sodium nitroprusside; SOD, superoxide dismutase; SV, simvastatin; TAS, total antioxidant status; TG, triglycerides; TX, thromboxane

Introduction

Ageing, independently of age-related diseases resulting from chronic exposure of the arteries to several risk factors, is characterised not only by reduced arterial compliance and alteration of the contractile properties of the vascular wall but also by endothelial dysfunction. Ageing is associated with endothelial dysfunction characterised as a progressive decline in endothelium-dependent vasodilatation in resistance and conductance arteries from both humans and different animal species (Egashira *et al*., 1993; Matz *et al*., 2000; Taddei *et al*., 2000). The mechanism underlying age-related dysfunction results from alteration in the equilibrium between the effect of relaxing and contracting factors released by the endothelium. This phenomenon includes progressive reduction of nitric oxide (NO)-mediated dilatation related to changes in expression and activity of endothelial NO-synthase (eNOS), increased production of oxygen-derived free radicals and increased release of cyclo-oxygenase (COX)-derived contracting factors (for a review; see Matz & Andriantsitohaina, 2003).

Simvastatin (SV) is an inhibitor of HMG-CoA reductase that belongs to lipid-lowering drugs. Recently, we reported that SV produces direct relaxation of conductance and small arteries of the rat through the mevalonate-sensitive pathway (Alvarez de Sotomayor *et al*., 2000). The endothelium-dependent relaxation to SV involves both NO and vasodilator eicosanoids by a mechanism sensitive to superoxide dismutase (SOD) and to tyrosine kinase inhibitors. An increase in endothelial cytosolic calcium involving the activation of Rho protein is essential for the action of SV (Alvarez de Sotomayor & Andriantsitohaina, 2001). Interestingly, SV is able to improve endothelial dysfunction associated with several cardiovascular diseases including hypercholesterolaemia and hypertension (Alvarez de Sotomayor *et al*., 1999; Carneado *et al*., 2002). Many experimental studies and clinical trials suggest that benefits observed using HMG-CoA reductase

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inhibitors appear to be greater than might be expected from changes in lipid metabolism, those benefits being explained by the 'pleiotropic' effects of statins. Accordingly, it has recently been demonstrated that, with equal lowering of LDL-cholesterol by SV or ezetimibe, only SV improved endothelial function in patients with congestive heart failure (Landmesser *et al*., 2005). Several facts may contribute to explain the effect of HMG-CoA reductase inhibitors, including improvement of endothelial NO pathway, increased antioxidant defence and anti-inflammatory properties (Laufs *et al*., 1998; Carneado *et al*., 2002; Dichtl *et al*., 2003).

The present study was designed to test the hypothesis that SV is able to improve age-related endothelial dysfunction in the rat aorta. Furthermore, the mechanisms involved were examined, including its effect on NO and COX pathways.

Methods

Animals

Adult (12–14 weeks) and old (60–80 weeks) male Wistar rats were bred in our institute from genitors provided by Iffa-Credo (Lyon, France). All experiments were performed according to the guidelines for the ethical treatment of animals of the European Union. Animals were housed at $24 \pm 2^{\circ}C$ with $60 \pm 20\%$ relative humidity, on a 12,12 L,D cycle. Rats were fed a standard diet and water *ad libitum*, and were weekly weighed. Systolic blood pressure (SBP) was measured before the start and at the end of the treatment by tail-cuff method with a pressure meter (Niprem 645, Cibertec, Madrid, Spain). Old rats were randomly divided into two groups ($n = 15$ in each group): the first group (67.89 ± 5.67-weeks old) in which SV was given in a dose of $1\,mg\,kg^{-1}$ body weight and the second group (65.15 ± 6.85-weeks old) receiving the vehicle (0.5% carboxymethylcellulose, $10\,ml\,kg^{-1}$ body weight). All substances were given by gavage. In the other series of experiments, two groups of six adult rats each received either SV ($1\,mg\,kg^{-1}$) or vehicle. The dose of SV used in the present study corresponded to the lowest dose that was effective in improving endothelial dysfunction in different models of experimental hypertension (Alvarez de Sotomayor *et al*., 1999; Carneado *et al*., 2002; Pérez-Guerrero *et al*., 2003). The treatments were administered daily during 12 weeks. The *in vitro* experiments were performed 3 days after withdrawing the treatments in order to study SV-induced long-term effect only. Animals were anaesthetised with pentobarbitone ($60\,mg\,kg^{-1}$) and blood was collected by intracardiac puncture for biochemical assays. At necropsy, no apparent pathology was noted in any animal.

Blood biochemical assays

Total antioxidant status (TAS), cholesterol, LDL-cholesterol, oxidised LDL, triglycerides and $NO_2 + NO_3$ were measured in serum. TAS was assayed using the kit, TAS (Randox Lab, Barcelona, Spain) based on the method reported by Miller *et al*. (1993). Serum cholesterol, triglycerides and LDL concentration were measured with a CHOD-PAP® kit (Roche Diagnostics, Barcelona, Spain). Oxidised LDL was assessed using an enzyme immunoassay kit. Plasma $NO_2 + NO_3$ was determined with a NO Colorimetric Assay® kit (Roche Diagnostics, Barcelona, Spain) (Green *et al*., 1982).

Arterial preparation and mounting

Thoracic aortas were carefully removed and cleaned of connective and fat tissue. Then, aortic segments (2–3 mm in length) were mounted on myographs filled with physiological salt solution (PSS) of the following composition (mM): NaCl 119, KCl 47, $MgSO_4$ 1.7, KH_2PO_4 1.18, $NaHCO_3$ 25, $CaCl_2$ 1.8 and glucose 11. The PSS was continuously kept at $37^{\circ}C$ and gassed with 95% O_2 and 5% CO_2 at pH 7.4. Aortic rings were stretched to 2.5 g, which yielded a maximal contractile response in aortic rings from old rats (Matz *et al*., 2000). Mechanical activity was recorded isometrically by a force transducer (Pioden UF-1) coupled to a Powerlab® data acquisition system (AD-Instruments, Castle Hill, Australia). Challenges with $10^{-6}\,mol\,l^{-1}$ phenylephrine (Phe) were performed in aorta in order to test its maximal contractile capacity and to elicit reproducible contractile response. The presence of functional endothelium was assessed by the ability of acetylcholine (ACh, $10^{-6}\,mol\,l^{-1}$) to induce relaxation of precontracted vessels.

Relaxation experiments

Arteries were precontracted at 80% of their maximal contraction with Phe. The concentration of Phe was adjusted for each preparation, being $3 \times 10^{-7}\,mol\,l^{-1}$. Precontaction values (being 1.84 ± 0.17 and 2.04 ± 0.31 g, $n = 6$ for the aorta from adult and old rats, respectively) were not significantly altered by SV treatment (being 1.90 ± 0.22 and 1.89 ± 0.21 g, $n = 6$ for aortic rings from adult and old rats, respectively). When the contraction reached a plateau, cumulative addition of vasodilator agents was performed (i.e., ACh from 3×10^{-8} to $10^{-5}\,mol\,l^{-1}$), calcium ionophore calcimycin (A-23187 from 3×10^{-9} to $10^{-5}\,mol\,l^{-1}$) and the NO donor, sodium nitroprusside (SNP from 3×10^{-10} to $10^{-6}\,mol\,l^{-1}$). Concentration–response curves to ACh were constructed in the absence or presence of indicated inhibitors: the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME $3 \times 10^{-4}\,mol\,l^{-1}$), the superoxide anion (SOD, $150\,UI\,ml^{-1}$), the hydrogen peroxide scavengers (catalase, $1000\,UI\,ml^{-1}$), the nonselective COX inhibitor, indomethacin ($10^{-5}\,mol\,l^{-1}$), the selective COX-2 inhibitor, *N*-(2-cyclohexyloxy-4-nitrophenyl) methane-sulphonamide (NS-398, $10^{-6}\,mol\,l^{-1}$) and the TXA_2/prostaglandin H_2 Tp receptor antagonist, 4(z)-6-(2-*o*-chlorophenyl-4-*o*-hydroxy-phenyl-1,3-dioxan-*cis*-5-yl) hexenoic acid (ICI-192,605, $10^{-6}\,mol\,l^{-1}$). All the inhibitors were used at a maximally active concentration (Matz *et al*., 2000) and were incubated with the tissue for 25 min before the precontraction with Phe.

Contraction to TX agonist

After the contractile capacity of the aorta with functional endothelium had been tested with Phe, concentration–response curves to the TX analogue, 9,11-di-deoxy-11α, 9α-epoxymethano-prostaglandin $F_{2\alpha}$ (U46619) were constructed. The effect of U46619 was expressed as percentage of KCl (80 mM)-induced contraction.

Measurement of TXA_2 and prostacyclin (PGI_2)

Both TXA_2 and PGI_2 are instable molecules that are quickly converted to the TXB_2 and 6-keto-PGF_1, respectively. Therefore, assays of TXB_2 and 6-keto-$PGF_{1\alpha}$ were performed in intact aorta as described previously (Matz *et al*., 2000). Briefly, intact aortas were incubated in PSS at 37°C and bubbled with a 95% O_2–5% CO_2 gas mixture and stimulated with Phe (10^{-6} mol l^{-1} for aorta) and ACh (10^{-6} mol l^{-1}) in order to stimulate the release of TXB_2 and 6-keto-$PGF_{1\alpha}$ in the medium. Concentrations of TXB_2 and 6-keto-$PGF_{1\alpha}$ were assessed by using competitive enzyme-immunoassay kits (Cayman Chemical Co., Ann Arbor, MI, U.S.A.) based on Pradelles method (Pradelles *et al*., 1985), and were expressed as pg mg^{-1} of dry tissue.

Western blotting of eNOS, COX-1 and COX-2

Aortic rings were homogenised in lysis buffer and 25 μg of protein fractions were loaded into 7% and 10% SDS–polyacrylamide gel to separate eNOS and COX-1 or COX-2, respectively. After electrophoresis, proteins were transferred onto nitrocellulose membrane. Immunostaining was achieved using specific monoclonal mouse anti-eNOS (Sigma-Aldrich), anti-COX-1 (Cayman Chemical) and anti-COX-2 (Calbiochem) antibodies, and reacted with peroxidase-conjugated antimouse antibodies. The blots were detected using an enhanced chemiluminescence assay (Pierce Chemical Company, Rockford, IL, U.S.A.) and evaluated by densitometry.

Drugs

SV was generously provided by MSD laboratories. ACh chloride, A-23187, catalase, indomethacin, L-NAME, Phe hydrochloride, SNP, SOD and U46619 were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). ICI-192,605 and NS-398 from Tocris (Biogen Cientifica SL, Madrid, Spain).

Statistical analysis

Results are expressed as relaxation percent of the initial precontraction level of means ± s.e.m. from n experiments, n represents the number of rats, which was at least equal to 6.

Table 1 Body weight, SBP, plasma cholesterol, LDL-cholesterol, oxidised LDL, triglycerides, TAS, and nitrites + nitrates ($NO_2 + NO_3$) from old rats treated with either placebo or simvastatin (1 mg kg^{-1})

	Placebo	*Simvastatin* (*1* mg kg^{-1})
Body weight (gm)	676.25 ± 15.80	702.86 ± 20.79
SBP (mmHg)	134.14 ± 16.67	135.31 ± 28.47
Cholesterol (mg dl^{-1})	147.2 ± 10.9	102.4 ± 16.0*
LDL-cholesterol (mg dl^{-1})	12.3 ± 1.4	10.1 ± 1.4
Oxidised LDL (mU ml^{-1})	23.8 ± 1.6	18.7 ± 1.3*
Triglycerides (mg dl^{-1})	208.2 ± 32.7	166.9 ± 30.8*
TAS (U gm^{-1})	905.5 ± 53.1	921.8 ± 50.9
$NO_2 + NO_3$ (μmol l^{-1})	11.3 ± 0.5	9.4 ± 0.3

Data represented are mean ± s.e.m. of $n = 10$. *$P < 0.05$ placebo *versus* simvastatin.

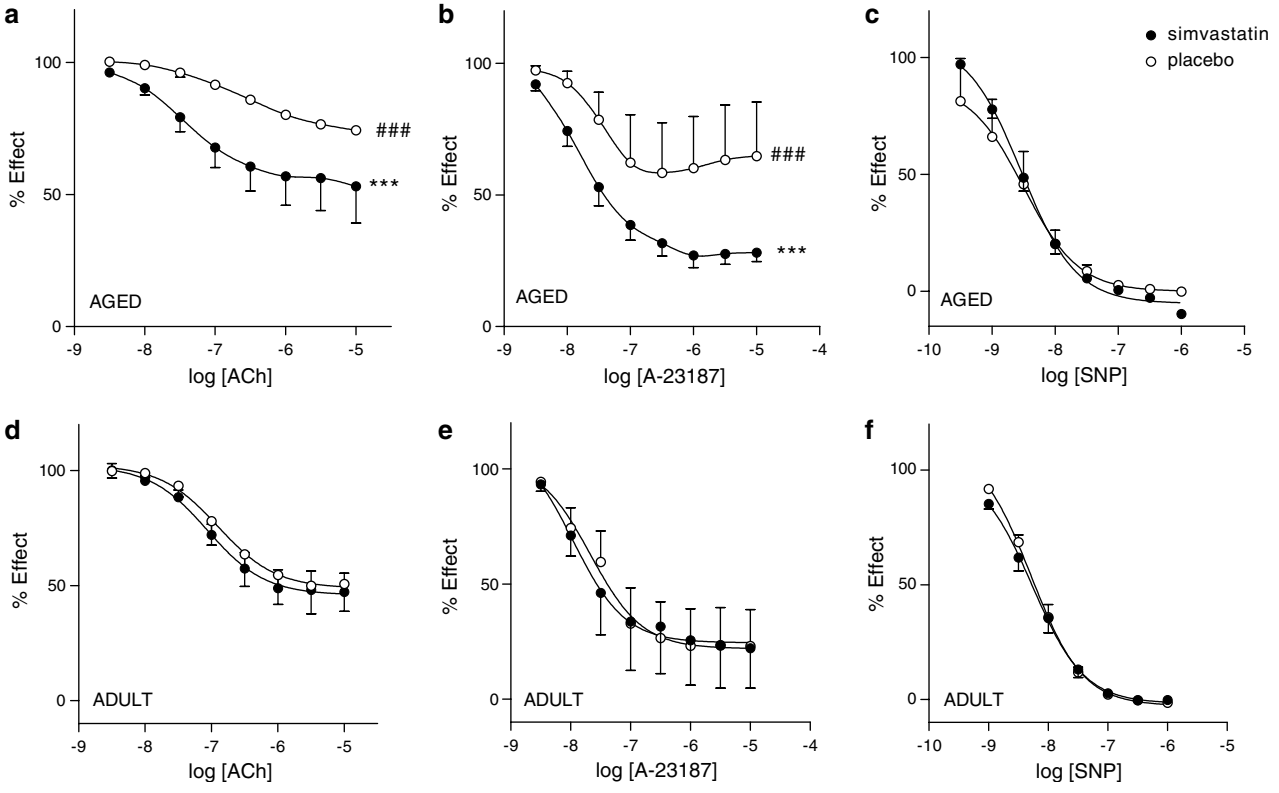

Figure 1 SV improves the endothelial function of aorta from aged (a–c, $n = 7$), but not in those from adult (d–f, $n = 6$), rats after 12 weeks of treatment. Concentration–response curves to ACh (a and d), A-23187 (b and e) and SNP (c and f) in aortic rings from placebo- and SV-treated rats ***$P < 0.001$ placebo *versus* SV-treated rats. ###$P < 0.001$ aged *versus* adult rats.

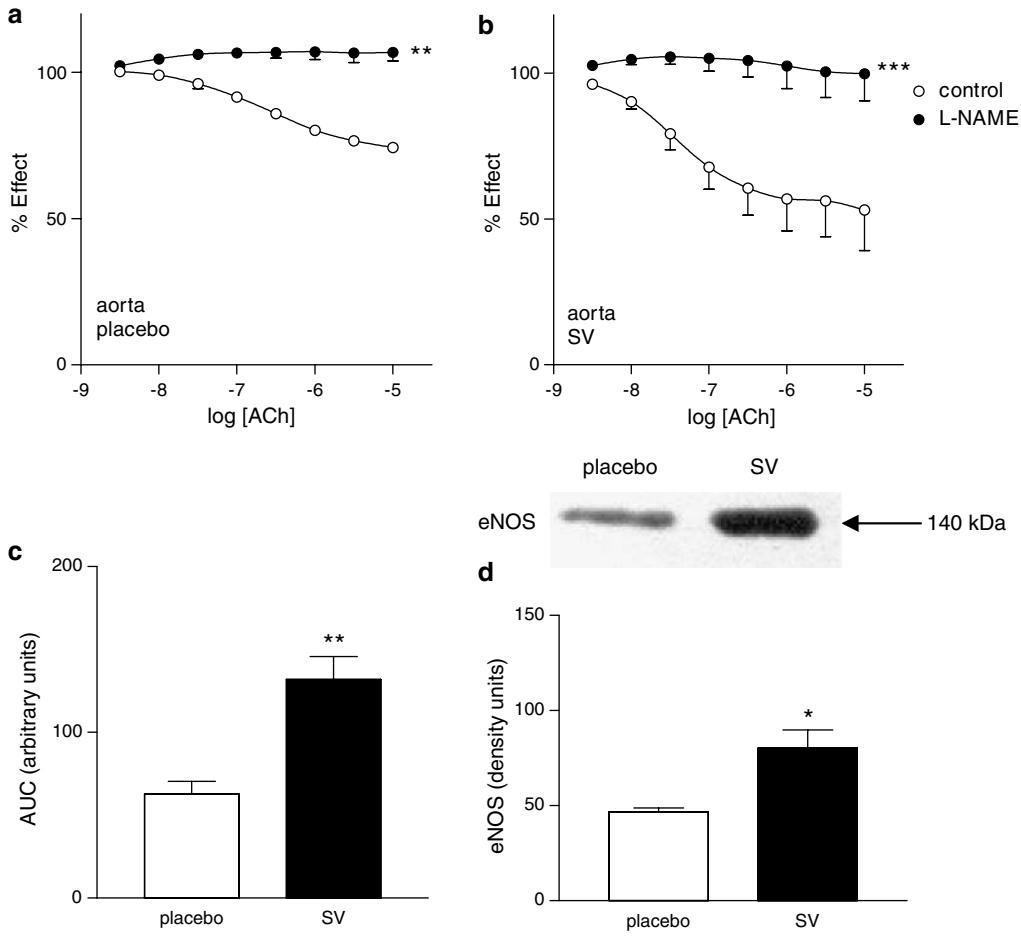

Figure 2 Concentration–response curves to ACh in aortic rings from placebo- (a) and SV-treated (b) rats in the absence and presence of L-NAME (3×10^{-4} mol l^{-1}). $^{**}P<0.01$; $^{***}P<0.001$ control *versus* curve in the presence of L-NAME. NO-mediated vasodilation expressed as difference between areas under the curve in the absence and presence of L-NAME (c). Data represented are mean ± s.e.m. of $n=7$. $^{**}P<0.01$ placebo *versus* SV. Representative Western blot of aortic eNOS and bars showing optic densitometry of $n=4$ blots of eNOS. (d) $^{*}P<0.05$ placebo- *versus* SV-treated rats.

Analysis of variance (ANOVA) and Tukey's multiple comparison test were used for statistical analysis. Differences were considered significant when $P<0.05$. Curves were fitted by a concentration–response nonlinear regression equation. Area under curve (AUC) was calculated from concentration–response curves in the absence or presence of L-NAME in order to evaluate the participation of the NO component of ACh-induced relaxation.

Results

Effect of SV on blood pressure, plasma cholesterol, TG and TAS in old rats (Table 1)

Body weight, SBP, plasma $NO_2 + NO_3$ and LDL-cholesterol were not affected by treatment with SV at the dosage used. Plasma cholesterol and triglycerides were significantly reduced ($P<0.05$). Interestingly, SV significantly reduced oxidised LDL even though it did not affect TAS.

Endothelium-dependent and NO donor-induced relaxation

ACh produced relaxation in a concentration-dependent manner in aortic rings with endothelium, but it failed to produce relaxation in endothelium-denuded arteries (not shown). As expected, the endothelium-dependent response to ACh and the calcium ionophore A-23187, but the relaxation to the NO donor SNP, were significantly decreased in vessels from old *versus* adult rats showing age-related endothelial dysfunction ($P<0.001$; Figure 1a, b, d and e). In old rats, treatment with 1 mg kg^{-1} of SV significantly enhanced relaxation of the aorta in response to both ACh and A-23187 ($P<0.001$ *versus* placebo; Figure 1a and b), but it did not affect relaxations to SNP (Figure 1c). Interestingly, the relaxations to ACh and A-23187 were restored towards that obtained in aorta from adult rats (Figure 1d and e). In aorta from adult rats, SV treatment did not alter either the endothelium-dependent (ACh- and A-23187) or -independent (SNP) relaxations (Figure 1d–f). Since SV improved age-related endothelial dysfunction, all the following experiments have been performed in vessels from old rats.

Effect of SV on endothelial NO pathways

The NO synthase inhibitor L-NAME (3×10^{-4} mol l^{-1}) almost completely abolished endothelium-dependent responses to

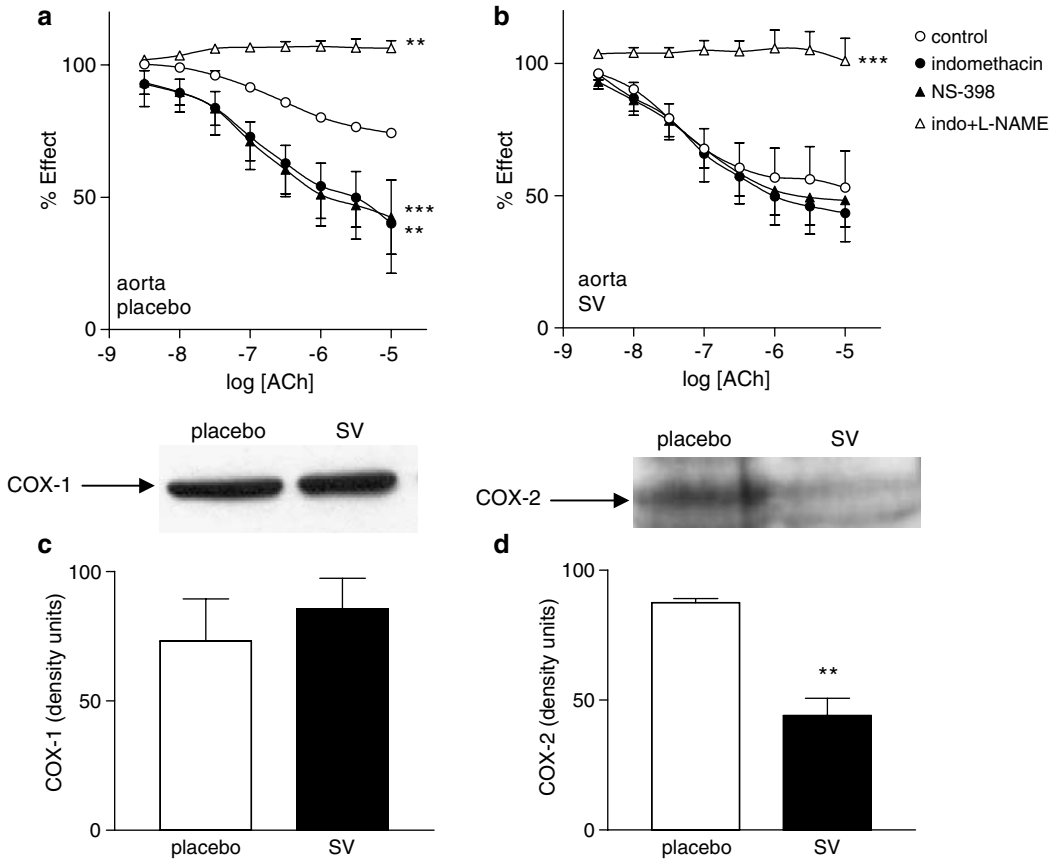

Figure 3 Concentration–response curves to ACh of aortic rings from placebo- (a) and SV-treated (b) rats in the absence and presence of indomethacin (10^{-5} mol l^{-1}), NS-398 (10^{-6} mol l^{-1}) or indomethacin (10^{-5} mol l^{-1}) plus L-NAME ($3\times\ 10^{-4}$ mol l^{-1}). Data represented are mean$\pm$s.e.m. of $n=7$. *$P<0.05$; **$P<0.01$; ***$P<0.001$ control *versus* curve in the presence of inhibitor. Representative Western blot and bars showing optic densitometry of $n=4$ blots of aortic COX-1 (c) and COX-2 (d). **$P<0.01$ placebo *versus* SV.

ACh in aorta from old rats treated with either placebo or SV (Figure 2a and b). In order to compare the participation of NO in relaxation of arteries from placebo and SV-treated rats, the areas under the curves of ACh-induced response were analysed. As shown in Figure 2c, treatment with SV increased the component sensitive to the NO inhibitor of ACh-induced relaxation ($P<0.01$). Interestingly, the 140 kDa eNOS isoform was expressed in aorta and its expression was significantly enhanced in vessels from old rats treated with SV compared to those from rats treated with the vehicle.

Effect of SV on endothelial COX pathways

In aorta from placebo-treated rats, both the nonselective COX inhibitor, indomethacin (10^{-6} mol l^{-1}) and the selective COX-2 inhibitor NS-398 (10^{-6} mol l^{-1}) significantly increased relaxation in responses to ACh (Figure 3a; $P<0.01$ and 0.001, respectively).

In contrast, in aorta from SV-treated rats, ACh-induced relaxations were affected neither by indomethacin nor NS-398 (Figure 3b). In both vehicle- and SV-treated rats, ACh failed to produce relaxation of aortic rings in the presence of indomethacin plus L-NAME (Figure 3a and b). Furthermore, ACh was not able to induce contractile response in L-NAME-treated vessels (data not shown).

SV treatment did not modify the expression of COX-1 enzyme, but it significantly reduced the expression of COX-2 in the aorta (Figure 3c and d).

To evaluate the role of prostanoids acting on Tp receptor, relaxant response to the ACh of aortic rings was studied in the presence of ICI-192,605 (10^{-6} mol l^{-1}). This inhibitor was able to significantly increase response to ACh in aortas from placebo- ($P<0.001$), but not in those from SV-treated rats (Figure 4a and b).

Interestingly, assay of TXB_2, the stable analogue of TXA_2, showed that SV treatment reduced the release of this metabolite in aorta from old rats (Figure 4c), although the release of vasodilatory PGI_2 using the assay of 6-keto-$PGF_{1\alpha}$ was not affected by SV treatment (Figure 4d). Finally, the concentration–response curves to U46619, acting on T_p receptors, were not altered either by ageing or by SV treatment (Figure 4e). Altogether, these data suggest that SV treatment is associated with reduced release of TXB_2, but not a down-regulation of smooth muscle T_p receptors (i.e. receptor expression or post receptor signalling).

Role of oxygen-free radicals in the effect of SV

The effect of the oxygen-free radical scavenger, SOD (150 UI ml^{-1}) plus catalase (1000 UI ml^{-1}) was tested on endothelial responses to ACh. SOD plus catalase significantly

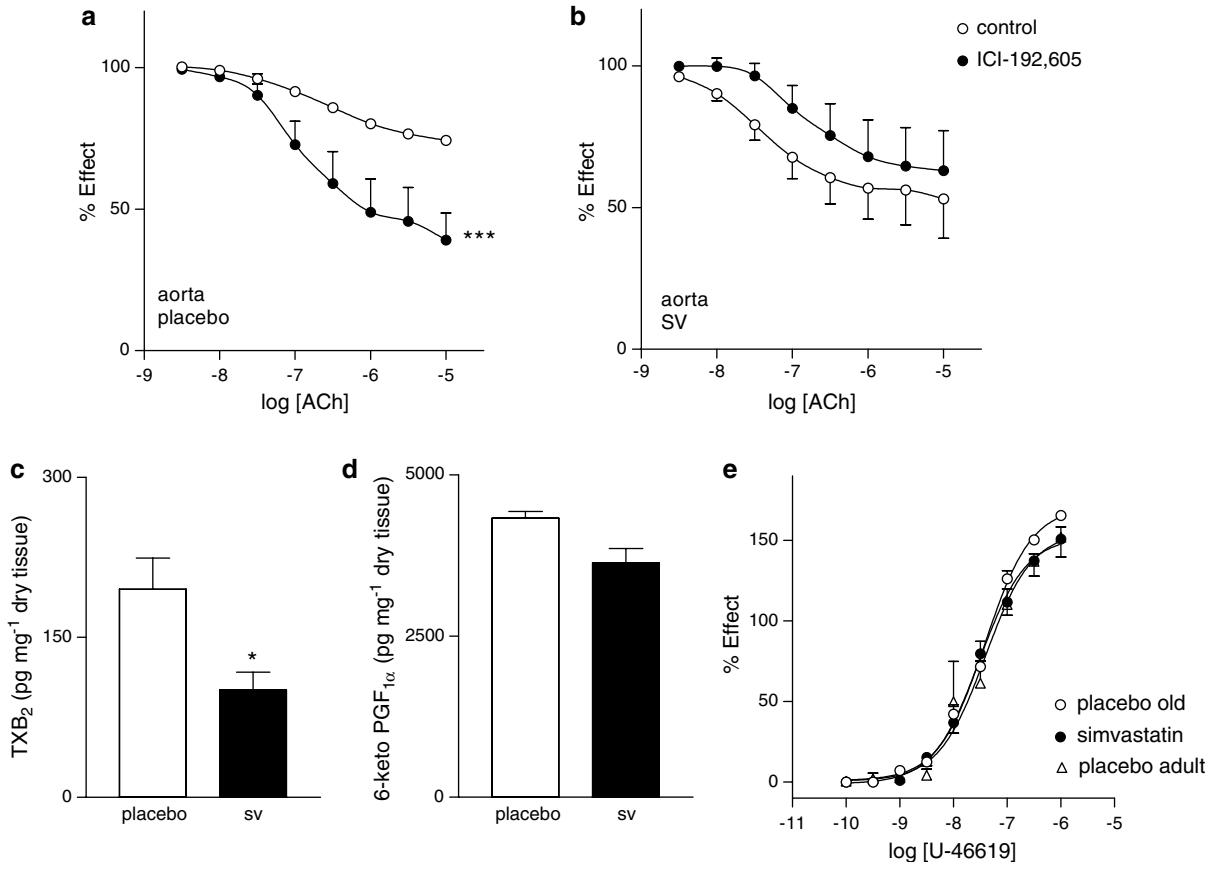

Figure 4 Concentration–response curves to ACh in aortic rings from placebo- (a) and SV-treated (b) rats in the absence and presence of the Tp receptor antagonist ICI-192,605 (10^{-6} mol l^{-1}). ***$P<0.001$ control *versus* curve in the presence of inhibitor. TXB_2 (c) and $PGF_{1\alpha}$ (d) released by aortic rings from placebo- (open bar) and SV-treated (closed bar). Data represented are mean ± s.e.m. of $n=7$. *$P<0.05$ placebo *versus* SV-treated rats. Concentration–response curves to the TX receptor agonist U46619 in endothelium-intact aortic rings from adult (open triangle) and old rats treated with either placebo (open circle) or SV (filled circle) (e). Data represented are mean ± s.e.m. of $n=6$.

enhanced ACh-induced endothelial relaxation in aorta from placebo-treated rats ($P<0.05$; Figure 5a). On the contrary, SOD plus catalase had no effect on ACh-induced relaxation of aortic rings taken from SV-treated rats (Figure 5b).

Discussion

The present study provides evidence that treatment with SV improved age-related endothelial dysfunction in aorta of the rat. The mechanisms involved enhanced endothelial NO vasodilatation through an increase of eNOS expression, decreased participation of TXA_2 associated with decreased expression of the COX-2 isoform and enhanced antioxidant properties of the vessel wall.

SV treatment reduced both total plasma cholesterol and triglycerides without significant decrease in LDL-cholesterol. Interestingly, SV reduced oxidised LDL. Reduced plasma cholesterol and triglycerides probably play a role in the beneficial effect of SV. However, previous data from the literature, including ours, reported that HMG-CoA inhibitors are known to exert several effects without decreasing plasma total cholesterol or LDL cholesterol, including a decrease of macrophage accumulation in atherosclerotic lesions (Baetta *et al*., 2002), a preservation of the structure of coronary adventitia (Wilson *et al*., 2002) and, interestingly, an improvement of endothelial function (Pérez-Guerrero *et al*., 2003). Finally, with equal LDL-cholesterol lowering, SV, but not ezetimide, improved flow-dependent dilation in the radial artery of chronic heart failure patients (Landmesser *et al*., 2005). Thus, reduced or unaltered cholesterol concentrations have been reported depending on the model and the study design. In the present study, the improvement of age-related endothelial dysfunction is associated with reduced plasma cholesterol.

SV has been shown to improve endothelial function and vasomotion in spontaneously hypertensive rats (Alvarez de Sotomayor *et al*., 1999; Carneado *et al*., 2002) and in hypertension induced by chronic inhibition of NO by L-NAME (Pérez-Guerrero *et al*., 2003). Interestingly, SV did not have any effect on endothelial function in normotensive young rats although it increased plasma nitrite concentration (Pérez-Guerrero *et al*., 2003). With regard to ageing, controversial data have been reported. Administration of another lipid-lowering drug such as the lipoprotein lipase activator NO-1886 for 3 months enhances endothelium-dependent relaxation of aorta from aged rats (Kusunoki *et al*., 2002). In contrast, no improvement of age-related endothelial dysfunction has been reported with atorvastatin in old patients (Weverling-Rijnsburger *et al*., 2004) or with cerivastatin in rats (Mukai *et al*.,

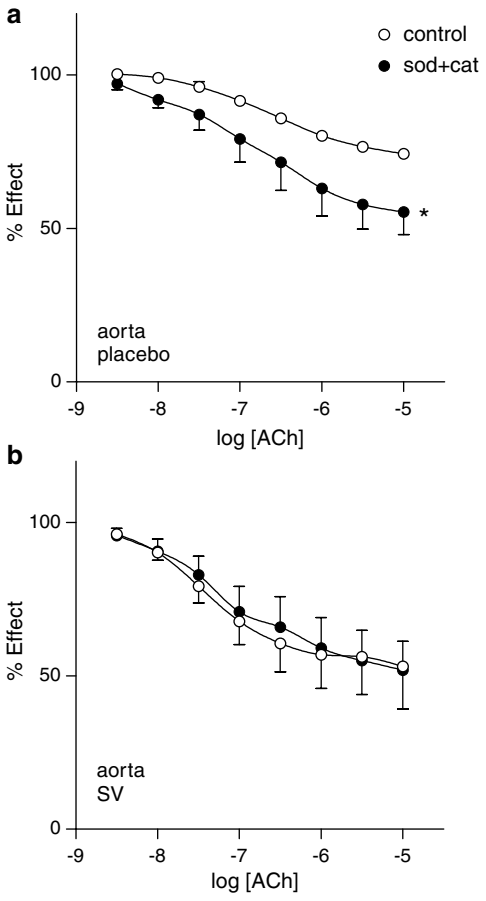

Figure 5 Concentration–response curves to ACh in aortic rings from placebo- (a) and SV-treated (b) rats in the absence and presence of ROS scavengers SOD (150 UI ml^{-1}) plus catalase (1000 UI ml^{-1}). Data represented are mean ± s.e.m. of $n=7$. *$P<0.05$; **$P<0.01$ control *versus* curve in the presence of inhibitor.

2002). Nevertheless, SV 1 mg kg^{-1} daily for 12 weeks was able to restore endothelial function in the present study. The low dose of SV used in the present study does not allow reaching a sufficient plasma concentration of SV able to stimulate endothelium-dependent relaxation. Also, it should be noted that experiments were carried out 3 days after the last dose of SV and the results are probably produced by the long-term effect of SV.

In accordance with our previous studies (Alvarez de Sotomayor *et al*., 1999; Carneado *et al*., 2002), the use of the same dose of SV (i.e. 1 mg kg^{-1}) did not affect SBP (Table 1) even though it improved age-related endothelial dysfunction. In line with our results, Ichihara *et al*. (2005) reported that SV decreased serum cholesterol levels without affecting blood pressure in hyperlipidaemic hypertensive patients (whose blood pressure was insufficiently controlled by antihypertensive therapy). However, it should be noted that the hypotensive effect of statin in humans has been shown with higher doses of this drug and with mildly hypertensive patients (Glorioso *et al*., 1999). Also, there are reports in which statins improve control of blood pressure in hypertensive patients (Borghi *et al*., 2004).

SV treatment improved both ACh- and A-23187-induced endothelium-dependent relaxation. Thus, its effect was not due to a change in agonist receptor number or a default in agonist signal transduction. The most likely hypothesis is that SV acts at the level of either the generation (i.e. synthesis or release) of or response to endothelial factors with ageing. Also, a restoration of the balance between endothelial relaxant (NO and PGI_2) and constricting (vasoconstrictor products from COX or reactive oxygen species (ROS)) factors can be advanced.

With regard to the endothelial NO pathway, the restoration of endothelial function by SV resulted in an enhanced endothelial NO component of the response to ACh. Some effects of HMG-CoA reductase inhibitors have been attributed to their ability to enhance eNOS expression, to improve eNOS mRNA stability and increase eNOS activity or NO availability (Laufs *et al*., 1998). Hence, HMG-CoA reductase inhibitors can reduce the caveolin content of endothelial cells and thus its association with eNOS, which enhances phosphorylation of eNOS by the complex heat shock protein 90-Akt (Brouet *et al*., 2001). In the present study, the improvement of endothelial NO relaxation was associated with an increased expression of eNOS in aorta taken from SV-treated old rats. An increase of smooth muscle sensitivity to NO was unlikely, since the relaxation induced by the NO donor SNP was not affected by SV.

Increased NO availability through a diminished NO breakdown by ROS or an increased antioxidant defence mechanism might contribute to the increased endothelial NO component of the response. The superoxide anions and hydrogen peroxide scavengers, SOD plus catalase, were able to enhance response to ACh in the aorta from placebo-treated old rats, suggesting the participation of ROS in the reduced endothelial NO component of the response. It is interesting to note that SOD plus catalase failed to affect response to ACh in the aorta taken from SV-treated rats. Thus, it might be possible that SV treatment enhances the antioxidant capacity of the vessel wall, resulting either from increased expression of Cu/Zn SOD and catalase or through another antioxidant enzyme. An antioxidant property of SV treatment has already been reported in the improvement of endothelial function in hypertensive animals (Carneado *et al*., 2002). Also, HMG-CoA reductase inhibitors can inhibit the gp91 phox-containing NADPH oxidase, a generator of superoxide anions, which are NO scavengers (Vecchione & Brandes, 2002). It is shown here that SV treatment was sufficient to blunt the potentiating effect of SOD plus catalase in aorta from old rats and this may partially contribute to the increased NO component of ACh-induced relaxation.

Another possible explanation of the restoration of endothelial function by SV is a decreased production of endothelium-derived contracting factors. In accordance with our previous study (Matz *et al*., 2000), we found that using both the nonselective inhibitor of the COX isoforms, indomethacin, and the reported specific inhibitor of COX-2, NS-398, were able to enhance ACh-induced relaxation in the aorta from placebo-treated old rats. Furthermore, increased expressions of COX-1 and COX-2 isoforms were found in these vessels. The nature of COX vasoconstrictor metabolites involved a mechanism sensitive to Tp receptor antagonist ICI-192,605, and was associated with an increased TXA_2 production. These results suggest that endothelial TXA_2 from COX-2 isoforms are involved in the reduced endothelial relaxation in aorta from old rats. SV did not affect contractile response after stimulation of Tp receptor by the Tp receptor agonist, U-46619. Altogether, these results support the hypothesis that

the effect of SV is related to a decrease of TXA_2 production, but not to an effect on either expression of Tp receptors or the downstream signalling pathway of smooth muscle. SV treatment abolished the participation of indomethacin- and NS-398-sensitive components of ACh-induced response. Furthermore, the Tp receptor antagonist ICI-192,605 was not able to potentiate ACh-induced relaxation, and the stimulation of TXA_2 production in response to the same agonist was significantly reduced in the aorta from SV-treated rats. Thus, SV treatment counteracts the participation of endothelial TXA_2 from COX isoforms in aorta from aged rats. This effect of SV had not previously been reported. The vasoactive product released through COX isoforms is shifted towards vasoconstrictor prostanoids with ageing. Here we found that SV reduced TXA_2 production, but it did not affect the release of 6-keto-$PGF_{1\alpha}$. Finally, these effects of SV were associated with reduced expression of COX-2, but not COX-1, isoforms in aorta from aged rats. Taken together, SV treatment reduced the capacity of the aorta from old rats to release vasoconstrictor products from the COX-2 isoforms without affecting the release of the vasodilatory prostanoid, PGI_2. The latter may participate in the restoration of endothelial dysfunction. These results were reinforced by the reports showing that HMG-CoA reductase inhibitor was able to reduce COX-2 expression in primary endothelial cells through the regulation of peroxisome proliferator-activated receptor-alpha (Inoue *et al*., 2000) and plaque macrophages (Cipollone *et al*., 2003). Further studies are required to determine the mechanisms by which SV affects the release of endothelial TXA_2 from COX-2 isoforms in aorta from old rats. Finally, ACh failed to produce relaxation, in L-NAME-treated vessels after blockade of the COX pathway with indomethacin. Furthermore, we did not find any contractile response to ACh in the presence of L-NAME in aged rats. Thus, the release of endothelial TXA_2 observed during ageing was able to counteract NO-induced relaxation but it might not be released in sufficient amount to induce contraction in the absence of NO. From these experiments, one can advance the hypothesis that both pathways, NO and prostanoids, may merge to a final common pathways.

In conclusion, we provide evidence that treatment with the HMG-CoA reductase inhibitor, SV, restored endothelial function in the aorta from aged rats. The results shed light on the potential therapeutic effect of SV in enhancing the endothelial NO vasodilatation linked to an increase expression of eNOS and in reducing the release of TXA_2 from COX-2 without affecting the release of PGI_2. Increased antioxidant capacity of the vessel wall may play a role in the beneficial effect of SV. Thus, treatment with SV may be useful in improving age-related endothelial dysfunction and may contribute to the explanation of some clinical features of this drug in old patients (Eaton *et al*., 2002).

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