Mechanisms of simvastatin-induced vasodilatation of rat superior mesenteric arteries

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Abstract. Independent of its lipid-lowering properties, simvastatin (Sim) induces vasorelaxation; however, the underlying mechanisms have remained elusive. The aim of the present study was to investigate the vasorelaxant effects of Sim on rat superior mesenteric arteries and the mechanisms involved. The isometric tension of rat superior mesenteric arterial rings was recorded in vitro on a myograph. The results showed that Sim concentration-dependently relaxed the superior mesenteric artery rings with endothelium pre-contracted by phenylephrine hydrochloride [maximum relaxation (E_{max})=51.05±4.09%; negative logarithm of the concentration that caused 50% of the maximum response $(pD_2)=4.17\pm0.18$] or KCl ($E_{max}=41.65\pm1.32\%$; $pD_2=3.55\pm0.1$). N ω -nitro-L-arginine methyl ester (100 μ M) significantly inhibited this effect, while it was not affected by 1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one (10 μ M) and indomethacin (5 μ M). In artery rings without endothelium, vasorelaxation induced by Sim was attenuated by 4-aminopyridine (100 μ M), but was not affected by barium chloride dehydrate (10 μ M), glibenclamide (10 μ M) and traethylammonium chloride (1 mM). Moreover, Sim also inhibited the contraction induced

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Abbreviations: PE, phenylephrine hydrochloride; Ach, acetylcholine chloride; 4-AP, 4-aminopyridine; eNOS, endothelial nitric oxide synthase; Gli, glibenclamide; Indo, indomethacin; BaCl₂, barium chloride dehydrate; L-NAME, N ω -nitro-L-arginine methyl ester; NO, nitric oxide; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a] quinoxalin-1-one; Sim, simvastatin; TEA, tetraethylammonium chloride; VSMCs, vascular smooth muscle cells

Key words: simvastatin, vasorelaxant effects, nitric oxide, K⁺ channel, Ca²⁺ influxs

by increasing external calcium in Ca^{2+} -free medium with added KCl (60 mM). These results suggested that Sim induces relaxation of superior mesenteric arterial rings through an endothelium-dependent pathway, involving nitric oxide release and also through an endothelium-independent pathway, involving the opening of voltage-dependent K⁺ channels and blockade of extracellular Ca²⁺ influx.

Introduction

Simvastatin (Sim) is a drug widely used for the treatment of cardiovascular disease (CVD) (1,2). Sim acts as an inhibitor of 3-hydroxy-methylglutaryl coenzyme A reductase, which is a rate-determining enzyme in the biosynthesis of cholesterol, and reduces the plasma levels of low-density lipoprotein (LDL) (3). Clinical studies have shown that treatment with Sim markedly decreased the incidence of cardiovascular events (4). While the lipid-lowering effect is a major mechanism of action of Sim against CVD, increasing evidence has demonstrated that other mechanisms are involved, including reduction of oxidative stress and vascular inflammation, improvement of endothelial function, and enhancement of the stability of atherosclerotic plaques (5). In addition, independent of its lipid-lowering properties, Sim induced vascular relaxation in the aorta and inferior mesenteric artery of rats (2). Moreover, Sim protected the vascular endothelium against damage induced by LDL or oxidized LDL, and relaxed the thoracic aorta in rats (6). However, the underlying mechanisms have remained to be fully elucidated.

Therefore, the present study was designed to explore the mechanisms by which Sim induces relaxation in the superior mesenteric artery of rats. It enhanced the current knowledge on the underlying mechanisms to contribute to the further development of cardiovascular drugs.

Materials and methods

Reagents. Phenylephrine hydrochloride (PE), acetylcholine chloride (ACh), N ω -nitro-L-arginine methyl ester (L-NAME), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), indomethacin (Indo), 4-aminopyridine (4-AP), barium chloride

dehydrate (BaCl₂), glibenclamide (Gli), tetraethylammonium chloride (TEA), Triton X-100 and Sim were obtained from Sigma-Aldrich (St. Louis, MO, USA). ODQ, TEA, Gli, 4-AP and Sim were dissolved in Dimethylsulfoxide. All other compounds were dissolved in distilled water.

In vitro pharmacology. Thirty Sprague-Dawley rats (male, 8 weeks old, 300-350 g), which were obtained from the Animal Center of Xi'an Jiaotong University (Xi'an, China), were sacrificed by CO₂ inhalation. The superior mesenteric artery was gently removed and freed from adhering tissue under a dissecting microscope. The endothelium was denuded by perfusion of the vessel with on Triton X-100 (0.1%, v/v)for 10 sec, followed by physiologic saline solution (PSS; NaCl 119 mM, KCl 4.6 mM, NaHCO₃ 15 mM, NaH₂PO₄ 1.2 mM, MgCl₂1.2 mM, CaCl₂1.5 mM and glucose 5.5 mM) for another 10 sec. The vessels were then cut into cylindrical segments of 1-3 mm in length. The segments were immersed in individual baths containing PSS (5 ml) in a temperature-controlled (37°C) myograph (Danish Myo Technology A/S, Aarhus, Denmark). The solution was continuously aerated with gas (containing 5% CO₂ and 95% O₂), resulting in a pH of 7.4. Following mounting of the arterial segments, isometric tension was continuously recorded of using Chart software (ADInstruments, Oxford, UK). The segments were allowed to stabilize at a resting tone of 2 mN for at least 1.5 h, followed by immersion in a K⁺-rich (60 mM) buffer solution with the same composition as the standard solution, except for NaCl being replaced by KCl to reach a final K⁺ concentration of 60 mM (KPSS). The potassium-induced contraction was used as a reference for contractile capacity, and only the segments which showed reproducible responses over 1.0 mN to potassium were used. In another group, PE (10 μ M) was used instead of KPSS. After a sustained tension was obtained, $Sim (10^{-10}-10^{-5} \text{ M})$ was cumulatively added to the baths and concentration-response curves to Sim were constructed.

In the experiment involving endothelium, Ach (10 μ M) was added after pre-contraction with KPSS to test the completeness of endothelium denudation. An effective functional removal of the endothelium was indicated by absence of relaxation in response to Ach. The rings with endothelium showing <30% relaxation in response to Ach were discarded (7). Furthermore, the artery rings with endothelium were pre-incubated with the cyclooxygenase inhibitor Indo (5 μ M), the guanylate cyclase inhibitor ODQ (10 μ M), the endothelial nitric oxide (NO) synthase (eNOS) inhibitor L-NAME (100 μ M), or with the with the K⁺ channel blockers 4-AP (100 μ M), BaCl₂ (10 μ M), Gli (10 μ M) or TEA (1 mM), respectively, for 20 min prior to addition of KCl (60 mM), followed by cumulative addition of Sim.

A further experiment was performed in the absence of Ca²⁺, for which the rings were washed with Ca²⁺-free PSS. After incubation with or without Sim (10 μ M) for 20 min in Ca²⁺-free PSS, PE (10 μ M) was added to stimulate the release of intracellular Ca²⁺ and the contraction was recorded (8). In another experiment, the rings were washed with Ca²⁺-free PSS containing ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA; 100 μ M; Sigma-Aldrich) and then rinsed with Ca²⁺-free PSS (without EGTA) containing KCl (60 mM K⁺). After incubation with or without Sim (10 μ M) for 20 min, CaCl₂ (2 mM) was added to contract the artery rings (8). All procedures involving animals were performed according to the Guide for the Care and Use of Laboratory Animals Published by the US National Institutes of Health (Publication no. 85-23, revised 1996) and the Guidelines for Animal Experimentation of Xi'an Medical University (Xi'an, China). The experimental protocols of the present study were approved by the Laboratory Animal Administration Committee of Xi'an Medical University (Xi'an, China).

Statistical analysis. Values are expressed as the mean \pm standard error of the mean. The effects of Sim are expressed as the percentage of relaxation with regard to the pre-contraction. For each agent, the negative logarithm of the concentration that caused 50% of the maximum response (pD₂) and the maximum relaxation (E_{max} %) were calculated. The unpaired Student's t-test was used to assess differences between groups. P<0.05 was considered to indicate a statistically significant difference between groups. The analysis was performed using the SPSS 16.0 software (SPSS Inc., Chicago, IL, USA).

Results

Simrelaxes rat superior mesenteric arteries pre-constricted by PE or KCl. In order to evaluate the vasodilative effects of Sim, the superior mesenteric artery rings of rats were pre-contracted with PE (10 μ M) or KCl (60 mM), and once a plateau was attained, concentration-response curves were obtained by adding cumulative doses of Sim to the bath. The results showed that Sim concentration-dependently relaxed the superior mesenteric artery rings with endothelium pre-contracted by PE [E_{max}=51.05±4.09% (Sim, 10⁻⁵ M); pD₂=4.17±0.18] or KCl [E_{max}=41.65±1.32% (Sim, 10⁻⁵ M); pD₂=3.55±0.10] (Fig. 1A and B).

Role of the endothelium in Sim-induced relaxation of rat superior mesenteric arteries. The vasorelaxant effects of Sim on superior mesenteric artery rings with endothelium pre-contracted by PE (10 μ M) were significantly stronger than those on artery rings without endothelium, with E_{max} =49.55±3.67 vs. 31.82±4.02% and pD₂=4.21±0.15 vs. 0.35±0.15 (P<0.01). Moreover, vasorelaxation induced by Sim in artery rings with endothelium pre-contracted by KCl (60 mM) also was significantly stronger than in artery rings without endothelium (E_{max} =40.79±1.49 vs. 31.68±1.76% and pD₂=3.56±0.09 vs. 3.57±0.08; P<0.01), while the effects were more marked in artery rings pre-contracted by PE (Fig. 2A and B).

To identify endothelial mediators associated with the vasodilative effects of Sim, the cyclooxygenase inhibitor Indo (5 μ M), the guanylate cyclase inhibitor ODQ (10 μ M) and the eNOS inhibitor L-NAME (100 μ M) were used, respectively. The results showed that in the artery rings with endothelium, L-NAME significantly inhibited the vasodilative effect of Sim, (E_{max}=13.72±1.12 vs. 38.46±1.36%; pD₂=1.22±0.18 vs. 3.72±0.09; P<0.01) (Fig. 3A). However, ODQ and Indo did not significantly affect the relaxation induced by Sim in the artery rings with endothelium (Fig. 3B and C).

Role of K^+ channels in Sim-induced relaxation of rat superior mesenteric arteries pre-constricted by KCl. To assess the role of K^+ channels in Sim-induced vasorelaxation, artery rings



Figure 1. Vasodilatation effects of simvastatin (Sim) on endothelium-intact superior mesenteric arterial rings pre-contracted with (A) KCl (60 mM) or (B) PE (10 mM). Values are expressed as the mean \pm standard error of the mean (n=6-8). *P<0.05; **P<0.01 vs. DMSO. DMSO, Dimethylsulfoxide; PE, phenylephrine hydrochloride.



Figure 2. Vasodilatation effects of simvastatin (Sim) on endothelium-intact and endothelium-denuded superior mesenteric arterial rings pre-contracted with (A) KCl (60 mM) or (B) PE (10 mM). Values are expressed as the mean \pm standard error of the mean (n=6-8). *P<0.05; **P<0.01 vs. Endo⁺. Endo⁺, artery ring with endothelium; Endo⁻, artery ring without endothelium; PE, phenylephrine hydrochloride.

ithout endothelium were pre-incubated with the K⁺ channel blockers 4-AP (100 μ M), BaCl₂ (10 μ M), Gli (10 μ M) or TEA (1 mM) for 20 min prior to addition of KCl (60 mM), following which Sim was added cumulatively. The results showed that 4-AP significantly reduced the relaxation induced by Sim in the artery rings without endothelium (E_{max}=13.02±1.24 vs. 33.08±0.91% and pD₂=1.36±0.28 vs. 3.77±0.28; P<0.01) (Fig. 4A). However, BaCl₂, Gli and TEA did not significantly affect the relaxation induced by Sim in the artery rings without endothelium (Fig. 4B-D).

Effect of Sim on the calcium release by the sarcoplasmic reticulum in rat superior mesenteric arteries pre-constricted by PE. To clarify whether the relaxation induced by Sim was associated with intracellular Ca²⁺ release, an experiment in Ca²⁺-free PSS was performed. After incubation with or without Sim (10 μ M) for 20 min, PE (10 μ M) was added to stimulate the release of intracellular Ca²⁺ and the contraction was recorded (8). The results showed that PE induced a transient contraction due to the release of intracellular Ca²⁺ into the Ca²⁺-free solution, while Sim did not attenuate this contraction (E_{max}=5.84±1.25 vs. 5.93±0.97%) (Fig. 5A).

Effect of Sim on extracellular Ca^{2+} -induced contraction activated in rat superior mesenteric arteries pre-constricted

by KCl. To determine whether the inhibition of extracellular Ca^{2+} influx affected the relaxation induced by Sim, an experiment was performed in Ca^{2+} -free PSS. Following immersion in Ca^{2+} -free PSS containing KCl (60 mM), the rings were incubated with or without Sim (10 μ M) for 20 min, followed by contraction of the artery rings by addition of $CaCl_2$ (2 mM) (8). The results showed that Sim significantly attenuated the contraction induced by addition of $CaCl_2$ to the Ca^{2+} -free PSS plus KCl (E_{max} =73.77±2.8 vs. 102.94±3.98%) (Fig. 5B). It was therefore suggested that Sim inhibits Ca^{2+} influx in the superior mesenteric artery.

Discussion

The present study revealed that Sim concentration-dependently relaxed the superior mesenteric artery rings with or without endothelium pre-contracted by PE or KCl. The results suggested that Sim exerted its vasorelaxation effects via endothelium-dependent and -independent pathways. Moreover, the vasorelaxation induced by Sim was inhibited by L-NAME, while it was not affected by ODQ and Indo in artery rings with endothelium. In addition, Sim-induced vasorelaxation was inhibited by 4-AP, while it was not affected by Gli, BaCl₂ and TEA in artery rings without endothelium. Finally, the vasorelaxation induced by Sim was



Figure 3. Vasodilatation effects of simvastatin (Sim) on endothelium-intact superior mesenteric arterial rings pre-contracted with KCl (60 mM) in the presence of (A) endothelial nitric oxide synthase inhibitor L-NAME (100 μ M), (B) guanylate cyclase inhibitor ODQ (10 μ M) or (C) cyclooxygenase inhibitor Indo (5 μ M). Values are expressed as the mean \pm standard error of the mean (n=6-8). *P<0.05; **P<0.01 vs. Sim. L-NAME, N ω -nitro-L-arginine methyl ester; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; Indo, indomethacin.

shown to be mediated through blockade of Ca²⁺ influx from extracellular medium.

Vascular endothelium located between vascular smooth muscle and circulating blood is known to be important in regulation of vascular tone. Vasorelaxation is mediated by vasorelaxant substances synthesized and released into the endothelium (9). In the present study, the relaxant effect induced by Sim was attenuated in the superior mesenteric artery rings without endothelium, suggesting that Sim also relaxes arteries through an endothelium-dependent pathway. Furthermore, the eNOS inhibitor L-NAME significantly reduced the vasorelaxation induced by Sim. However, cyclooxygenase inhibitor Indo and guanylate cyclase inhibitor ODQ did not affect the action of Sim. These results suggested that NO is involved in the relaxation of Sim in the superior mesenteric artery with endothelium, whereas the effect was not attributed to or prostanoids (Indo inhibits prostaglandin-endoperoxide synthase) or the cyclic guanosine monophosphate pathway. In accordance with the results of the present study, a previous study reported that in the aorta and small mesenteric artery, the efficacy of Sim was closely associated with the NO system in endothelial cells (2). However, the study also identified an association with prostanoids, which may therefore require clarification by further studies.

The present study further revealed that Sim also exerted vasorelaxant effect in superior mesenteric arteries without



Figure 4. Vasodilatation effects of simvastatin (Sim) on endothelium-denuded superior mesenteric arteries. Vasodilatation effects of simvastatin on endothelium-denuded superior mesenteric arterial rings pre-contracted with KCl (60 mM) in the presence of K⁺ channel blockers (A) 4-AP (100 μ M), (B) BaCl₂ (10 μ M), (C) Gli (10 μ M) or (D) TEA (1 mM). Values are expressed as the mean ± standard error of the mean (n=6-8). 4-AP, 4-aminopyridine; Gli, gliben-clamide; TEA, tetraethylammonium chloride; BaCl₂, barium chloride dehydrate.



Figure 5. Inhibitory effects of simvastatin on (A) extracellular Ca^{2+} influx induced by KCl (60 mM) and (B) the sarcoplasmic reticulum Ca^{2+} release induced by PE in Ca^{2+} -free solutionin endothelium-denuded superior mesenteric arterial rings. Values are expressed as the mean \pm standard error of the mean (n=6-8). *P<0.05 vs. control. PE, phenylephrine hydrochloride.

endothelium, suggesting that Sim has a direct effect on vascular smooth muscle cells (VSMCs). The opening of K⁺ channels in these cells causes hyperpolarization of the membrane potential and a decreased Ca^{2+} influx through voltage-operated Ca^{2+} channels, resulting in vasorelaxation (10,11). Several types of K⁺ channel have been identified in vascular smooth muscle, the most abundant ones being the large conductance Ca^{2+} -activated K⁺ channel, the voltage-sensitive K⁺ channel, the adenosine triphosphate (ATP)-sensitive K⁺ channel and inward-rectifyer K⁺ channels. In order to detect the contribution of different types of K⁺ channel to endothelium-independent

relaxation induced by Sim in superior mesenteric artery rings, various K⁺ channel-blocking agents were used, including the voltage-dependent K⁺ channel blocker 4-AP, inward-rectifying potassium channel blocker BaCl₂, ATP-sensitive K⁺ channel blocker Gli and Ca²⁺-activated K⁺ channel blocker TEA (12,13). The results revealed that 4-AP significantly inhibited the effect of Sim, indicating that the voltage-dependent K⁺ channel was involved in the mechanism of the vasorelaxant action of Sim. However, BaCl₂, Gli and TEA did not affect the concentration-response curves of Sim, suggesting that the inward-rectifyer, ATP-sensitive and Ca²⁺-activated K⁺ channels were not involved in Sim-mediated vasorelaxation.

Accumulation of intracellular calcium is associated with vascular smooth muscle contraction. Moreover, intracellular calcium levels may increase via extracellular Ca²⁺ influx through the receptor-operated or voltage-dependent calcium channels, as well as intracellular Ca²⁺ release (14). Contractions of VSMCs induced by KCl almost exclusively rely on Ca²⁺ influx through activation of voltage-sensitive channels (15), whereas PE-induced contractions are mediated via increasing the Ca²⁺ influx through receptor-operated (16) as well as voltage-sensitive channels (17). The results of the present study showed that Sim inhibited the contractile effects induced by PE or KCl on the superior mesenteric artery without endothelium, suggesting that Sim may interfere with receptor-operated as well as voltage-sensitive potassium channels. The release of intracellular stored Ca²⁺ is mainly regulated by the ryanodine and inositol triphosphate (IP3) receptor systems. In Ca2+-free medium, PE induces vascular contraction via inducing intracellular Ca²⁺ release through sarcoplasmic reticulum Ca²⁺ channels activated by IP3 (18). In the present study, Sim was shown to significantly inhibit CaCl₂-induced contraction of superior mesenteric artery rings without endothelium in Ca²⁺-free PSS containing KCl (60 mM), indicating that Sim is able to block Ca^{2+} influx. However, Sim did not inhibit the contraction triggered by PE in Ca²⁺-free PSS, suggesting that Sim does not affect Ca²⁺ mobilization from intracellular stores. It can therefore be concluded that in the superior mesenteric artery, Sim induces vasorelaxation via inhibition of extracellular calcium influx into VSMCs.

In conclusion, the results of the present study suggested that Sim induced relaxation of superior mesenteric arteries of rats through an endothelium-dependent pathway involving NO release, as well as an endothelium-independent pathway, opening of voltage-dependent K⁺ channels and blockade of extracellular Ca²⁺ influx. These findings may support the further development of treatments for CVD.

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