Preventive effects of pravastatin on thrombin-triggered vascular responses via Akt/eNOS and RhoA/Rac1 pathways in vivo

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Aims
Small GTPases RhoA and Rac1 play crucial roles in endothelial dysfunction and reactive oxygen species (ROS) generation. We reported evidence that in thrombin-stimulated endothelial cells, rapid geranylgeranylation is an essential process for full activation of unprocessed RhoA, which is blocked by statin. In this study, we examined the effects of intravenous administration of pravastatin on thrombin-triggered vascular responses in vivo, as well as on the lipid modification of unprocessed forms of RhoA and Rac1 and their activation induced by thrombin.

Methods and results
Thrombin (50 U/kg) was intravenously injected with or without 0.3 mg/kg pravastatin into Wistar and spontaneously hypertensive rats. Coadministration of pravastatin prevented thrombin-induced impaired endothelium-dependent coronary vasodilation and down-regulated Akt/endothelial nitric oxide synthase (eNOS) phosphorylation within 1 h, as well as the down-regulation of eNOS protein expression within 4 h. In addition, thrombin increased Rac1/p47phox-dependent NAD(P)H oxidase activities of rat aortas within 1 h, resulting in ROS generation, which was prevented by the coadministration of pravastatin. Furthermore, the coadministration of pravastatin prevented thrombin-induced conversion of unprocessed RhoA and Rac1 into the geranylgeranylated forms as well as GTP-loading and membrane translocation within 1 h.

Conclusion
Intravenous injection of pravastatin prevents impaired NO-dependent vasodilation and Rac1/NAD(P)H oxidase-mediated-ROS generation by blocking the down-regulation of Akt/eNOS pathways and the full activation of unprocessed RhoA and Rac1 in vivo.

Keywords
Statin • Akt • eNOS • RhoA • Rac1

1. Introduction
Endothelial dysfunction in response to various stimuli initiates the development of atherosclerosis, and reactive oxygen species (ROS) accelerate atherosclerotic lesion formation and plaque instability.1,2 Small GTPase RhoA plays a crucial role in endothelial dysfunction including the down-regulation of endothelial nitric oxide synthase (eNOS) protein expression, whereas small GTPase Rac1 induces the assembly of the NADPH oxidase complex, the most important source of the primordial oxygen radical in the vessel wall.3–5 Statin inhibits the synthesis of isoprenoid intermediates of the cholesterol synthesis pathway, such as geranylgeranylprophosphate and farnesylpyrophosphate. These intermediates serve as important lipid attachments for the post-translational modification of a variety of proteins, including the small GTPases such as RhoA and Rac1. Intracoronary thrombin formation reflects the pathogenesis of acute coronary syndrome which is based on impaired endothelial dysfunction and increased ROS generation.6 Thrombin activates RhoA, and Rac1 and p47phox, the components of NADPH oxidase, in the vascular cell components.7–9 We have shown that thrombin-induced
rapid geranylation is essential for the full activation of unprocessed RhoA in vitro, which is blocked by acute short-term pretreatment with statin. Therefore, it will be of importance to determine whether or not thrombin induces a rapid activation of unprocessed RhoA and Rac1 in vivo and statin reverses it.

Although pravastatin has beneficial effects on primary and secondary cardiovascular prevention mainly mediated via cholesterol-lowering, pleiotropic effects of pravastatin independent of cholesterol-lowering may also play a role in the outcomes of primary and secondary prevention. Pravastatin rapidly improves endothelium-dependent coronary vasomotion within 24 h in patients with stable angina pectoris. However, the mechanism(s) by which pravastatin exerts acute effects on impaired coronary endothelial function beyond cholesterol-lowering in vivo needs to be clarified.

This study investigates the effects of intravenous pravastatin administration on thrombin-induced vascular responses such as impaired nitric oxide (NO)-related endothelium-dependent vasodilation and increased NAD(P)H oxidase-dependent ROS generation in vivo in rats, as well as on the rapid activation of unprocessed RhoA and Rac1.

2. Methods

2.1 Study protocol

Adult male Wistar rats and spontaneously hypertensive rats (SHR) weighing 180–200 g were obtained from Japan SLC, Inc. (Shizuoka, Japan). Rats were anesthetized by an intraperitoneal injection of 25 mg/kg sodium pentobarbital. Fifty unit per kilogram thrombin (Sigma Chemical Co., St. Louis, MO, USA) with or without 0.3 mg/kg pravastatin (Sankyo Pharmaceutical Co., Tokyo, Japan) in phosphate-buffered saline (PBS, pH 7.4) was intravenously injected into the tail veins of the rats. In some experiments, 1 mg/kg/day pravastatin was orally administered to rats for 5 days, followed by the single injection of thrombin. The thoracic aortas were removed within 1 h after the single injection of thrombin with or without pravastatin, and the unprocessed and geranylated forms of RhoA and Rac1 of the aortas were separated by the Triton X-114 partition method. The GDP/GTP exchange (GTP-loading) of RhoA and Rac1 was determined by pull-down assays, whereas we performed membrane translocation to determine the activities of Rac1 and p47phox. We also measured the activity of geranylgeranyl transferase I (GGTase I) responsible for geranylgeranylation, the first step of unprocessed small GTPases, 1 h after injection. Coronary vasodilation and eNOS and Akt phosphorylation were examined 1 h after injection, whereas the protein expression of eNOS in the rat aortas was determined 4 h after injection, followed by western blotting and immunohistochemistry. The activities of NAD(P)H oxidase of the aortas were quantitatively measured, and ROS generation was determined by dihydroethidine staining. These experiments were carried out under the control of the Animal Research Committee in accordance with the Guidelines on Animal Experiments of Fukushima Medical University and the Japanese Government Animal Protection and Management Law (No. 105), as well as the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

We performed time course experiments (30 min, 1, 2, 4, 12, and 18 h) to investigate the effects of thrombin (various doses from 10 to 200 U/kg) on eNOS protein expression in aortas as determined by western blotting. These indicated that a stable maximum decrease in eNOS expression was observed 4 h after 50 U/kg thrombin injection, so this dose was used in subsequent experiments. We also examined the time course effect of thrombin on the levels of the active GTP-bound form of RhoA as determined by pull-down assays (30 min, 1, 2, and 4 h). Since the increase in GTP-bound RhoA were similar 30 min to 2 h after the single injection of 50 U/kg thrombin, RhoA and Rac1 activation and GGTase I activity were assessed 1 h after injection. Since the time course experiments of endothelium-dependent vasodilation in response to acetylcholine (ACH) demonstrated that thrombin-induced a similar impairment between 1 and 2 h after injection, we herein present the results of 1 h regarding physiological assessment of coronary arterioles after injection.

In addition, the reason why we selected 0.3 mg/kg pravastatin employed in the present study is as follows. In clinical practice, we usually use oral 10–20 mg/day of pravastatin, which means ~0.17–0.34 mg/kg. Gehr et al. reported that a single dose of 20 mg intravenous dose of pravastatin was administered to patients on chronic haemodialysis and the circulating concentrations of pravastatin after equilibrium of plasma concentrations was achieved were similar to the maximal concentrations of orally administered 10 mg pravastatin in healthy volunteers reported by Koitabashi et al. Therefore, we preliminarily tested the effect of intravenous injection of 0.03 and 0.3 mg/kg pravastatin on thrombin-induced RhoA activation of rat aortas as determined by membrane translocation and pull-down assay. The result showed that intravenous injection of 0.3 mg/kg pravastatin appeared to be enough to attenuate thrombin-triggered RhoA activation, although Kivisto et al. injected 5 mg/kg pravastatin into rats.

2.2 Functional assessment of isolated coronary arterioles

The heart was excised and immediately placed in cold (4°C) physiological salt solution (PSS, mM: NaCl 145.0, KCl 4.7, CaCl₂ 2.0, MgSO₄ 1.17, NaH₂PO₄ 1.2, glucose 5.0, pyruvate 2.0, EDTA 0.02, and Mops 3.0). A branch of the septal coronary artery (40–98 μm in internal diameter, 0.5 mm in length) was carefully isolated 1 h after the injection of thrombin with or without pravastatin as described previously and then used in the functional study described below. The inner diameters of coronary arterioles were measured using the microscope technique. To determine the response of coronary arteries to ACh or sodium nitroprusside (SNP), vessels were cannulated with glass micropipettes and pressurized to 60 mmHg intraluminal pressures without flow. The cannulated vessel was bathed in PSS containing 1% bovine serum albumin (Sigma) at 37°C. After developing a stable basal tone (i.e. spontaneous constriction to 60–70% of maximal diameter), the experimental interventions were performed. The concentration–diameter relationships for ACh (10⁻⁶–10⁻⁵ M) and SNP (10⁻¹⁰–10⁻⁸ M) were established. ACh was used as an activator of NO-mediated vasodilation. The contributions of the NO pathway in these vasodilations were examined by treating the vessels with the NOS inhibitor L-nitro-arginine-methyl-ester (L-NAME, 10⁻⁴ M, 30 min incubation). All drugs were administered extraluminally in these functional studies.

2.3 Determination of eNOS and Akt phosphorylation

Akt and eNOS phosphorylation in response to thrombin in rat aortas was determined 1 h after a single injection of thrombin with or without pravastatin by western blotting and fluorescence immunohistochemistry, respectively, as described previously. Anti-phospho-Akt (Ser-473, dilution 1:200, Cell Signaling Technology, Inc., Danvers, MA, USA) and anti-phospho-eNOS (Ser-1177, dilution 1:200, Cell signaling Technology) antibodies were used.

2.4 Determination of aortic eNOS expression

Protein expression of eNOS in rat aortas was determined by western blotting and immunohistochemistry as described previously. Rats were treated with a single injection of thrombin with or without pravastatin. The aortas were removed 4 h after the injection, cut into small pieces, and solubilized with a lysis buffer. Immunoblotting was performed using a mouse monoclonal antibody to human eNOS (Transduction Laboratories, Lexington, KY, USA) diluted 1:1000. Anti-GAPDH-antibody
was used for the normalization of eNOS expression. Additionally, the removed aortas were fixed with 10% formaldehyde and embedded in paraffin, and 4 μm-thick cross sections were cut from each paraffin block. The sections were stained immunohistochemically with a rabbit polyclonal antibody against human eNOS antigen (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit IgG as a negative control at 4°C overnight and for 30 min with a biotinylated gout F(ab′)2 IgG against rabbit IgG (Dako, Carpinteria, CA, USA). A streptavidin-biotinylated horseradish peroxidase system (StreptABComplex/HRP, Dako) was used, and antibody binding was visualized with 3,3′-diaminobenzidine (Dako) and hydrogen peroxide. The slides were counterstained with hematoxylin.

2.5 Measurement of NAD(P)H oxidase activity

The aortas were isolated 1 h after injection and immediately placed in cold (4°C) PBS, and the adventitia was completely removed. The enzymatic activities of NAD(P)H oxidase of homogenates of the aortas was assessed by lucigenin-enhanced chemiluminescence (L-CL) as described previously.4,18,20 Briefly, the assay solution contained 50 mM HEPES (pH 7.4), 1.0 mM EDTA, 6.5 mM MgCl2, 5.0 μM lucigenin as an electron acceptor, and either 1 mM NADH or 1 mM NADPH as a substrate. After pre-incubation at 37°C for 10 min, the reaction was started by adding 50 μg of homogenate. Final volume of the reaction solution was 1.0 mL. Photon emission was continuously recorded for 15 min with a CL reader (ALOKA, BLR-201, Mitaka, Tokyo). The chemiluminescent signals observed in the absence of homogenates were subtracted from the chemiluminescence signals of the samples. The chemiluminescence signal corrected for the protein concentration of each cell homogenate and expressed as counts per minute (cpm) per milligram protein for a 15 min period. In some experiments, the homogenates were pre-incubated with 10 μmol/L diphenyleneiodium (DPI), a selective NAD(P)H oxidase inhibitor, for 20 min before L-CL measurement.

2.6 ROS generation in rat aortas and quantitative image analysis

Rat aortas were isolated 1 h after intravenous injection of thrombin with or without pravastatin, and 30-μm frozen sections of aortic segments were prepared. The fluorescence was determined by 20 μM dihydroethidine staining as described previously.21 The sections were incubated with 20 μM dihydroethidine for 30 min in PBS solution at 37°C and then washed with PBS. The fluorescence was determined using a confocal microscope (Olympus, Tokyo, Japan) at excitation and emission wavelengths of 520 and 610 nm, respectively. Images were stored in a computer and the intensity of fluorescence of rat aortas was quantitatively analysed by the NIH Image Program (ImageJ).18

2.7 Separation of unprocessed and geranylgeranylated RhoA and Rac1

Unprocessed and geranylgeranylated forms of RhoA and Rac1 were separated by the Triton X-114 partition method as described previously.9 Briefly, the aortas were cut into small pieces and solubilized with a lysis buffer and then sonicated. Triton X-114 (11% (w/v)) was added to the lysates to a final concentration of 1% (v/v). The lysates were mixed for 10 min at 4°C and centrifuged at 15 000 g at 4°C for 30 min to remove insoluble materials. The supernatant was warmed at 37°C for 2 min until it became cloudy, and then centrifuged at 400 g for 4 min at room temperature to separate the upper (unprocessed) phase from the lower (geranylgeranylated) phase. Both phases were adjusted to 1% (v/v) Triton X-114 on ice and the protein levels of RhoA and Rac1 in each phase were determined by western blotting. We used a mouse monoclonal antibody to RhoA diluted 1:250 (Santa Cruz Biotechnology), and a mouse monoclonal antibody to Rac1 (Upstate Biotechnology, Lake Placid, NY, USA) diluted 1:500 for immunoblotting.

2.8 GTP/GDP exchange of RhoA and Rac1

The levels of the GTP-bound forms of RhoA and Rac1 of the aortas were determined by pull-down assays as described previously.9,22 Bound RhoA and Rac1 proteins were quantified by western blotting.

2.9 Determination of Rac1 and p47phox activities by membrane translocation

The levels of Rac1 and p47phox in membrane fractions of the rat aortas were determined by western blotting.22,23 We used a goat polyclonal antibody to p47phox (Santa Cruz Biotechnology) diluted 1:500 for immunoblotting.

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Figure 1 Functional assessment of isolated coronary arterioles in response to thrombin with or without pravastatin injection. A branch of septal coronary artery was isolated 1 h after injection and subjected to functional assessment. (A) Effects of thrombin with or without pravastatin on ACh-induced vasodilation. Isolated coronary arterioles from the rat heart injected with 50 U/kg thrombin with or without 0.3 mg/kg pravastatin were treated with 10⁻¹⁰–10⁻⁵ M ACh. (B) Effects of L-NAME on ACh-induced vasodilatation in response to thrombin with or without pravastatin. Arterioles were pre-treated with 10⁻⁸ M L-NAME for 30 min before exposure to ACh. (C) Effects of thrombin with or without pravastatin on SNP-induced vasodilation. Isolated coronary arterioles were treated with 10⁻¹⁰–10⁻⁵ M SNP. Data are expressed as means ± SD. ***p < 0.001.
Figure 2  Effects of pravastatin on Akt-dependent eNOS phosphorylation in response to thrombin.  (A and B) Effects of thrombin and pravastatin plus thrombin on Akt (A) and eNOS (B) phosphorylation of rat aortas in vivo 1 h after injection as determined by western blotting. Data are expressed as means ± SD of ratio of each phosphorylated expression to non-phosphorylated expression of densitometric analyses from four separate experiments. Representative immunoblots are shown. *P < 0.05; **P < 0.01.  (C) Effects of intravenous coadministration of pravastatin on thrombin-induced reduction in eNOS phosphorylation in the branches of rat thoracic aortas 1 h after injection as determined by fluorescence immunohistochemistry. Photomicrographs are from an experiment representative of three independent experiments [a: control; b: thrombin (50 U/kg) alone; c: thrombin + pravastatin (0.3 mg/kg); d: pravastatin alone].  (D and E) Effects of oral pre-treatment with stain (1 mg/kg of pravastatin per day for 5 days) on thrombin-induced decrease in Akt and eNOS phosphorylation of rat aortas in vivo. Data are expressed as means ± SD of ratio of each phosphorylated expression to non-phosphorylated expression of densitometric analyses from three separate experiments. Representative immunoblots are shown. *P < 0.05.  (F and G) Effects of intravenous coadministration of pravastatin on thrombin-induced down-regulation of eNOS protein expression in rat aortas as determined by western blotting (F) and immunohistochemistry (G). Rat aortas were removed 4 h after 50 U/kg thrombin injection with or without 0.3 mg/kg pravastatin, followed by western blotting and immunohistochemistry.  (F) Results of western blotting of eNOS in response to pravastatin. eNOS expression was normalized by GAPDH expression. Representative immunoblots of eNOS of rat aortas are shown above. Bars are means ± SD of quantitative densitometric analysis in each group. **P < 0.01.  (G) eNOS protein expression of rat aortas by immunohistochemical staining. Arrows indicate eNOS expression in rat aortic endothelial cells (original magnification, ×100; a, control; b, thrombin alone; and c, pravastatin plus thrombin).
2.10 Measurement of GGTase I activity
The isolated aortas within 1 h after injection were cut into small pieces and solubilized with a lysis buffer. The activity of GGTase I of the aortas was determined as described previously.9

2.11 Densitometric analysis of immunoblots
After scanning immunoblots onto a computer (EPSON GT5500ART, Tokyo, Japan), individual bands were analysed for optical density using NIH image.4,9

2.12 Statistical analysis
Statistical analysis was performed using ANOVA with Scheffé's post hoc test, as appropriate. A level of $P < 0.05$ was considered significant. Data are expressed as means ± SD.

3. Results
3.1 Rapid preventive effects of pravastatin on thrombin-induced impaired coronary vasodilation
To investigate endothelium-dependent coronary vasomotion, we assessed NO-related vasodilation in response to ACh. Intravenous thrombin injection profoundly impaired ACh-induced vasodilation of coronary arterioles which were isolated 1 h after injection (Figure 1A, $n = 7$, $P < 0.001$). However, coadministration of pravastatin prevented the thrombin-triggered impairment of vasodilation (Figure 1A, $n = 6$, $P < 0.001$). Pravastatin injection alone had negligible effect on ACh-induced vasodilation compared with control. Exposure of isolated coronary arterioles to L-NAME abolished ACh-induced vasodilation, which was not altered by coadministration of pravastatin (Figure 1B). In contrast, neither thrombin nor thrombin plus pravastatin affected SNP-induced vasodilation (Figure 1C). These findings suggest that the acute effect of pravastatin on thrombin-triggered impaired vasodilation is NO-related endothelium-dependent.

3.2 Effects of pravastatin on Akt/eNOS phosphorylation and eNOS protein expression in response to thrombin
Next, we examined whether the preventive effect of pravastatin on vasodilation is mediated via Akt/eNOS pathway. Intravenous coadministration of pravastatin attenuated thrombin-induced decreased levels of phosphorylated Akt and eNOS in rat aortas within 1 h as determined by western blotting (Figure 2A and B, $n = 4$, $P < 0.01$ and $P < 0.05$, respectively). Furthermore, fluorescence immunohistochemistry shows that intravenous injection of thrombin alone decreased the levels of phosphorylated eNOS in the branches...
of rat thoracic aortas, which was prevented by coadministration of pravastatin within 1 h (Figure 2C). These results suggested that pravastatin rapidly prevented thrombin-triggered impaired vasodilation by increasing NO production through Akt-dependent eNOS phosphorylation. Furthermore, pre-treatment with pravastatin orally for 5 days prevented the down-regulation of Akt and eNOS phosphorylation 1 h after thrombin injection (Figure 2D and E).

In addition, we examined the protein expression of eNOS in response to thrombin with or without pravastatin. A significant reduction of eNOS protein expression in rat aortas was observed 4 h after thrombin injection (Figure 2F, 59 ± 15% of control, *P < 0.01). The coadministration of pravastatin prevented the decreased levels of eNOS expression caused by thrombin (Figure 2F, 110 ± 13% of control, *P < 0.01 vs. thrombin). Immunohistochemistry shows that the protein expression of eNOS in rat aortas was decreased 4 h after the thrombin injection alone (Figure 2G-b), which was prevented by the coadministration of pravastatin (Figure 2G-c).

### 3.3 Effects of pravastatin on thrombin-induced NAD(P)H oxidase activities and ROS generation

Intravenous thrombin injection markedly increased the activities of NADH and NADPH oxidase of rat aortas after 1 h (Figure 3A, n = 6, *P < 0.001 and *P < 0.001, respectively). L-CL assay demonstrated that pre-treatment of homogenates of the aortas with DPI abrogated the increased activities of NADH and NADPH oxidase induced by thrombin (Figure 3A). Coadministration of pravastatin and thrombin markedly attenuated the increased activities of NADH and NADPH oxidase caused by thrombin (Figure 3A, n = 6, *P < 0.01 and *P < 0.001, respectively). In addition, the levels of ROS generation in rat aortas were increased 1 h after the single injection of thrombin, which was blocked by coadministration of pravastatin (Figure 3B, n = 6, *P < 0.01). Pre-treatment with pravastatin orally for 5 days also markedly prevented thrombin-induced ROS generation (Figure 3C, n = 3, *P < 0.01).

### 3.4 Preventive effects of pravastatin on Akt and eNOS phosphorylation and ROS generation in SHR

Next, we examined the actions of pravastatin in an animal disease model SHR. Intravenous coadministration of pravastatin prevented thrombin-induced down-regulation of Akt and eNOS phosphorylation (Figure 4A and B, n = 3, *P < 0.05, each) and up-regulation of ROS generation (Figure 4C, n = 3, *P < 0.01) in the aortas of SHR within 1 h.

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**Figure 4** (A and B) Effects of pravastatin on Akt-dependent eNOS phosphorylation in response to a single injection of thrombin in SHR 1 h after injection as determined by western blotting. Data are expressed as means ± SD of ratio of each phosphorylated expression to non-phosphorylated expression of densitometric analyses from three separate experiments. Representative immunoblots are shown. *P < 0.05. (C) Effects of intravenous coadministration of pravastatin on thrombin-induced ROS generation in rat aortas of SHR 1 h after injection. Representative photomicrographs are shown [a: control; b: pravastatin (0.3 mg/kg); c: thrombin (50 U/kg) alone; d: thrombin plus pravastatin]. Data are expressed as means ± SD (n = 3) quantitatively analysed by ImageJ.
3.5 Effects of pravastatin on thrombin-induced geranylgeranylation and GTP-loading of RhoA in vivo

The proportions of the unprocessed and geranylgeranylated forms of RhoA in rat aortas were determined by the Triton X-114 partition method, indicating that the percentages of the unprocessed and geranylgeranylated forms of RhoA in untreated rat aortas were 42 ± 6 and 58 ± 6%, respectively (Figure 5A, n = 4, P < 0.01). The thrombin injection significantly induced a significant increase in the conversion of the unprocessed form of RhoA into its geranylgeranylated form within 1 h in rat aortas (Figure 5B, n = 4, P < 0.05). Thrombin-induced conversion was blocked by the coadministration of pravastatin (Figure 5B, n = 4, P < 0.05). Pull-down assays revealed that the thrombin injection increased the levels of the GTP-bound form of RhoA (GTP-RhoA) in the aortas within 1 h, which was prevented by the coadministration of pravastatin and thrombin (Figure 5C, n = 5, P < 0.05, each). Pravastatin alone had a negligible effect on GTP-loading (Figure 5D).

3.6 Prevention of thrombin-induced geranylgeranylation and GTP-loading of Rac1 by pravastatin

The proportions of unprocessed and geranylgeranylated forms of Rac1 in untreated rat aortas were 32 ± 12 and 68 ± 12%, respectively (Figure 6A, n = 4, P < 0.05). The unprocessed form of Rac1 was significantly converted into the mature geranylgeranylated form by the single injection of thrombin within 1 h (Figure 6B, n = 4, P < 0.05), which was

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**Figure 5** (A) Proportions of unprocessed (UP) and geranylgeranylated (GG) forms of RhoA in rat aortas determined by Triton X-114 partition method. Amounts of RhoA protein were determined by western blotting. Representative immunoblots from four independent experiments are shown at the top. Values are the means ± SD of quantitative densitometric analyses from four separate experiments. **P < 0.01. (B) Effects of intravenous injection of thrombin (50 U/kg) with or without pravastatin (0.3 mg/kg) on the conversion of unprocessed RhoA into geranylgeranylated RhoA in vivo 1 h after injection. Levels of unprocessed GDP-RhoA and geranylgeranylated RhoA in rat aortas were determined by western blotting. Representative immunoblots from four independent experiments are shown. Bars are the means ± SD of quantitative densitometric analyses from four separate experiments. *P < 0.05. (C) Effects of intravenous coadministration of pravastatin on thrombin-triggered GTP/GDP exchange 1 h after injection as determined by pull-down assays. Data are results of densitometric analysis of the GTP-bound form of RhoA, indicating the means ± SD of five rats in each group. Representative blots are shown in upper column. *P < 0.05. (D) Effects of intravenous administration of pravastatin alone on RhoA GTP/GDP exchange 1 h after injection as determined by pull-down assays. Data are expressed as the means ± SD of three rats. Representative blots are shown. NS, not significant.
prevented by the coadministration of pravastatin and thrombin (Figure 6B, n = 4, P < 0.05). We further investigated the effect of thrombin with or without pravastatin on the activation of Rac1 and p47phox in vivo in rats. Membrane translocation (Figure 6C) and pull-down assays (D). Bars are the means ± SD of four separate experiments. *P < 0.05. (E) Effects of administration of pravastatin alone on Rac1 GTP/GDP exchange in rat aortas. Results represent means ± SD of three separate experiments and immunoblots are from an experiment representative. (F) p47phox activation was determined by membrane translocation, followed by western blotting. Data are expressed as means ± SD of three separate experiments. *P < 0.05.

3.7 GGTase I activity

We finally measured the enzymatic activity of the GGTase I responsible for geranylgeranylation of RhoA and Rac1 1 h after injection. The single injection of thrombin significantly increased the activity of GGTase I of rat aortas within 1 h, whereas coadministration of pravastatin did not alter the increased activity of GGTase I (data not shown, n = 7, P < 0.001). These results indicated that thrombin-induced the rapid geranylgeranylation of unprocessed RhoA and Rac1-mediated via increased GGTase I activity in vivo, which were then converted into GTP-bound active RhoA.

4. Discussion

The present study showed that within 1 h in vivo in rats intravenous coadministration of pravastatin prevented thrombin-induced endothelial dysfunction such as impaired NO-related vasodilation and NAD(P)H oxidase-dependent ROS generation. We emphasize here possible mechanisms by which coadministration of pravastatin rapidly prevents thrombin-induced down-regulation of Akt/eNOS phosphorylation and up-regulation of ROS generation as well as conversion of unprocessed RhoA and Rac1 into mature geranylgeranylated forms, which appears to be essential for full activation of unprocessed RhoA and Rac1.

In the present study, a single intravenous injection of thrombin-induced a conversion of unprocessed RhoA and Rac1 into GTP-bound geranylgeranylated forms, which then translocated to membrane, within 1 h in vivo in rat aortas. We clearly showed that simultaneous injection of pravastatin antagonized thrombin-induced conversion of the unprocessed RhoA and Rac1 into mature geranylgeranylated proteins as well as the GTP-loading and membrane translocation in vivo. To our knowledge, this is the first study to demonstrate that pravastatin prevented agonist-induced rapid
activation of the unprocessed forms of RhoA and Rac1 via geranylgeranylation through GTP-loading and membrane translocation in vivo. In addition, we observed that thrombin-induced increase in GGTase I activity was partially blocked by inhibition of Gi, suggesting the signaling pathway via Gi.

Importantly, we provided evidence of the acute physiological effect of pravastatin on NO-mediated endothelium-dependent coronary vasodilation to ACh. This finding was associated with the profile of Akt/eNOS phosphorylation, showing that coadministration of pravastatin prevented thrombin-induced down-regulation of Akt/eNOS phosphorylation within 1 h after injection in normal rats. In fact, we electrochemically measured serum NO levels using NO-sensitive electrode. Thrombin decreased the serum levels of NO 1 h after injection, which was prevented by the coadministration of pravastatin. Lipophilic statins have shown to stimulate the Akt/eNOS pathway in endothelial cells in vitro. The present study suggests that hydrophilic pravastatin rapidly enhanced NO-dependent vasodilation appears to be based on increased NO bioavailability in vivo in rats. Although Wassmann et al. reported that atorvastatin blocked the Rac1 activation responsible for NADPH oxidase-dependent ROS generation in rat aortic smooth muscle cells in vitro and in rats aortas in vivo, they did not clarify the detailed metabolism of the inhibition of Rac1 by statin. The present study clearly demonstrated that thrombin injection induced a rapid Rac1 activation including the conversion of the unprocessed Rac1 into the geranylgeranylated form, GTP-loading, and membrane translocation, accompanied by p47phox activation, in rat aortas. Moreover, Price et al. reported that active Rac1 induces a translocation of p47phox and p67phox to the membrane in COS cells. Rac1-mediated superoxide formation has been shown to be dependent on the guanine nucleotide exchange factor (GEF) for GTP-loading in COS cells and phagocytes. In the present study, we found that coadministration of pravastatin blocked a thrombin-triggered series of Rac1 and p47phox activations and decreased thrombin-triggered NAD(P)H oxidase activities of the rat aortas by ~60% in vivo, which probably accounts for 60% of the increased NAD(P)H oxidase activity derived from the unprocessed Rac1 and 40% increase from the mature geranylgeranylated form. In addition to GEF activity, our findings in this study based on the conversion assay of unprocessed Rac1 into mature form suggest that the geranylgeranylation of unprocessed Rac1 may play a key role in the assembly of NAD(P)H oxidase, NAD(P)H oxidase activities and ROS generation in vivo.

In addition to the single injection of pravastatin, we pre-treated rats with pravastatin orally for 5 days before administration of thrombin, indicating the similar preventive effects of pravastatin on Akt/eNOS phosphorylation and ROS generation. In terms of the translation of our findings to clinical situation, the present study supports the beneficial effects of pravastatin for primary and secondary cardiovascular prevention such as MEGA and MUSASHI trials. Furthermore, the findings of SHR support the clinical trial of ALLHAT that statin treatment is beneficial for hypertensive patients with relatively normocholesterolemia.

In conclusion, we show that the rapid prevention of the down-regulation of Akt/eNOS and the conversion of unprocessed RhoA and Rac1 into their geranylgeranylated forms by pravastatin improve NO-related endothelium-dependent vasodilation, and reduces NAD(P)H oxidase-dependent ROS generation in thrombin stimulation in vivo in rats. This clarifies part of the acute beneficial effects of pravastatin on endothelial dysfunction and oxidant stress.

Conflict of interest: none declared.

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