

EXPERIMENTAL STUDY

Rosuvastatin-induced responses in calf cardiac vein

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ABSTRACT

OBJECTIVE: The effects of Rho-kinase inhibitors on vasodilatation induced by 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor rosuvastatin (10^{-9} – 10^{-4} M) on 5-HT-precontracted calf cardiac vein and the role of endothelium in these effects were analyzed.

MATERIAL AND METHODS: Cardiac vein ring preparations were suspended in organ baths containing 25 ml of Krebs–Henseleit solution, maintained at 37 °C and continuously gassed with 95 % O₂–5 % CO₂. At the end of the resting period, the cardiac vein preparations were contracted with 10^{-6} M 5-HT. After the contraction had reached a steady state, rosuvastatin was added to the organ bath cumulatively (10^{-9} – 10^{-4} M).

RESULTS: Rosuvastatin relaxed the cardiac vein rings in general while the degree of relaxation was greater in those with endothelium and lower in those without it. HA1077 [1-(5-isoquinolinesulfonyl)-homopiperazine] (Fasudil, 10^{-6} M) and Y-27632 [(+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexane carboxamide dihydrochloride] (10^{-6} M) incubation increased the rosuvastatin-induced relaxation only in the presence of endothelium.

CONCLUSIONS: The results demonstrate for the first time that in calf cardiac vein, rosuvastatin induced endothelium-dependent relaxations while Rho-kinase inhibition increased these relaxations in the presence of endothelium layer (Fig. 3, Ref. 44). Text in PDF www.elis.sk.

KEY WORDS: calf cardiac vein, endothelium-dependent relaxation, Rho-kinase, rosuvastatin.

Introduction

In addition to their beneficial effects on lipid modulation, statins exert a variety of “pleiotropic” actions that may be clinically beneficial (1, 2). Indeed, the pleiotropic effects of statins have been considered to be as important as their hypolipemiant effects in the prevention of cardiovascular diseases (3). Examples of the pleiotropic actions of statins such as pravastatin, cerivastatin, atorvastatin, simvastatin, and rosuvastatin (4–6) include direct acute vascular effects associated with increased production of either endothelial nitric oxide synthase (eNOS or NOS-3) in endothelial cells (7) or inducible NOS (iNOS or NOS-2) in smooth muscle cells (8), antithrombotic effects, and anti-inflammatory effects (9).

A substantial number of experimental and clinical studies have demonstrated the favorable cardiovascular effects of rosuvastatin, one of the most commonly employed statins. These effects are generally associated with endothelial function. Among the endogenous regulatory systems of vascular tone, the vascular endothelial tissue plays a very important role. The cells of this tissue produce vasoactive substances in response to diverse chemical and hemodynamic

factors that act on their receptors (10, 11). The balance between endothelial-derived contractile and relaxant factors under normal and pathological conditions determines the tone and the physical state of the vascular smooth muscle. The endothelium-dependent vascular relaxation is mainly mediated by nitric oxide (NO) and to a lesser extent by prostacyclin and endothelial-derived hyperpolarizing factor (EDHF). Thus, endothelial-derived NO plays an important physiological role in the regulation of blood pressure and blood flow and is commonly used as a marker of endothelial function (12). Cardiovascular diseases such as arteriosclerosis are closely associated with endothelial dysfunction. Thus, it is important to identify drugs such as statins, which are able to restore the normal activity of dysfunctional endothelium, prevent damage to this tissue, or stimulate its relaxant and antithrombotic activity. It is well known that statins can block cholesterol synthesis and thus reduce cholesterol levels, which justifies their therapeutic use to lower the risk of cardiovascular disease (13–16). However, the effects of statins appear to be diverse and depend upon the vasculature studied (17, 18). Studies also found that Rho/ROCK pathway inhibition might contribute to the pleiotropic effects of statin therapy (19).

Recent advances in molecular biology have elucidated the important role of small GTP binding proteins (G proteins) such as Rho, Ras, Rab, Sar1/Arf, and Ran families in mediating various cellular functions (17). Rho is known to modulate Ca²⁺ sensitization of vascular smooth muscle (VSMC) and is thought to act by inhibiting myosin phosphatase activity (17, 18). Rho-kinase plays an important role in mediating various cellular functions, for example VSMC contraction (17, 20). Rho-kinase is involved

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in endothelial contraction that increases endothelial permeability and hence enhances atherosclerosis (21, 22). Taken together, the accumulation of evidence suggests that Rho-kinase is substantially involved in the pathogenesis of a wide spectrum of cardiovascular diseases. Antagonism of Rho/Rho-kinase pathway may result in smooth muscle relaxation (23, 24). Endogenous NO raises levels of cGMP, vascular smooth muscle relaxes, and the vessel lumen enlarges. This leads to increased expression of protein kinase G (PKG). The vasodilator action of the cGMP/PKG pathway also decreases cytosolic Ca^{2+} and causes Ca^{2+} desensitization by increased MLCP activity (25). Moreover, statins increase eNOS mRNA stability by inhibiting Rho GTPases, including RhoA, to increase the production and bioavailability of endothelium-derived NO (26). The cGMP-dependent inhibition of RhoA/Rho-kinase has a similar pleiotropic effect to statins. The eNOS allows the local regulation of vascular smooth muscle tone and cell proliferation by inhibiting the expression of Rho-kinase. Thus, eNOS and Rho-kinase act in an opposing, regulatory relationship (27). In this regard, Rho kinase inhibitors are useful for the treatment of various cardiovascular diseases associated with VSMC hypercontraction and it is highly possible that long-term inhibition of Rho-kinase suppresses the development of arteriosclerosis/atherosclerosis. Several Rho-kinase inhibitors have been identified, including Y-27632 and fasudil (28). Clinical trials also determined that Rho/ROCK pathway inhibition might contribute to the pleiotropic effects of statin therapy (29). Although, the involvement of NO in the vascular effect of statins is known, some investigators have reported that the effects of statins appear to be diverse and depend upon the vasculature studied (30, 31). No investigations have been carried out so far to assess the statin-induced relaxations in the presence of Rho-kinase inhibitors. Furthermore the effect of statin treatment of the cardiac vasculature has not yet been investigated.

Thus, in the current study we aimed to determine the effects of rosuvastatin in calf cardiac vein preparations and analyze the role of Rho-kinase inhibitors, fasudil and Y-27632. This vessel was selected because it is easily accessible and appropriate for studying the direct effect of agents on the vascular smooth muscle.

Materials and methods

Materials

Serotonin (5-HT) creatinine sulfate, acetylcholine (ACh) chloride, N^G nitro-L-arginine methyl ester (L-NAME), fasudil, Y-27632 (all dissolved in distilled water) and rosuvastatin (dissolved in dimethyl sulphoxide; DMSO) were used. The concentration of DMSO in the tissue bath was always kept below 0.4%. Rosuvastatin was a gift of Abdi İbrahim Drug Company, Istanbul, Turkey. The other material was obtained from Sigma (St. Louis, MO, USA).

Preparations

Calf hearts were obtained from a slaughterhouse and immediately placed in Krebs-Henseleit solution. Segments of the great

cardiac vein were removed and cut into rings 2.5 mm in length. Care was taken not to damage the endothelium. Each ring was mounted in 25 ml organ baths containing Krebs-Henseleit solution (KHS), aerated with 95% O_2 and 5% CO_2 . KHS was composed of (mM): NaCl 119, KCl 4.70, $MgSO_4$ 1.50, KH_2PO_4 1.20, $CaCl_2$ 2.50, $NaHCO_3$ 25, and glucose 11. Changes in isometric tension were recorded by a force-displacement transducer (BIOPAC MP36, Santa Barbara, California, USA) connected through amplifiers to ITBS08 Integrated Tissue Bath System (Commat, Ankara, Turkey). The tissues were allowed to equilibrate for 60 min under a resting tension of 1 g with repeated washing every 15 min.

The endothelial cell integrity was determined in each ring before all experiments. Relaxation responses to ACh (10^{-6} M) in rings pre-constricted with 5-HT (10^{-6} M) were used to test endothelial cell integrity. Preparations that had been relaxed by >70% of 5-HT-induced tone after addition of ACh were considered to have had undamaged endothelium. In some experiments we employed denuded rings. Endothelium-denuded rings were prepared by turning the rings gently several times on the distal portion of small forceps. Endothelial integrity was pharmacologically assessed by ACh-induced vasodilatation (10^{-6} M); segments showing no relaxation were considered to be denuded of endothelium. Thereafter, experimental procedures were performed as describe below.

Experimental protocol

After the equilibration period, cardiac vein preparations were contracted with 10^{-6} M 5-HT. After the contraction had reached steady state, rosuvastatin was added to the organ bath cumulatively (10^{-9} – 10^{-4} M). The maximal 5-HT contraction was used as a standard by which subsequent responses of the tissue could be expressed (as a percentage of this contraction).

As rosuvastatin induced a relaxant effect in the presence of endothelium, the concentration-response curves were obtained

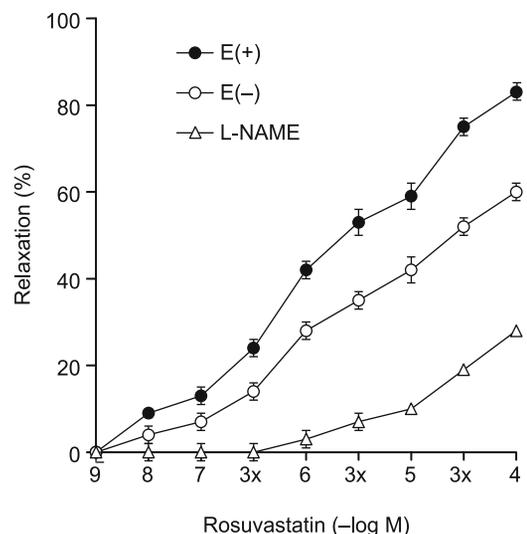


Fig. 1. Responses of rosuvastatin (10^{-9} – 10^{-4} M) in 5-HT (10^{-6} M)-precontracted calf cardiac vein with E(+) and without E(-) endothelium, $n = 6$.

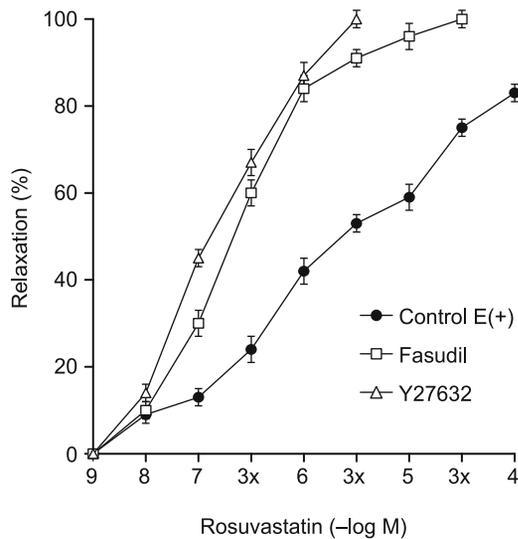


Fig. 2. Responses of rosvastatin (10^{-9} – 10^{-4} M) in 5-HT (10^{-6} M)-precontracted calf cardiac vein with endothelium (control) and in presence of fasudil (10^{-6} M) or Y-27632 (10^{-6} M), $n = 6$.

after treatment with the nitric oxide synthase inhibitor N^G nitro-L-arginine methyl ester (L-NAME, 10^{-4} M). L-NAME had been added to the organ bath 20 min before rosvastatin concentration-response curves were obtained.

After the first concentration-response curve had been completed, preparations were washed and allowed to reestablish the resting tension. To determine the role of Rho-kinase action on rosvastatin-induced relaxations in absence as well as in presence of endothelium, aortic rings precontracted with 5-HT were tested in the presence of fasudil (10^{-6} M) or Y-27632 (10^{-6} M) and concentration-response curves were obtained.

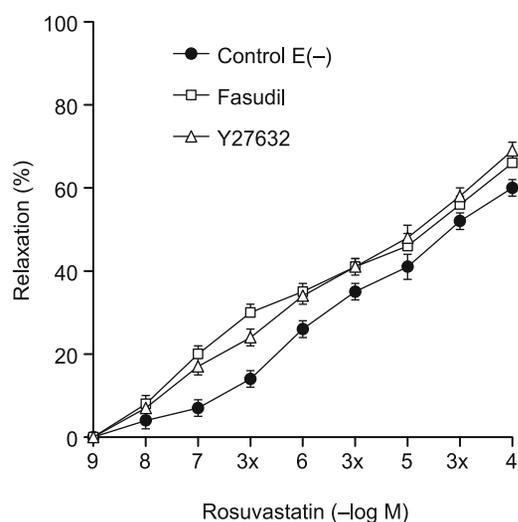


Fig. 3. Responses of rosvastatin (10^{-9} – 10^{-4} M) in 5-HT (10^{-6} M)-precontracted calf cardiac vein without endothelium (control) and in presence of fasudil (10^{-6} M) or Y-27632 (10^{-6} M), $n = 6$.

Statistical analysis

Relaxation responses to rosvastatin were expressed as percentages of 5-HT (10^{-6} M)-induced contraction. Concentrations of rosvastatin causing 50 % of the maximal response (IC_{50}) were calculated from each individual concentration-response curves. Maximal responses (E_{max}) and pIC_{50} ($-\log IC_{50}$) values for curves were compared by using Student's *t* test. Data are presented as mean \pm SE. Statistical significance was set at $p < 0.05$.

Results

Effect of rosvastatin on cardiac vein rings with and without endothelium precontracted with 5-HT

As shown in Figure 1, rosvastatin (10^{-9} – 10^{-4} M) elicited a concentration-dependent relaxation of 5-HT-induced contractions in cardiac vein rings both with and without endothelium. However, the relaxation was lesser in the absence of endothelium. The pIC_{50} values were 5.65 ± 0.23 vs 4.59 ± 0.20 , and maximal relaxations were 83.0 % vs 60.0 % in the presence and absence of endothelium, respectively. The pIC_{50} value of rosvastatin was significantly lower in the preparations denuded of endothelium ($p < 0.05$).

Effect of L-NAME on the relaxation induced by rosvastatin in cardiac vein

As shown in Figure 1, pretreatment with L-NAME produced a significant rightward shift in the relaxation curve obtained by rosvastatin. The maximal relaxant effect was reduced significantly in the presence of L-NAME (28.0 %).

Effects of fasudil and Y-27632 on the relaxant effect induced by rosvastatin in cardiac vein rings with and without endothelium

As shown in Figure 2, pretreatment with Rho-kinase inhibitor fasudil or Y-27632 produced a significant leftward shift in the relaxation curve for rosvastatin in rings with endothelium. The pIC_{50} values were 6.66 ± 0.02 and 6.89 ± 0.01 , respectively, and the maximal relaxations were 100 % in the presence of fasudil or Y-27632.

Furthermore, as shown in Figure 3, the pretreatment with fasudil or Y-27632 in rings denuded of endothelium significantly decreased both the pIC_{50} value and maximal relaxation of rosvastatin. The pIC_{50} values were 4.41 ± 0.24 and 4.69 ± 0.60 , and the maximal relaxations were 66.0 % to 69.0 % in the presence of fasudil or Y-27632 in the absence of endothelium, respectively. Compared with endothelium-denuded rings, endothelium-intact vessels exhibited increased vasodilator sensitivity to rosvastatin in the presence of fasudil or Y-27632, suggesting the endogenous inhibition of Rho-kinase activity by an endothelium-derived agent.

Discussion

The results of the present study clearly show that rosvastatin has an acute relaxant effect on calf cardiac vein smooth muscle, which is in agreement with previous reports evaluating the vascular effects of different statins on isolated rat aortic rings (3, 4, 17). It even has been reported that the effects of statins appear to

be diverse and depend upon the vasculature studied (30, 31). To our knowledge, this is the first report showing that rosuvastatin-induced endothelium-dependent relaxations and Rho-kinase inhibition increased these relaxations in the presence of endothelium layer.

As in rat aorta with simvastatin, cerivastatin and rosuvastatin (6, 32, 33), in calf cardiac vein, we found that the vascular endothelium layer plays a very important role in the relaxant effect of rosuvastatin. In the absence of endothelium, the relaxant effect of this statin was strongly but not totally inhibited, which clearly demonstrates that the endothelium participates in this relaxation. Our results also demonstrated that in the presence of NO synthase inhibitor L-NAME, the relaxant effect of rosuvastatin was strongly inhibited. This clearly demonstrates that the endothelium participates in this relaxation through the liberation of NO. This finding is in line with the finding of Lopez et al. (6) in rat aorta. The facts that endothelium-mediated relaxation in cardiac vein rings relies greatly on the production of NO, and that the inhibitory effect of L-NAME in our study seemed to be higher than that obtained with the removal of endothelium suggest that the vascular smooth muscle may also produce NO in response to rosuvastatin, although in smaller quantities than those produced by endothelium.

Numerous studies have suggested that the inhibition of RhoA/Rho-kinase-mediated Ca^{2+} sensitization induces the relaxation of vascular smooth muscle (31, 34). And because Rho-kinase isoforms are expressed not just in vascular smooth muscle but also in endothelial cells (35), and because RhoA/Rho-kinase activation and NO production/signaling pathways may be interconnected (36), we further assessed whether endothelium and especially NO could be mediating the responses of fasudil and Y-27632 in calf cardiac vein. In the present study, it is apparent that both Rho-kinase inhibitors increased the rosuvastatin-induced relaxations in the presence of endothelium. It is known that inhibition of Rho-kinase may contribute to some of cholesterol-independent effects of statins by increasing the expression of endothelial nitric oxide synthase (eNOS) (37, 38). Specifically, the reduction in geranylgeranyl pyrophosphate after statin treatment reduces the activity of RhoA and Rho-kinase. This results in stabilization of eNOS mRNA and increased bioavailability of nitric oxide (NO) (37, 39). Another important pathway of eNOS activation in endothelial cells is the post-translational phosphorylation of eNOS by protein kinase Akt (40, 41). Because the increased Rho-kinase activity is associated with cardiovascular risks such as hypertension (42), it is quite likely that eNOS regulation by Rho-kinase may play an important role in the pathogenesis of cardiovascular diseases. Moreover, Wolfrum et al (43) reported that acute inhibition of Rho-kinase leads to cardiovascular protection mediated by rapid activation of eNOS. As in our study, Chitale and Webb (44) also reported that in the absence of endothelium, Y-27632 was an ineffective inhibitor of phenylephrine-induced contraction.

In summary, the findings of the present study support the hypothesis that endogenous NO-induced relaxation occurs principally through the inhibition of RhoA/Rho-kinase activity in the intact calf cardiac vein. This provides a novel insight into a prominent vasodilation pathway.

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