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Characterization of endothelial factors involved in the vasodilatory effect of simvastatin in aorta and small mesenteric artery of the rat

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1 Vascular effects of the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor, simvastatin, were studied in conductance (aorta) and resistance vessels (branch II or III of superior mesenteric artery, SMA) of the rat (12–14 weeks old).

2 Simvastatin produced relaxation of both aorta and SMA, with and without functional endothelium. These responses were inhibited by the product of HMG-CoA reductase, mevalonate (1 mmol l^{-1}).

3 In vessels with functional endothelium, the NO-synthase inhibitor, L-N^G-nitroarginine (L-NOARG, 30 μmol l^{-1}), inhibited simvastatin-induced relaxation. In the presence of L-NOARG, relaxation to simvastatin was lower in vessels with endothelium than in endothelium-denuded arteries without L-NOARG.

4 The cyclo-oxygenase inhibitor, indomethacin (10 μmol l^{-1}), abolished endothelium-dependent component of the response to simvastatin in both arteries. The combination of L-NOARG plus indomethacin did not produce further inhibition. The T_p receptor antagonist, GR 32191B (3 μmol l^{-1}), did not affect relaxation in aorta but it reduced response to low concentrations of simvastatin in SMA. However, the inhibitory effect of L-NOARG was less marked in the presence of GR 32191B in aorta but not in SMA.

5 The endothelium-dependent relaxation to simvastatin was inhibited by the superoxide dismutase (SOD, 100 u ml^{-1}) or by the tyrosine kinase inhibitor, genistein (30 μmol l^{-1}) in the two arteries.

6 The present study shows that simvastatin produces relaxation of conductance and small arteries through mevalonate-sensitive pathway. The endothelium-dependent relaxation to simvastatin involves both NO and vasodilator eicosanoids by a mechanism sensitive to SOD, and to genistein. Also, the results highlighted participation in the aorta of endothelial vasoconstrictor eicosanoids acting on the T_p receptor after blockage of NO synthase only.

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Keywords: 3-Hydroxy-3-methylglutaryl-coenzyme A reductase; simvastatin; endothelium; nitric oxide; cyclo-oxygenase products; tyrosine kinase; rat aorta; small mesenteric artery

Abbreviations: ACh, acetylcholine; CHAPS, 3-[3-cholamidopropyl) dimethylammonio]-1, propane sulphonate; COX, cyclo-oxygenase; DMSO, dimethylsulphoxide; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; LDL, low density lipoprotein, L-NOARG, L-N^G-nitroarginine; NA, norepinephrine; PSS, physiological salt solution; SMA, superior mesenteric artery; SOD, superoxide dismutase

Introduction

Simvastatin is a drug widely used in the treatment of hypercholesterolemia. Simvastatin acts as an inhibitor of the rate-determining enzyme in the biosynthesis of cholesterol, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase and has been proved useful in the reduction of plasma low density lipoprotein (LDL) (Mauro & Macdonald, 1991). Clinical trials have demonstrated that inhibitors of HMG-CoA reductase reduce cardiovascular-related morbidity and mortality in patients with and without coronary artery disease (Scandinavian Simvastatin Survival Study Group, 1994) and that endothelium-mediated responses were improved in arteries from patients treated with HMG-CoA reductase inhibitors (Treasure *et al*., 1995), this improvement persisted with continued administration of simvastatin despite the absence of further reduction in serum cholesterol levels (O'Driscoll *et al*., 1997).

HMG-CoA reductase is also involved in the biosynthetic pathway of isoprenoids from mevalonate (Goldstein & Brown, 1990). Isoprenoids have been shown to play a role in the mechanisms leading to vascular smooth muscle cells proliferation and migration. Thus, the antiatherosclerotic effect of simvastatin might in part results from its inhibitory effect on the synthesis of isoprenoids, independently of its hypocholesterolemic properties (Soma *et al*., 1995; Raiteri *et al*., 1997). Clinical benefits of simvastatin therapy have been reported as being best explained by their direct effects on each component of the triad, on atherosclerotic and thrombotic mechanisms within arteries, as well as through the more conventionally accepted way of decreasing plasma LDL concentrations (Vaughan *et al*., 1996).

In previous studies, we found that another HMG-CoA reductase inhibitor, lovastatin was able to decrease blood

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pressure and produce relaxation of the isolated rat thoracic aorta (Bravo *et al*., 1998). We also found that chronic treatment with simvastatin improved endothelial dysfunction in spontaneously hypertensive rats (Alvarez de Sotomayor *et al*., 1999).

The present study was carried out to determine the vascular effect of simvastatin in both the conductance and resistance arteries of the rat. Also the mechanism of action and the relative contribution of endothelial nitric oxide (NO) and cyclo-oxygenase (COX) metabolites in relaxation to simvastatin was examined.

Methods

Animals

Male Wistar rats from 12–14 (adult) weeks old were bred in our institute from genitors provided by Iffa-Credo (Lyon, France). All rats were maintained in a colony room with fixed dark : light cycles and constant humidity and temperature. They were provided with rodent chow AO4 from U.A.R. (Villemoisson, France) and tap water *ad libidum*. This investigation conforms to authorization (number 01918) for the use of laboratory animals given by the French government (Department of Agriculture).

Arterial preparation and mounting

The animals were killed by cervical dislocation and exsanguinated. The thoracic aorta and branch II or III of the superior mesenteric artery (SMA) were carefully removed and cleaned of fat and connective tissue. Then, artery segments (2–3 mm or 1.6–2.0 mm in length for the aorta and the SMA, respectively) were mounted on myographs filled with physiological salt solution (PSS) of the following composition (in mmol l^{-1}): NaCl 119, KCl 4.7, $MgSO_4$ 1.17, KH_2PO_4 (1.18 and 0.4), $NaHCO_3$ (25 and 14.9), $CaCl_2$ (1.8 and 2.5) and glucose (11 and 5.5) for the aorta and the SMA, respectively. The PSS was continuously kept at 37°C and gassed with 95% O_2 and 5% CO_2 at pH 7.4. Resting tension was adjusted to 2 g for the aorta and 200 mg for the SMA as previously described (Andriambeloson *et al*., 1997; Martinez *et al*., 1996). Mechanical activity was recorded isometrically by a force transducer (Kistler-Morse, DSG BE4). In some experiments, the endothelial layer was removed immediately after dissection either by gently rubbing the intimal surface with curved forceps for the aorta or by intraluminal perfusion with 0.5% 3-[(3-cholamidopropyl) dimethylammonio]-1 propane sulphonate (CHAPS) in PSS for 25 s followed by repeated washing with PSS for the SMA.

After setting the vessel to its working length, challenges with 10 μmol l^{-1} norepinephrine (NA) were performed in both aorta and SMA to test their maximal contractile capacity and to elicit reproducible contractile response.

The presence of functional endothelium was assessed, in adult rats, by the ability of acetylcholine (ACh, 1 μmol l^{-1}) to induce more than 50% relaxation of vessels pre-contracted with NA (10 μmol l^{-1}). The absence of a relaxation response to ACh was taken as evidence that the vessel segments were functionally denuded of endothelium.

Relaxation experiments

Arteries with and without functional endothelium were pre-contracted at 80% of their maximal contraction with NA. The concentration of NA was adjusted for each preparation. In vessels with functional endothelium, NA concentration used was 1 μmol l^{-1} for the aorta and 3 μmol l^{-1} for the SMA. For each preparation was checked that NA-induced contractions were stable during all the experiments. When the contraction reached a plateau, cumulative addition of simvastatin was performed. Concentration-response curves were constructed in the absence or in the presence of the indicated inhibitor(s). The following inhibitors were used: the product of HMG-CoA reductase, mevalonate (1 mmol l^{-1}), the NO synthase inhibitor, L-N^G-nitroarginine (L-NOARG, 30 μmol l^{-1}), the COX inhibitor, indomethacin (10 μmol l^{-1}), the thromboxane A_2/prostaglandin H_2, Tp receptor antagonist, [1R-[1α(z),2β,3βm 5α]]-(+)-7-[5-[[(1,1′-biphenyl)-4-yl] methoxy]-3-hydroxy-2-(1-piperidinyl) cyclopentyl]-4-heptenoic acid, hydrochloride, (GR 32191B, 3μmol l^{-1}), the superoxide anion (O_2^-) scavenger, superoxide dismutase (SOD, 100 u ml l^{-1}) and the tyrosine kinase inhibitor, genistein (30 μmol l^{-1}). The concentration of L-NOARG used in the present study was 30 μmol l^{-1} in order to avoid non specific effect as previously reported (Wang & Pang, 1994). All inhibitors were used at a maximally active concentration and were incubated with the tissue for 20 min before the pre-contraction with NA except for mevalonate (i.e. 2 h prior to pre-contraction with NA) and genistein (added at the same time as NA). Indomethacin was used at maximally active concentration as previously reported (Cortes *et al*., 1996). GR 32191B at used concentrations completely abolished contractile response produced by the thromboxane A2 analogue, U46619 up to 1 μM (a concentration that produced maximal contraction both in the aorta and SMA). The efficacy of SOD has been functionally tested on rat aortic rings using pyrogallol as a generator of superoxide anions. On vessels pre-contracted with 10 nM noradrenaline, pyrogallol (100 μM) produced further contraction. When the response to pyrogallol reached a steady-state level, addition of SOD (100 u/ml) completely inhibits the pyrogallol-induced contractile response. Finally, the contraction of genistein reduced responses linked to the activation of the tyrosine kinase pathway (Akiyama *et al*., 1987) without affecting the contractile capacity of the vessels.

Drugs

GR 32191 B was generously provided by Glaxo Research and Development (Hertfordshire, U.K.) and simvastatin by Merck laboratories (New Jersey, U.S.A.). All other chemicals were purchased from Sigma Chemical Co (St. Louis, MO, U.S.A.). All drugs were dissolved in distilled water except glybenclamide, indomethacin, mevalonate and simvastatin which were dissolved in dimethylsulphoxide (DMSO). The final DMSO concentration in the bath was less than (0.01%) and preliminary experiments showed that this concentration of DMSO neither significantly affected the NA pre-contraction nor the relaxation to simvastatin.

Expression of results and statistical analysis

Control experiments showed that two consecutive concentration-response curves of simvastatin were not significantly different in the absence of other treatments. The first curve was thus taken as control. The maximal contractions obtained with NA (10 μmol l^{-1}) were not significantly different in vessels with or without endothelium, being respectively 1.94 ± 0.23 g ($n=9$) and 2.19 ± 0.31 g ($n=9$) for the aorta, and 1.33 ± 0.08 g ($n=9$) and 1.17 ± 0.07 g ($n=9$)

for the SMA. Results were expressed as a percentage of the relaxation from the initial NA-induced pre-contraction level. The values of contractions obtained with NA (1 μmol l^{-1} for aorta and 3 μmol l^{-1} for SMA) were 1.72 ± 0.20 ($n=9$) in the aorta and 1.06 ± 0.12 ($n=9$) g in the SMA. All results are expressed as mean $\pm$ s.e.mean of n experiments, n representing the number of rats. Analysis of variance (MANOVA) followed by Tukey's Multiple Comparison test were used as appropriate for statistical analysis. Differences were considered significant when $P<0.05$.

Results

Characterization of the relaxant effect of simvastatin

As illustrated in Figure 1, during the period needed for constructing concentration-response to simvastatin, the plateau of the pre-contraction obtained with NA was stable without significant spontaneous loss of contraction both in the aorta and in the SMA. Under these experimental conditions, simvastatin produced relaxation in a concentration-dependent manner both in aorta and SMA with and without functional endothelium (Figures 1a,c, 2a,b). In endothelium denuded vessels, relaxation occurred at a concentration 10 fold higher than that necessary to obtain relaxation in vessels with functional endothelium in both types of arteries. In the SMA, the relaxation to simvastatin occurred at lower concentrations than that necessary to obtain a response in the aorta (1 nmol l^{-1} *versus* 1 μmol l^{-1}). Furthermore, in the SMA, the concentration-response curve to simvastatin showed a biphasic profile both in the presence or in the absence of functional endothelium (Figure 2b). Thus, the first phase of relaxation started from 1 nmol l^{-1} and plateaued at 1 μmol l^{-1} and the second phase of the response begun at 1 μmol l^{-1}, the maximal relaxation being reached at 100 μmol l^{-1}.

The product of HMG-CoA reductase, mevalonate (1 mmol l^{-1}) significantly inhibited the relaxation to simvastatin in both types of arteries with and without endothelium ($P<0.001$) (Figure 3). In the SMA, mevalonate completely abolished the first phase and significantly reduced the second phase of simvastatin-induced relaxation (Figure 3b).

Characterization of endothelial factors released by simvastatin

To test whether NO is involved in the endothelium-dependent relaxation to simvastatin the effect of the NO-synthase inhibitor, L-NOARG (30 μmol l^{-1}) was studied. In these conditions, L-NOARG produced a significant rightward shift of the relaxation to simvastatin both in the aorta and in the SMA with endothelium (Figure 4a,b). As expected, L-NOARG did not affect the relaxation to simvastatin in the absence of functional endothelium in both types of arteries (not shown). Furthermore in the presence of L-NOARG, the relaxation to simvastatin was significantly lower in vessels with intact endothelium than in the relaxation obtained in endothelium-denuded arteries without L-NOARG ($P<0.05$) in both types of arteries (compare Figure 4a,b, with Figure 2a,b). These data suggest that in the presence of endothelium, simvastatin-induced relaxation is partly mediated by NO. In addition, they also indicate that blockage of endothelial NO production either inhibits the release of other endothelial relaxant factors, reveals release of endothelial constricting factors, or both.

The COX inhibitor, indomethacin (10 μmol l^{-1}), completely abolished endothelium-dependent relaxation but it did not affect the endothelium-independent response to simvastatin in either type of arteries (Figure 4c,d). Exposure to

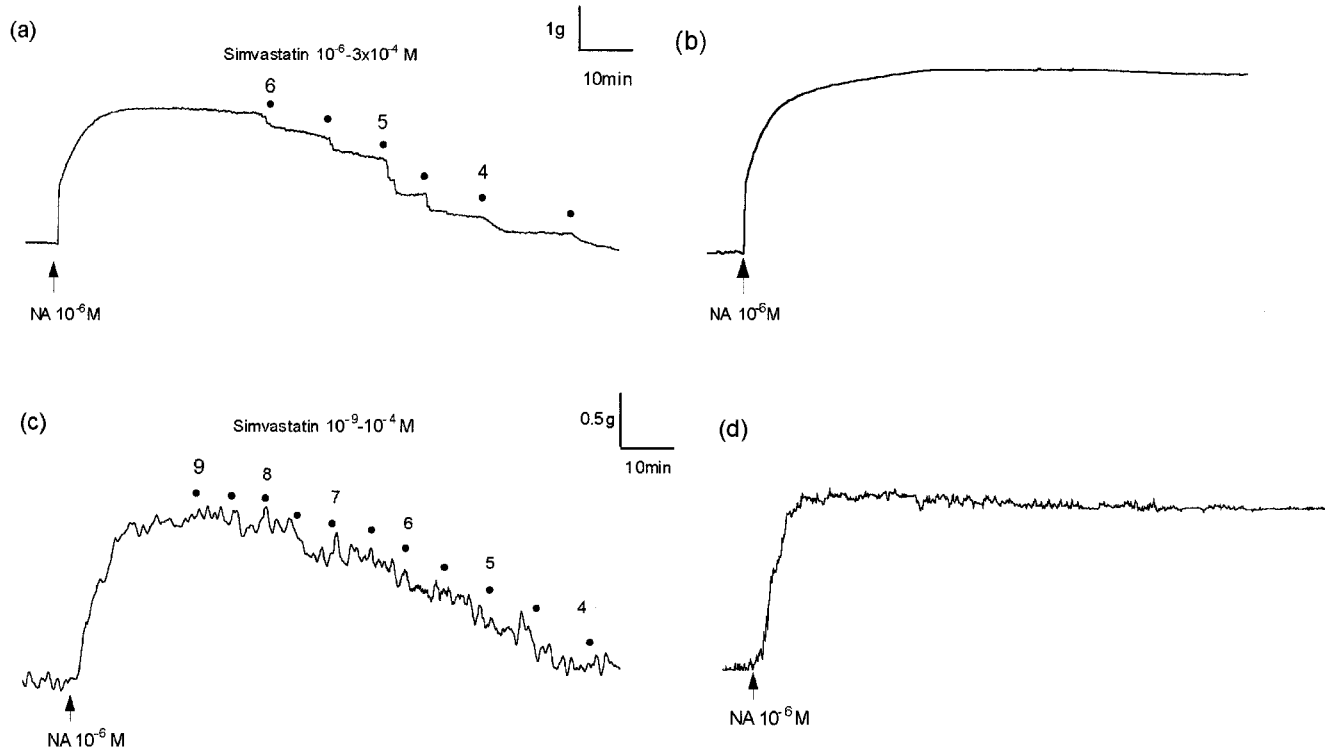

Figure 1 Representative original record of the relaxation induced by simvastatin in NA-contracted aortic rings (a) and SMA (c), and the contractile effect of NA during all the experiment (b and d).

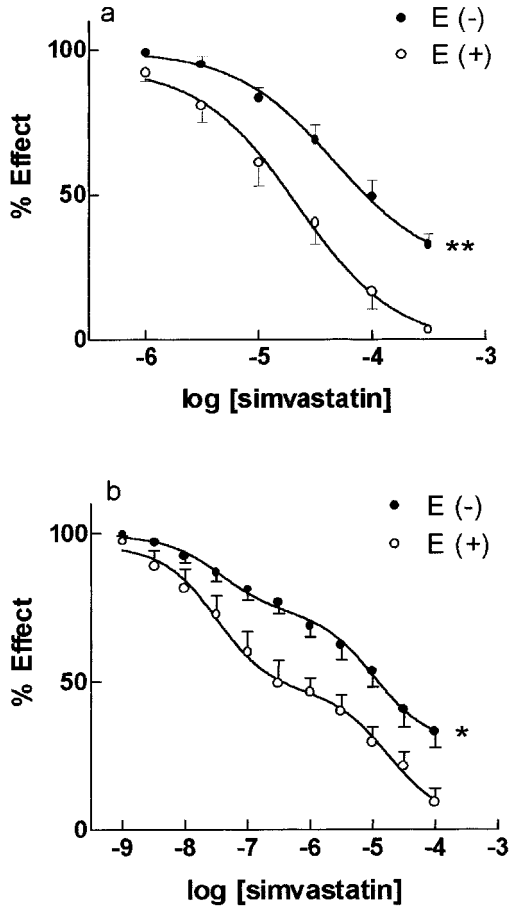

Figure 2 Simvastatin-induced relaxation in aortic rings (a) and SMA (b) with (E (+)) and without endothelium (E (−)) precontracted with NA. Data represent means ± s.e.mean of $n=9$ experiments. $^{**}P<0.01$; $^{*}P<0.05$ *versus* values obtained in intact arteries.

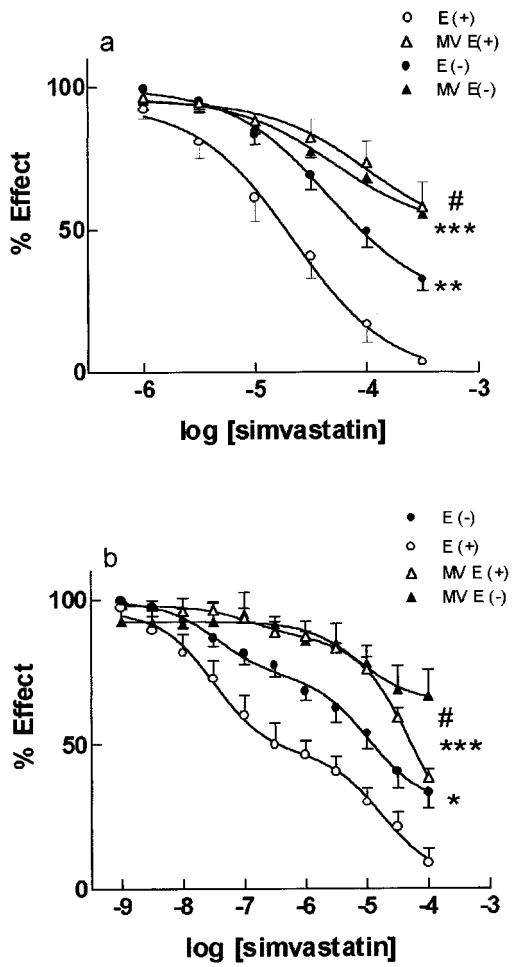

Figure 3 Effect of mevalonate (MV 1 mmol l^{-1}) on simvastatin-induced relaxation of aortic rings (a) and SMA (b) precontracted with NA, compared with relaxation induced by simvastatin in endothelium intact (E (+)) and denuded (E (−)) arteries. Data represent means ± s.e.mean of $n=6$ experiments. $^{*}P<0.05$, $^{**}P<0.01$. $^{***}P<0.001$ *versus* values obtained in intact arteries. $\#P<0.05$ values obtained in endothelium denuded arteries.

indomethacin (10 μmol l^{-1}) plus L-NOARG (30 μmol l^{-1}) did not result in further alteration in relaxation to simvastatin compared to indomethacin alone in both arteries (Figure 4e,f). However, the inhibitory effect of L-NOARG (Figure 4a,b) was less marked in the presence of indomethacin (Figure 4e,f). Taken together, these data suggest that simvastatin-induced relaxation involved vasodilatory products from COX. In addition, comparison between the effect of L-NOARG alone and L-NOARG plus indomethacin shows that blockage of endothelial NO unmasked the participation of endothelial vasoconstrictor products upon simvastatin stimulation.

To verify the nature of endothelial vasoconstrictor products from the COX involved, the effect of the thromboxane A_2/prostaglandin H_2 receptor antagonist, GR 32191 B (3 μmol l^{-1}) was investigated on simvastatin-induced relaxation (Figure 5). In the aorta, GR 32191 B did not significantly affect the concentration-response curve to simvastatin (Figure 5a). However, in the SMA, GR 32191 B modified the biphasic concentration-response curve to simvastatin into a monophasic profile (Figure 5b). Furthermore, the inhibitory effect of L-NOARG alone was less marked in the presence of GR 32191 B in the aorta. The concentration-response curve to simvastatin obtained in the presence of GR 32191 B plus L-NOARG (Figure 5c) was not significantly different from that obtained in the presence of indomethacin plus L-NOARG. In contrast to the aorta, the concentration-response curve to simvastatin was not significantly different in the presence L-NOARG alone or in combination with GR 32191 B in the SMA (Figure 5d). Taken together, these data suggest that the endothelial vasoconstrictor products from COX released upon simvastatin stimulation involves TXA_2 or other prostanoids acting on T_p receptors in the aorta but not in the SMA.

Mechanism of action involved in the endothelium-dependent relaxation to simvastatin

To investigate whether an augmented production of O_2^- is involved in the mechanism by which simvastatin produced endothelium-dependent relaxation, the effect of SOD (100 u ml^{-1}) was studied. In these conditions SOD completely inhibited the endothelium-dependent relaxation to simvastatin in the aorta (Figure 6a). In the SMA, SOD modified the biphasic concentration-response curve to simvastatin into a monophasic profile (Figure 6b). Thus, SOD inhibit the response to low concentrations (<1 μmol l^{-1}) of simvastatin. These data suggest that production of O_2^- is involved in the simvastatin-induced

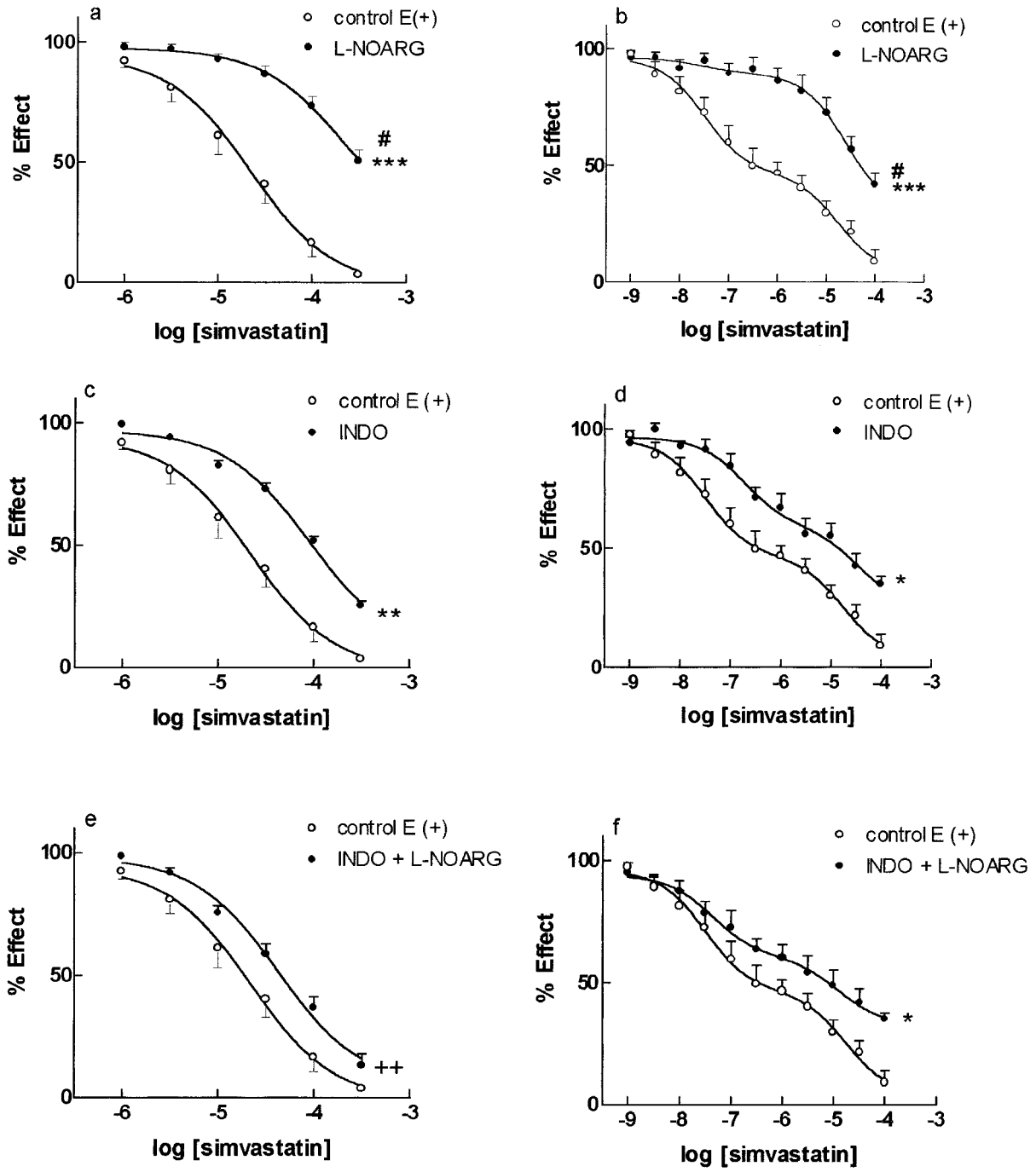

Figure 4 Effect of NO synthase inhibitor, L-N^G-nitroarginine (L-NOARG 30 μmol l^{-1}), cyclooxygenase inhibitor, indomethacin (INDO 10 μmol l^{-1}) or L-NOARG plus indomethacin on simvastatin-induced relaxation of aortic rings (a, c and e) and SMA (b, d and f) precontracted with NA, compared with the relaxant effect of simvastatin in arteries with functional endothelium (control E (+)). Data represent means ± s.e.mean of $n=6$ experiments. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ *versus* values obtained in intact arteries $\#P<0.05$ values obtained in endothelium denuded arteries. $++P<0.01$ *versus* curve made in arteries in the presence of L-NOARG (30 μmol l^{-1}).

endothelium-dependent relaxation in the aorta. The latter mechanism is also involved in response to low concentrations of simvastatin in the SMA.

Finally, to test whether the tyrosine kinase pathway is involved in the endothelium-dependent relaxation, the effect of the tyrosine kinase inhibitor, genistein (30 μmol l^{-1}), was studied. Both in the aorta and in the SMA, genistein inhibited the relaxation to simvastatin in vessels with but not in those without functional endothelium (Figure 7). These data suggest that a mechanism sensitive to the tyrosine kinase inhibitor, genistein, plays a major role in the endothelium-dependent relaxation to simvastatin in both types of arteries.

Discussion

The present study shows that simvastatin is able to produce vascular relaxation, independent of it lipid lowering property, both in conductance and small arteries, by acting on smooth muscle and endothelium. The endothelium-dependent relaxation to simvastatin involves the release of both NO and

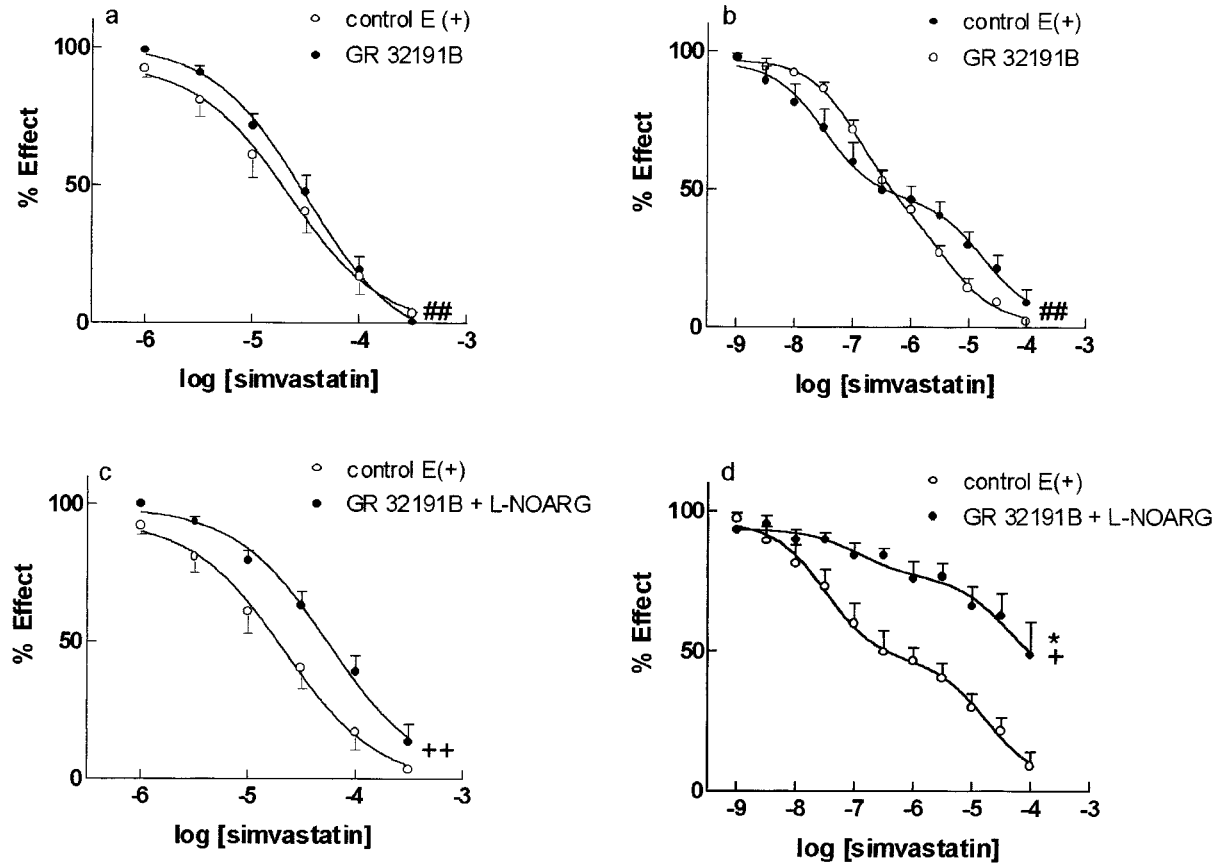

Figure 5 Effect of T_p receptor antagonist, GR 32191B (3 μmol l^{-1}) and GR 32191B plus L-NOARG on simvastatin-induced relaxation of aortic rings (a and c) and SMA (b and d), compared with relaxant effect of simvastatin in arteries with endothelium (control E (+)) precontracted with NA. Data represent means ± s.e.mean of $n=6$ experiments. $^{**}P<0.01$; $^{*}P<0.05$ *versus* values obtained in intact arteries. $\#\#P<0.01$ *versus* curve made in endothelium denuded arteries. $+P<0.05$; $++P<0.01$ *versus* curve made in arteries in the presence of L-NOARG (30 μmol l^{-1}).

vasodilator products from COX by a mechanism sensitive to O_2^- scavengers, SOD. Also, a complex interaction between NO and the release of endothelial vasoconstrictor products from COX in the effect of simvastatin is observed in the two arteries. Meanwhile, the nature of the vasoconstrictor products from COX are different in the two arteries. Finally, the relaxation to simvastatin involved both mevalonate and tyrosine kinase pathways.

Clinical trials showed that inhibitors of HMG-CoA reductase reduce cardiovascular-related morbidity and mortality in patients with and without coronary artery disease (Scandinavian Simvastatin Survival Group, 1994). The clinical benefit of these inhibitors appears early in the course of lipid lowering therapy before the occurrence of plaque regression and is best explained by the direct effects of statins on atherosclerotic and thrombotic mechanisms within arteries, as well as decreasing LDL concentrations. Improvement in endothelial function and vasomotion have been demonstrated not only in hypercholesterolaemic patients treated with statins (Treasure *et al*., 1995; O'Driscoll *et al*., 1997) but also in spontaneously hypertensive rats (SHR) (Alvarez de Sotomayor *et al*., 1999).

HMG-CoA reductase inhibitors have shown some effects in cell experiments and animal models that could help to explain the results of the clinical trials. In patients treated with therapeutic dose of HMG-CoA reductase inhibitors (from 20 to 60 mg daily), the plasma concentration of simvastatin was ranged between 0.01 and 1 μM (Laufs *et al*., 1998a; Lilja *et al*., 1998; Kantola *et al*., 1998). Thus, sufficient concentration of simvastatin that could promote endothelial-dependent relaxation (i.e. the present study) might be achieved in the plasma of patient receiving high therapeutical dose of simvastatin. One possible mechanism by which statins might improve vasomotion is their vasodilator properties at the level of smooth muscle. Indeed in the present study, simvastatin was able to produce relaxation of both aorta and small arteries in the absence of functional endothelium. These effects of simvastatin might take place in the mechanism of agonist-induced increase in cytosolic calcium involved in vascular smooth muscle contraction. These mechanisms include a decrease in the release of Ca^{2+} from thapsigargin-sensitive pool, inhibition of inositol triphosphate-dependent Ca^{2+} mobilisation (Ng *et al*., 1994; Escobales *et al*., 1996) or blockade of L-type Ca^{2+} channels (Yada *et al*., 1999).

Another relevant hypothesis on the vasodilator properties of simvastatin might involve the participation of endothelial factors including NO and vasoactive products from COX. In the present work, relaxation produced by low concentrations of simvastatin required the presence of the endothelium both in the aorta and the SMA suggesting the involvement of endothelial factors. Turning to NO, HMG-CoA reductase inhibitors have been reported to up-regulate the expression of NO synthase (Laufs *et al*., 1998a) and to improve endothelial NO synthase mRNA stability (Laufs *et al*., 1998b). Also, HMG-CoA reductase inhibitors have been shown to prevent the inhibitory action exerted by oxidized LDL on the expression and activity of endothelial NO synthase mRNA

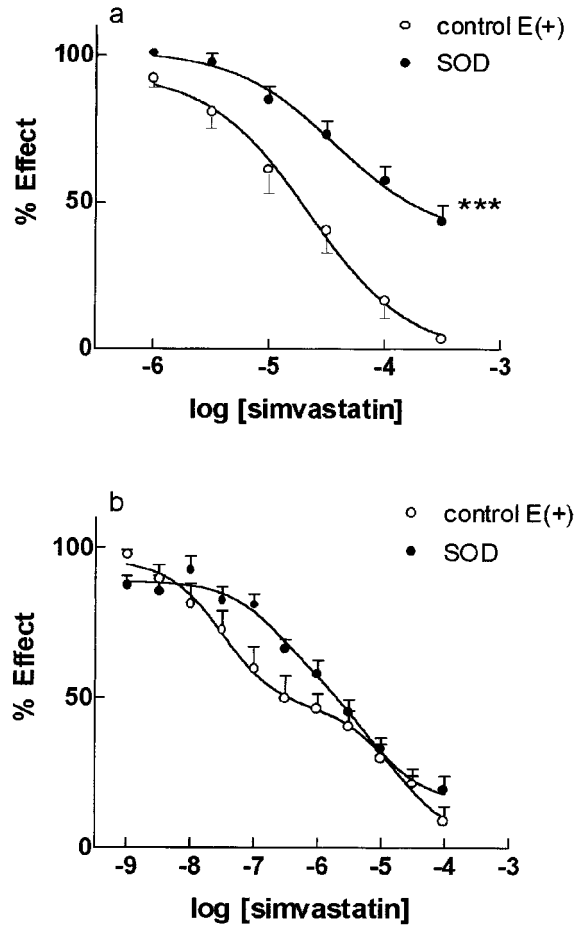

Figure 6 Effect of superoxide dismutase (100 u ml^{-1}) on simvastatin-induced relaxation of aortic rings (a) and SMA (b), compared with the effect induced by simvastatin in arteries with endothelium (control E(+)) precontracted with NA. Data represent means ±s.e.mean of $n=6$ experiment. ***$P<0.001$; **$P<0.01$; *$P<0.05$ *versus* values obtained in intact arteries.

and protein (Hernández-Perera *et al*., 1998). The above mentioned effects of HMG-CoA reductase inhibitors might not contribute to the vasodilatation produced by simvastatin in the present study. Indeed, the time course of the relaxation produced by simvastatin occurred within a minute whereas modulation of endothelial NO expression or endothelial NO synthase stability required a much longer time period. Thus, the simplest explanation about the participation of NO in simvastatin-induced relaxation is the activation by the statin of endothelial NO synthase and therefore NO release. Indeed, such an effect has been previously reported for another HMG-CoA reductase inhibitor, pravastatin (Kaesemeyer *et al*., 1999).

Another hypothesis is to consider that simvastatin mediates its effect *via* protection of NO breakdown by O_2^-, since this drug has shown anti-oxidant properties (Aviram *et al*., 1998; Suzumura *et al*., 1999). Moreover, it has been shown recently that HMG-CoA reductase inhibitors can improve NO-mediated relaxation by a mechanism sensitive to the O_2^- scavenger, SOD. Indeed, the authors found HMG-CoA reductase inhibitors can attenuate endothelial O_2^- formation by preventing isoprenylation of p21 Rac (Wagner *et al*., 2000). However, in the present study, SOD did not improve relaxation to simvastatin in the two arteries studied. In contrast, SOD completely inhibited the endothelium-dependent relaxation to simvastatin in the aorta. Also, SOD inhibited the endothelium-dependent relaxation produced by low concentrations of simvastatin in SMA.

The observation that SOD inhibited rather than potentiated the endothelium-dependent relaxation to simvastatin was surprising since O_2^--sensitive to SOD are known to be implicated in the breakdown of NO. Two hypotheses can be advanced in order to explain the observed data. Both NO and O_2^- are released from the endothelium under the experimental condition used in the present study. Any drug scavenging O_2^- or preventing its release would enhance the availability of endothelial NO, thereby improving endothelium-dependent relaxation. In the presence of sufficient concentration of SOD, however, a NO-mediated dilatory mechanism might no longer be demonstrable because SOD blunted the effect of stimulated endothelial NO. Indeed in the present work, NA-induced contraction was lower in the presence than in the absence of SOD (i.e. in the aorta and in the SMA) although the difference was not statistically significant probably as a consequence of an increased availability of endothelial NO by SOD. On the other hand, there are some data reported in the literature, showing that O_2^- can induce an increase of cytosolic calcium and thereby the production of endothelial relaxant factor including NO in human umbilical vein and aortic endothelial cells (Dreher *et al*., 1995; Hu *et al*., 1998). In addition, induction of O_2^- production by high glucose concentrations enhances agonist-stimulated Ca^{2+}/NO signalling in porcine aortic endothelial cells (Graier *et al*., 1996). Therefore, the results from the present study might suggest that an increase of O_2^--sensitive to SOD may play a role in the simvastatin-induced vasodilatation. We cannot currently distinguish among these possibilities and further studies are needed to sort out the underlying pathway. Nevertheless, one can conclude that the endothelium-dependent relaxation to simvastatin involves a mechanism sensitive to SOD under the experimental condition used in the present study.

The use of the COX inhibitor, indomethacin, revealed the involvement of vasodilatory products from COX in the endothelium-dependent relaxation to simvastatin in the two arteries. In addition, the comparison between the effect of L-NOARG alone and L-NOARG plus indomethacin shows that blockage of endothelial NO synthesis unmasked the participation of endothelial vasoconstrictor products upon simvastatin stimulation. The most likely candidate for endothelial vasoconstrictor factor from COX is probably TXA_2 or other prostanoids acting on the T_p receptor. Participation of such prostanoids appear likely in the aorta but not in the SMA as revealed by the use of the combination of T_p receptor antagonist, GR 32191B, and L-NOARG. Indeed, the inhibitory effect of L-NOARG alone was less marked in the presence of GR 32191 B in the aorta. The concentration-response curve to simvastatin obtained in the presence of GR 32191 B plus L-NOARG was not significantly different from that obtained in the presence of indomethacin plus L-NOARG. In contrast to the aorta, the concentration-response curve to simvastatin was not significantly different in the presence of L-NOARG alone or in combination with GR 32191 B in SMA. Under certain circumstances with increasing lipid accumulation, the patten of COX products might change, due to the different lipid substrates, and many more hydroxyoctadecadienoic (HODE), hydroxyeicosatetraenoic (HETE) acids and isoprostane vasoconstrictor metabolites may be produced (Maclouf *et al*., 1998). This could be the case for endothelial COX metabolites released by simvastatin in the SMA.

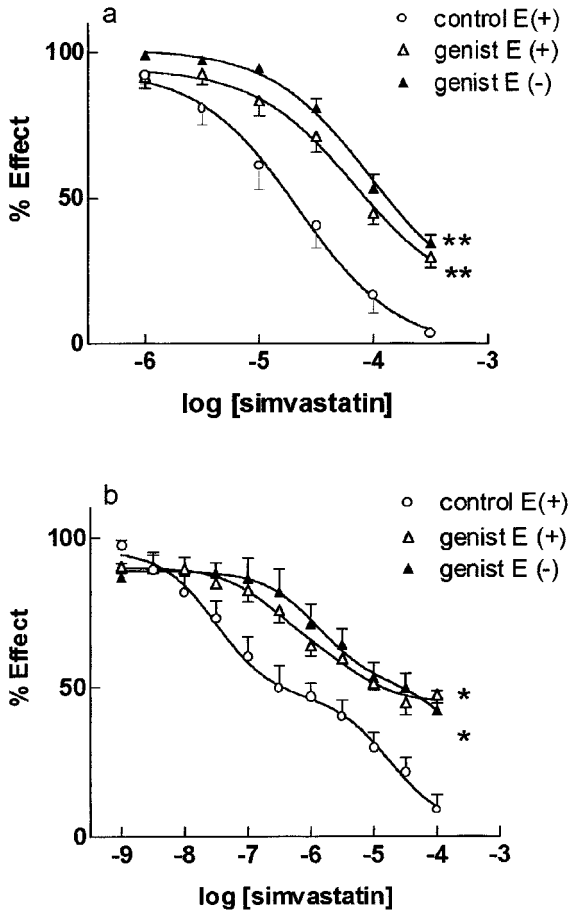

Figure 7 Effect of tyrosine kinase inhibitor, genistein (30 μmol l^{-1}) on simvastatin-induced relaxation of aortic rings (a) and SMA (b) precontracted with NA, compared with simvastatin-induced relaxation in endothelium intact arteries (control E (+)). Data represent means ± s.e.mean of $n=6$ experiments. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ *versus* values obtained in intact arteries.

Taken together, the present study reports for the first time the participation of COX metabolites in the endothelium-dependent relaxation to simvastatin both in the aorta and the SMA. In addition, the results highlight the involvement of both vasodilatory and vasoconstrictor eicosanoids upon stimulation with simvastatin, the vasoconstrictor products being unmasked after blockage of NO synthase only. Induction of COX-2 has been reported after chronic blockage of NO synthase by administration of L-NAME (Henrion *et al*., 1997) and the release of vasoconstrictor prostanoids by endothelial cells has been shown to depend on COX-2 activity (Camacho *et al*., 1998). However, it is unlikely that the release of vasoconstrictor eicosanoids produced by simvastatin results from COX-2 in the presence of L-NOARG in the present study since induction of this enzyme requires a longer time period due to the activation of the transcription factor, NF-kappa B.

The mechanism by which simvastatin produces vasorelaxation was further examined. Some of the properties of simvastatin including antiproliferative or anti-oxidant have been reported to be the consequence of its inhibitory effect on the synthesis isoprenoid intermediates, such as dolichol, ubiquinone and farnesyl pyrophosphate (Goldstein & Brown, 1990; Doyle *et al*., 1993; Soma *et al*., 1995; Raiteri *et al*., 1997). Indeed, isoprenylation (with farnesyl or geranyl-geranyl chains) is one process of post-translational modification of proteins like gamma subunit of heterotrimeric G (Fukada *et al*., 1990), Rho (Casey, 1995) and p21 Rac (Lim *et al*., 1996) proteins that have been involved in simvastatin effect. One way to test this hypothesis is the use of mevalonate which can restore the synthesis of these isoprenoids intermediates and therefore reverse the action of simvastatin. In the present study, the product of HMG-CoA reductase, mevalonate, inhibited the relaxation to simvastatin both in the aorta and in the SMA with and without endothelium. These results suggest that, like other properties of simvastatin, isoprenoid intermediates are also involved in the relaxant effect of simvastatin in both types of arteries,

Finally, the involvement of the tyrosine kinase pathway in the mechanism of action of simvastatin was tested using the tyrosine kinase inhibitor genistein. Both in the aorta and in the SMA, genistein inhibited relaxation to simvastatin in vessels with, but not in those without, functional endothelium. These data suggest that tyrosine kinase inhibitor-sensitive mechanism plays a major role in the endothelium-dependent relaxation to simvastatin in both types of arteries. Tyrosine phosphorylation of phospholipase C gamma 1 has been shown to be involved in the mobilization of Ca^{2+} from the intracellular Ca^{2+} pool in the pathway leading to cell death of myoblasts induced by simvastatin (Mutoh *et al*., 1999). In additon, tyrosine phosphorylation has been involved in calcium entry in endothelial cells (Hisayama *et al*., 1995). Thus, it can be hypothesized that simvastatin might promote an increase of Ca^{2+} signalling through activation of the tyrosine kinase pathway that leads to release of endothelial factors both in the aorta and the SMA.

In summary, simvastatin is able to produce vascular relaxation, independent of its lipid lowering property, both in conductance and small arteries of the rat by acting on smooth muscle and endothelium. The endothelium-dependent relaxation to simvastatin involves the release of both NO and vasodilator products from COX by a mechanism sensitive to O_2^- scavengers, SOD. Also, a complex interaction between NO and the release of endothelial vasoconstrictor products from COX in the effect of simvastatin is observed in the two arteries. Meanwhile, the nature of the vasoconstrictor products from COX are different in the two arteries. Finally, the relaxation to simvastatin involves both mevalonate and tyrosine kinase pathways. These findings might help to better understand the beneficial effects of HMG-CoA reductase inhibitors on the endothelial function.

References

AKIYAMA, T., ISHIDA, J., NAKAGAWA, S., OGAWARA, H., WATANABE, S., ITOH, N., SHIBUYA, M. & FUKAMI, Y. (1987). Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J. Biol. Chem.*, **262,** 5592 – 5595.

ALVAREZ DE SOTOMAYOR, M., PÉREZ-GUERRERO, C., HERRERA, M.D. & MARHUENDA, E. (1999). Effects of chronic treatment with simvastatin on endothelial dysfunction in spontaneously hypertensive rats. *J. Hypertens.*, **17,** 769 – 776.

ANDRIAMBELOSON, E., KLESCHYOV, A.L., MULLER, B., BERETZ, A., STOCLET, J.C. & ANDRIANTSITOHAINA, R. (1997). Nitric oxide production and endothelium-dependent vasorelaxation induced by wine polyphenols in rat aorta. *Br. J. Pharmacol.*, **120,** 1053 – 1058.

AVIRAM, M., HUSSEIN, O., ROSENBLAT, M., SCHLEZINGER, S., HAYEK, T. & KEIDAR, S. (1998). Interactions of platelets, macrophages, and lipoproteins in hypercholesterolemia: antiatherogenic effects of HMG-CoA reductase inhibitor therapy. *J. Cardiovasc. Pharmacol.,* **31,** 39 – 45.

BRAVO, L., HERRERA, M.D., MARHUENDA, E. & PEREZ-GUERRERO, C. (1998). Cardiovascular effects of lovastatin in normotensive and spontaneously hypertensive rats. *Gen. Pharmacol.,* **30,** 331 – 336.

CAMACHO, M., LOPEZ-BELMONTE, J. & VILA, L. (1998). Rate of vasoconstrictor prostanoids released by endothelial cells depends on cyclooxygenase-2 expression and prostaglandin I synthase activity. *Circ. Res.,* **83,** 353 – 365.

CASEY, P.J. (1995). Protein lipidation in cell signaling. *Science,* **268,** 221 – 225.

CORTES, S.D., ANDRIANTSITOHAINA, R. & STOCLET, J.C. (1996). Alterations of cyclo-oxygenase products and NO in responses to angiotensin II of resistance arteries from the spontaneously hypertensive rat. *Br. J. Pharmacol.,* **119,** 1635 – 1641.

DOYLE, J.W., WARD-BAILEY, P.F. & KANDUTSCH, A.A. (1993). Effects of growth factors on cell cycle arrest in dolichyl phosphate-depleted cultures. *J. Cell. Physiol.,* **155,** 170 – 178.

DREHER, D., JORNOT, L. & JUNOD, A.F. (1995). Effects of hypoxanthine-xanthine oxidase on Ca^{2+} stores and protein synthesis in human endothelial cells. *Circ. Res.* **76,** 388 – 395.

ESCOBALES, N., CASTRO, M., ALTIERI, P.I. & SANABRIA, P. (1996). Simvastatin release Ca^{2+} from a Thapsigargin-sensitive pool and inhibits $InsP_3$-dependent Ca^{2+} mobilization in vascular smooth muscle cells. *J. Cardiovasc. Pharmacol.,* **27,** 383 – 391.

FUKADA, Y., TAKAO, T., OHGURO, H., YOSHIZAWA, T., AKINO, T. & SHIMONISHI, Y. (1990). Farnesylated-γsubunit of photoreceptor G protein indispensable for GTP-binding. *Nature,* **346,** 658 – 660.

GOLDSTEIN, J.L. & BROWN, M.S. (1990). Regulation of the mevalonate pathway. *Nature,* **343,** 425 – 430.

GRAIER, W.F., SIMECEK, S., KUKOVETZ, W.R. & KOSTNER, G.M. (1996). High D-glucose-induced changes in endothelial Ca^{2+}/EDRF signalling are due to generation of superoxide anions. *Diabetes,* **45,** 1386 – 1395.

HENRION, D., DECHAUX, E., DOWELL, F.J., MACLOUR, J., SAMUEL, J.L., LEVY, B.I. & MICHEL, J.B. (1997). Alteration of flow-induced dilatation in mesenteric resistance arteries of L-NAME treated rats and its partial association with induction of cyclo oxygenase-2. *Br. J. Pharmacol.,* **121,** 83 – 90.

HERNÁNDEZ-PERERA, O., PÉREZ-SALA, D., NAVARRO-ANTOLIN, J., SÁNCHEZ-PASCUALA, R., HERNÁNDEZ, G., DÍAZ, C. & LAMAS, S. (1998). Effects of 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors, atorvastatin and simvastatin, on the expression of endothelin-1 and endothelial nitric oxide synthase in vascular endothelial cells. *J. Clin. Invest.,* **101,** 2711 – 2719.

HISAYAMA, T., KIDA, K., IMADA, K. & MORITOKI, H. (1995). Tyrosine kinase may participate in Ca^{2+} entry for endothelial nitric oxide production. *Jpn. J. Pharmacol.,* **67,** 181 – 183.

HU, Q., CORDA, S., ZWEIER, J.L., CAPOGROSSI, M.C. & ZIEGELSTEIN, R.C. (1998). Hydrogen peroxide induces intracellular calcium oscillations in human aortic endothelial cells. *Circulation,* **97,** 268 – 275.

KAESEMEYER, W.H., CAIDWELL, R.B., HUANG, J. & CALDWELL, R.W. (1999). Pravastatin sodium activates endothelial nitric oxide synthase independent of its cholesterol-lowering actions. *J. Am. Coll. Cardiol.,* **33,** 234 – 241.

KANTOLA, T., KIVISTO, K.T. & NEUVOVEN, P.J. (1998). Erythromicin and verapamil considerably increase serum simvastatin and simvastatin acid concentrations. *Clin. Pharmacol. Ther.,* **64,** 177 – 182.

LAUFS, U., LA FATA, V., PLUTZKY, J. & LIAO, J.K. (1998a). Upregulation of endothelial nitric oxide synthase by HMG-CoA reductase inhibitors. *Circulation,* **97,** 1129 – 1135.

LAUFS, U. & LIAO, J.K. (1998b). Post-transcriptional regulation of endothelial nitric oxide synthase mRNA stability by Rho GTPase. *J. Biol. Chem.,* **273,** 24266 – 24271.

LILJA, J.J., KIVISTO, K.T. & NEUVOVEN, P.J. (1998). Grapefruit juice-simvastatin interaction: effect of serum concentrations of simvastatin, simvastatin acid and HMG-CoA reductase inhibitors. *Clin. Pharmacol. Ther.,* **64,** 477 – 483.

LIM, L., MANSER, E., LEUNG, T. & HALL, C. (1996). Regulation of phosphorylation pathways by p21 GTPases: the p21 Ras-related Rho subfamily and its roles in phosphorylation signalling pathways. *Eur. J. Biochem.,* **242,** 171 – 185.

LINDBERG, R.A., SCHUSCHKE, D.A. & MILLER, F.N. (1995). Spontaneously hypertensive rats are resistant to the development of hypercholesterolemia. *Am. J. Hypertens,* **8,** 1001 – 1008.

MACLOUF, J., FOLCO, G. & PATRONO, C. (1998). Eicosanoids and iso-eicosanoids: constitutive, inducible and transcellular synthesis in vascular disease. *Thomb. Haemost.,* **79,** 691 – 705.

MARTINEZ, M.C., MULLER, B., STOCLET, J.C. & ANDRIANTSITOHAINA, R. (1996). Alteration by lipopolysaccharide of the relationship between intracellular calcium levels and contraction in rat mesenteric artery. *Br. J. Pharmacol.,* **118,** 1218 – 1222.

MAURO, V.F. & MACDONALD, J.L. (1991). Simvastatin: a review of its pharmacology and clinical use. *DICP Ann. Pharmacother.,* **25,** 257 – 264.

MUTOH, T., KUMANO T., NAKAGAWA, H. & KURIYAMA, M. (1999). Role of tyrosine phosphorylation of phospholipase C gamma 1 in the signalling pathway of HMG-CoA reductase inhibitor-induced cell death of L6 myoblast. *FEBS Lett.,* **446,** 91 – 94.

NG, L.L., DAVIES, J.E. & WOJCIKIEWICZ, R.J.H. (1994). 3-Hydroxy-3-methyl Glutaryl Coenzyme A reductase inhibition modulates Vasopressin-stimulated Ca^{2+} response in rat A10 vascular smooth muscle cells. *Circ. Res.,* **74,** 173 – 181.

O'DRISCOLL, G., GREEN, D. & TAYLOR, R.R. (1997). Simvastatin, an HMG-coenzyme A reductase inhibitor, improves endothelial function within 1 month. *Circulation,* **95,** 1126 – 1130.

RAITERI, M., ARNABOLDI, L., MCGEADY, P., GELB, M., VERRI, D., TAGLIABUE, C., QUARATO, P., FERRABOSHI, P., SANTANIELLO, E., PAOLLETI, R., FUMAGALLI, R. & CORSINI, A. (1997). Pharmacological control of the mevalonate pathway: effect on arterial smooth muscle cell proliferation. *J. Pharmacol. Exp. Ther.,* **281,** 1144 – 1153.

SCANDINAVIAN SIMVASTATIN SURVIVAL STUDY GROUP. (1994). Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). *Lancet,* **344,** 1383 – 1389.

SOMA, M.R., PAROLINI, C., DONETTI, E., FUMAGALLI, R. & PAOLETTI, R. (1995). Inhibition of isoprenoid biosynthesis and arterial smooth-muscle cell proliferation. *J. Cardiovasc. Pharmacol.,* **25** (Suppl 4), S20 – S24.

SUZUMURA, K., YASUHARA, M., TANAKA, K., ODAWARA, A., NARITA, H. & SUZUKI, T. (1999). An in vitro study of the hydroxyl radical scavenging property of fluvastatin, an HMG-CoA reductase inhibitor. *Chem. Pharm. Bull.,* **47,** 1010 – 1012.

TREASURE, C.B., KLEIN, J.L., WEINTRAUB, W.S., TALLEY, J.D., STILLABOWER, M.E., KOSINSKI, A.S., ZHANG, J., BOCCUZZI, S.J., CEDARHOLM, J.C. & ALEXANDER R.W. (1995). Beneficial effects of cholesterol-lowering therapy on the coronary artery diseases. *N. Eng. J. Med.,* **332,** 481 – 487.

VAUGHAN, C.J., MURPHY, M.B. & BUCKLEY, B.M. (1996). Statins do more than just lower cholesterol. *Lancet,* **348,** 1079 – 1082.

WAGNER, A.H., KOHLER, T., RUCKCHLOSS U. & HECKER, M. (2000). Improvement of nitric oxide-dependent vasodilatation by HMG-CoA reductase inhibitor through attenuation of endothelial superoxide anion formation. *Arterioscler. Thromb. Vasc. Biol.,* **20,** 61 – 69.

WANG, Y.X. & PANG, C.C.Y. (1994). N^G-nitro-L-arginine contracts vascular smooth muscle by an endothelium-independent mechanism. *J. Cardiovasc. Pharmacol.,* **24,** 59 – 63.

YADA, T., NAKATA, M., SHIRAISHI, T & KAKEI, M. (1999). Inhibition by simvastatin, but not pravastatin, of glucose-induced cytosolic Ca^{2+} signalling and insulin secretion due to blockade of L-type Ca^{2+} channels in rat islet β-cells. *Br. J. Pharmacol.,* **126,** 1205 – 1213.

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