Long-Term Inhibition of RhoA Attenuates Vascular Contractility by Enhancing Endothelial NO Production in an Intact Rabbit Mesenteric Artery

Noriko Shiga, Katsuya Hirano, Mayumi Hirano, Junji Nishimura, Hajime Nawata, Hideo Kanaide

Abstract—RhoA plays a critical role in regulating NO production in cultured endothelial cells. To determine its role in situ endothelial cells, we investigated the effects of 3-hydroxy-3-methyl-glutaral coenzyme A reductase inhibitors and a RhoA-binding domain of Rho-kinase (RB) on vascular contractility in the isolated rabbit mesenteric artery. Ex vivo treatment of the strips with $3 \times 10^{-5}$ mol/L simvastatin and fluvastatin for $\approx 24$ to 30 hours significantly attenuated the contractile response to phenylephrine and high K$^+$ in the presence of endothelium. The addition of $N^\omega$-nitro-L-arginine methyl ester and the removal of endothelium abolished the attenuation of the contractile response. The cotreatment with geranylgeranyl pyrophosphate prevented the statin-induced attenuation of the contractile response, whereas geranylgeranyl transferase inhibitor mimicked the effect of simvastatin. Treatment with simvastatin enhanced the bradykinin-induced endothelium-dependent relaxation in the mesenteric artery, whereas it had no effect on the bradykinin-induced [Ca$^{2+}$], elevation in endothelial cells of the aortic valves. Introduction of RB to the strips using a cell-penetrating peptide of Tat protein (TATHA-RB) attenuated the contractile responses in a NO-dependent manner. However, a Rac1/Cdc42-binding fragment of p21-activated protein kinase, RB without Tat peptide or TATHA-protein A had no effect. The in vivo treatment of rabbit with simvastatin and TATHA-RB attenuated the contractility in a NO-dependent manner. Simvastatin and TATHA-RB significantly upregulated eNOS in the rabbit mesenteric artery. The present study provides the first evidence that RhoA plays a physiological role in suppressing NO production in situ endothelial cells. (Circ Res. 2005;96:1014-1021.)

Key Words: endothelial cells ■ RhoA ■ nitric oxide ■ statins ■ molecular biology

Endothelial dysfunction is associated with such vascular diseases as atherosclerosis and hypertension, and it also plays a critical role in the early development of atherosclerotic lesions.1–4 Endothelial dysfunction is often characterized by an impairment of the production and release of the endothelium-derived vasorelaxing factors including nitric oxide (NO). It is thus important to elucidate the mechanism regulating NO production for in situ endothelial cells to develop new therapeutic strategies for the treatment of impaired endothelial function associated with the vascular diseases. Small GTP-binding protein RhoA has been shown to regulate the expression of endothelial nitric oxide synthase (eNOS), thereby regulating the production of NO.5 The treatment of endothelial cells with Clostridium botulinum C3 exoenzyme and the overexpression of a dominant-negative mutant of RhoA in the endothelial cells were demonstrated to upregulate the expression of eNOS mRNA mainly by stabilizing mRNA.5 On the other hand, the activation of RhoA by Escherichia coli cytotoxic necrotizing factor-1 downregulated eNOS mRNA.5 Furthermore, RhoA and Rho-kinase were shown to inhibit the phosphorylation of eNOS at Ser1177, thereby inhibiting NO production in endothelial cells.6 However, such experimental evidence was obtained in cultured endothelial cells. The physiological role of RhoA in the regulation of NO production using an intact vascular tissue thus remains to be established.

It is well known that 3-hydroxy-3-methyl-glutaral coenzyme A (HMG-CoA) reductase inhibitors, statins, inhibit not only cholesterol synthesis but also protein isoprenylation because of the reduction of the isoprenoid intermediates of the cholesterol biosynthesis pathway.7–9 Because protein isoprenylation is essential for the function of small GTP-binding proteins,10 statins have been used to inhibit the intracellular signaling mediated by the small GTP-binding proteins.7–9 Indeed, statins have been shown to upregulate the expression of eNOS, enhance the basal and stimulated NO production, and improve endothelium-dependent vasorelaxation, in a manner sensitive to geranylgeranyl pyrophosphate.
in both human and animal studies.7–9,11 These observations strongly suggest that these small GTP-binding proteins play a physiological role in the regulation of NO production in the vascular tissue. However, these observations could not specify which small GTP-binding proteins are involved.

To establish the role of the small GTP-binding proteins in the regulation of NO production in the intact vascular tissue, it is essential to examine the effect of the dominant-negative mutants of the small GTP-binding proteins. However, it has up to now been technically difficult to efficiently introduce these mutants and examine their effect on the contractility in the intact vascular tissue. In the present study, we utilized 2 methods to inhibit the intracellular signaling mediated by RhoA and Rac1 in the isolated rabbit mesenteric artery. First, we treated the isolated vascular tissues with statins for ≈24 to 30 hours and examined their effects on vascular contractility. Second, we utilized a cell-penetrating peptide found in human immunodeficiency viral transactivator of transcription (Tat) protein22,23 to introduce the RhoA-binding domain of Rho-kinase14 and the Rac1/Cdc42-binding domain of p21-activated protein kinase-1 (PAK1).15 Such fragments were shown to exert a dominant-negative effect on the endogenous RhoA and Rac1/Cdc42, respectively.16 The present study provides the first evidence of the physiological role that RhoA plays in the regulation of endothelial NO production in the intact vascular tissue.

**Materials and Methods**

An expanded Materials and Methods section can be found in the online data supplement available at http://circres.ahajournals.org.

**Tissue Preparation**

Japanese white male rabbits (Kyudo, Saga, Japan) were euthanized according to the protocol approved by the Animal Care and the Committee of the Research Institute of Angiocardiology, Graduate School of Medical Sciences, Kyushu University. The mesenteric artery was isolated to evaluate the contractile response, and the aortic strips were recovered with Ni2+ in situ endothelial cells.17 The experimental number indicates the number of rabbits.

**Recombinant Proteins**

The RhoA-binding domain (RB) of human Rho-kinase14 and the Rac1/Cdc42-binding domain (PBD) of human PAK115 were prepared as a fusion protein with a (His)_6 tag, a protein transduction domain (PTD) of Tat and a hemagglutinin tag (TATHA-RB and TATHA-PBD).14,15 RB and PBD only with a (His)_6 tag [(His)_6-RB and (His)_6-PBD], and the Tat PTD-tagged IgG binding region of Staphylococcal protein A20 (TATHA-protein A) were constructed as control proteins. The recombinant proteins were expressed and purified as previously described.19

**Measurement of Force Development in the Isolated Rabbit Mesenteric Artery**

The strips were mounted vertically to a force transducer, and the contractile response was measured at 37°C as previously described.21

In some arterial strips, the luminal surface was rubbed off with a cotton swab to remove the endothelium.

**Cell Culture**

Bovine aortic endothelial cells (BAECs) were cultured as previously described,25 and used for experiments at passages 11 to 18.

**Front-Surface Fluorometry of [Ca^{2+}]_i in Valvular Strips**

The front-surface fluorometry and the fura-PE3-loaded strips of the rabbit aortic valves were used to monitor changes in [Ca^{2+}]_i in situ endothelial cells as previously described.17,23

**Long-Term Treatment of Arterial and Valvular Strips**

The strips of the mesenteric artery and the aortic valves were treated for ≈24 to 30 hours at 37°C without (control) and with various reagents and recombinant proteins, as indicated, in the serum-free Dulbecco modified Eagle medium (DMEM) containing streptomycin and penicillin, unless otherwise specified. The strips were then washed and equilibrated in physiological salt solution at least 1 hour before performing functional study. To avoid the withdrawal effect of statins,24 the functional study was completed within 2 hours after terminating the treatment with statins.

**Immunoblot Detection of Intracellular Protein Transduction and Endogenous RhoA, Rac1, Cdc42, and eNOS in the Intact Strips of Mesenteric Artery**

The transduction of Tat PTD-tagged recombinant proteins into the arterial strips was evaluated by an immunoblot analysis as previously described.19 The extracts of the arterial strips were also subjected to immunoblot detection of the endogenous RhoA, Rac1, Cdc42, and eNOS.

**Pull-Down Assay**

The interaction of TATHA-RB and TATHA-PBD with endogenous RhoA and Rac1 was examined by pull-down assay as previously described.23–26 The extract of the arterial strips were incubated with TATHA-RB and TATHA-PBD, and the recombinant proteins were recovered with Ni^{2+}-nitrilo acetate resin (Qiagen). The bound protein was then subjected to immunoblotting to detect RhoA and Rac1.

**In Vivo Treatment With Simvastatin and TATHA-RB**

Rabbits received intravenous injection of 0.33 mg/kg per day of simvastatin or 0.7 mg/kg per day of TATHA-RB for 3 days. The mesenteric artery was then isolated and its contractility was examined ex vivo.

**Statistical Analysis**

The data are the mean±SEM. The unpaired Student t test and an analysis of variance (ANOVA) evaluated statistical significance. Probability values of less than 0.05 were considered to be significant.

**Results**

**Endothelium-Dependent, N^\text{G}\text{-Nitro-L-Arginine Methyl Ester–Sensitive Attenuation of the Vascular Contractility by Statins in the Rabbit Mesenteric Artery**

The ≈24 to 30–hour treatment of the isolated artery with 3×10^{-5} and 1×10^{-4} mol/L simvastatin attenuated the contractile response to the cumulative applications of phenylephrine in the absence of N^\text{G}\text{-nitro-L-arginine methyl ester (L-NAME) (Figure 1a). However, when the contractile response was examined in the presence of 1×10^{-4} mol/L L-NAME, the response of the strips treated with 3×10^{-3} mol/L simvastatin was similar to that seen in the control. On the other hand, the attenuation of the phenylephrine-induced contraction seen with 1×10^{-4} mol/L simvastatin was resistant to 1×10^{-4} mol/L L-NAME (Figure 1a). The similar L-NAME–
sensitive attenuation of the contractile response to phenylephrine was observed with $3 \times 10^{-5}$ mol/L fluvastatin (online Figure S1 in the online data supplement). However, pravastatin, a hydrophilic statin, demonstrated no significant effect on the contractile response even at $1 \times 10^{-4}$ mol/L (data not shown). Similarly, $3 \times 10^{-5}$ mol/L simvastatin attenuated the contraction induced by 118 mmol/L K$^+$ in a L-NAME–sensitive manner, whereas the attenuation of the 118 mmol/L K$^+$–induced contraction by $1 \times 10^{-4}$ mol/L simvastatin was resistant to L-NAME (Figure 1b). A similar L-NAME–sensitive attenuation of the contractile response to 118 mmol/L K$^+$ was also observed with $3 \times 10^{-5}$ mol/L fluvastatin (data not shown).

On the other hand, the treatment of the endothelium-denuded strips with $3 \times 10^{-5}$ mol/L simvastatin or fluvastatin had no effect on the contractile response to phenylephrine and 118 mmol/L K$^+$ (Figure 1c), whereas $1 \times 10^{-4}$ mol/L simvastatin and fluvastatin also attenuated the contractility (data not shown). As a result, the attenuation of the contractile response seen with $3 \times 10^{-5}$ mol/L statins was dependent on the endothelium and sensitive to L-NAME, whereas those seen with $1 \times 10^{-4}$ mol/L statins were independent of the endothelium and resistant to L-NAME.

### Involvement of Protein Geranylgeranylation in the Statin-Induced Attenuation of the Contractility

To determine the involvement of isoprenoid intermediates of the cholesterol biosynthetic pathway in the attenuation of contractility seen with lipophilic statins, the effect of geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) on the statin-induced attenuation of contractility were studied (Figure 2). The treatment of the arterial strips with $3 \times 10^{-5}$ mol/L simvastatin or fluvastatin in the presence of $1 \times 10^{-5}$ mol/L GGPP or FPP failed to attenuate the contractile response to both phenylephrine and 118 mmol/L K$^+$ (Figure 2). On the other hand, the $\approx 24$ to $30$–hour treatment with $1 \times 10^{-5}$ mol/L geranylgeranyl transferase inhibitor (GGTI-298) but not with farnesyl transferase inhibitor (FTI-276) attenuated the contractile response to phenylephrine and 118 mmol/L K$^+$ in an L-NAME–sensitive manner (Figure 2), as in the case with lipophilic statins (Figure 1). The endothelium-independent, L-NAME–resistant attenuation of the contractile responses seen with $1 \times 10^{-5}$ mol/L simvastatin was only partially inhibited by treatment with $1 \times 10^{-5}$ mol/L GGPP (data not shown).

### Transduction of TATHA-RB Attenuated the Contractility of Rabbit Mesenteric Artery in an Endothelium-Dependent, L-NAME–Sensitive Manner

To investigate the involvement of Rho proteins, RhoA, Rac1, and Cdc42, in the statin-induced attenuation of the contractile response of rabbit mesenteric artery, we utilized cell-penetrating peptide-mediated protein transduction technique, and introduced the RhoA-binding domain (RB) of Rho-kinase and the Rac1/Cdc42-binding domain (PBD) of PAK1 into the vascular strips, as previously described. Such fragments were shown to exert a dominant-negative effect on the endogenous RhoA and Rac1/Cdc42 signaling, respectively. Treatment with $1 \times 10^{-6}$ mol/L TATHA-RB for $\approx 24$ to $30$ hours attenuated the contractile response to phenylephrine and 118 mmol/L K$^+$ to the similar extent to that seen with $3 \times 10^{-5}$ mol/L simvastatin. However, the 30-minute treatment with TATHA-RB failed to attenuate the contractile response (data not shown). L-NAME ($1 \times 10^{-4}$ mol/L) abolished the TATHA-RB–induced attenuation of the contractile response as in the case with lipophilic statins (Figure 3). The treatment of the endothelium-denuded strips with TATHA-RB for 24 hours had no effect on the contractile response to phenylephrine and 118 mmol/L K$^+$ (data not shown). On the other hand, $1 \times 10^{-6}$ mol/L TATHA-PBD
had no effect on the contractile response to phenylephrine (Figure 3). The control recombinant proteins, (His)6-RB, (His)6-PBD, and TATHA-protein A, at 10^-6 mol/L demonstrated no significant effect on the contractile response to phenylephrine (Figure 3). Rho-kinase is one of the effector proteins of the RhoA signaling. However, the 24 to 30-hour treatment of the strips with 10^-5 mol/L Y27632 had no effect on the contractile responses (data not shown).

Immunoblot Verification of Protein Transduction and Specific Interaction of TATHA-RB and TATHA-PBD With Endogenous Targets in the Rabbit Mesenteric Artery

As shown in Figure 4a, only TATHA-RB but not (His)_6-RB was detected in the strips exposed to the recombinant proteins for 15 minutes. TATHA-RB was also detected after 24-hour exposure, with no substantial degradation. However, TATHA-RB was scarcely detected after 24-hour exposure and subsequent wash and 1-hour equilibration in physiological salt solution. This observation indicated that a negligible amount of protein remained during the evaluation of the contractile response. The transduction of TATHA-PBD and TATHA-protein A also confirmed by an immunoblot analysis (online Figure S2). The treatment of the strips with TATHA-RB and TATHA-PBD exhibited no effect on the expression level of endogenous RhoA and Rac1 (Figure 4a).
Cdc42 was scarcely detected in the rabbit mesenteric artery under any conditions (data not shown). The pull-down assay demonstrated a specific interaction of TATHA-RB and TATHA-PBD with endogenous RhoA and Rac1, respectively, and no cross-reactivity was observed (Figure 4b).

**Treatment With Simvastatin and TATHA-RB Upregulated the Expression of eNOS in the Rabbit Mesenteric Artery**

A Western blot analysis revealed that the treatment of the strips with $3 \times 10^{-5}$ mol/L simvastatin or $1 \times 10^{-6}$ mol/L TATHA-RB significantly increased the expression of eNOS in the rabbit mesenteric artery (Figure 5). A 3-hour wash in saline decreased the level of eNOS to the control level in the simvastatin- and TATHA-RB–treated strips (Figure 5).

**Reversibility of the Effect of Simvastatin and TATHA-RB Treatment on the Contractility**

The contractile response to phenylephrine was significantly attenuated after $\approx 1$-hour equilibration in the resting buffer after terminating the 24-hour treatment with simvastatin and TATHA-RB. After further $\approx 1$ hour equilibration, the contraction induced by the second application of phenylephrine in the simvastatin- and TATHA-RB–treated strips did not significantly differ from that seen in the control. However, the control untreated strips exhibited similar responses to both the first and second applications of phenylephrine (Figure 6).

**Simvastatin Enhanced the Bradykinin-Induced Endothelium-Dependent Relaxation in the Arterial Strips With No Effect on the [Ca$^{2+}$], Elevation in Endothelial Cells**

In the control rabbit mesenteric artery, bradykinin induced concentration-dependent relaxation during the sustained phase of the $1 \times 10^{-5}$ mol/L phenylephrine-induced contraction (Figure 7a). This relaxation was enhanced in the arterial strips treated with $3 \times 10^{-5}$ mol/L simvastatin for $\approx 24$ to 30 hours (Figure 7a). On the other hand, the relaxation induced by sodium nitroprusside in the presence of $1 \times 10^{-5}$ mol/L L-NAME was similar between the control and simvastatin-treated strip (Figure 7b). The bradykinin-induced elevation of...
[Ca\(^{2+}\)], seen in the strips of the aortic valve treated with 3×10\(^{-5}\) mol/L simvastatin did not significantly differ from that seen in the control strips (Figure 7c).

**In Vivo Treatment With Simvastatin and TATHA-RB Attenuated the Contractile Responses of the Rabbit Mesenteric Artery in a NO-Dependent Manner**

In vivo treatment of rabbits with simvastatin and TATHA-RB attenuated the contractile response induced by cumulative applications of phenylephrine (Figure 8a) and 118 mmol/L K\(^+\) (Figure 8b) in a manner sensitive to the 1×10\(^{-5}\) mol/L L-NAME.

**Discussion**

We herein for the first time demonstrated that the long-term inhibition of RhoA signaling attenuated the vascular contractility by augmenting the endothelial NO production in the isolated, intact rabbit mesenteric artery. We first utilized statins to inhibit the activity of Rho proteins and observed that lipophilic statins attenuated the contractile response to both phenylephrine and high K\(^+\) depolarization, which was abolished by the supplementation of GGPP. Furthermore, the bradykinin-induced endothelium-dependent relaxation was enhanced in the arteries treated with lipophilic statins, with no enhancement of the bradykinin-induced [Ca\(^{2+}\)], elevation in the endothelial cells. However, the relaxation induced by sodium nitroprusside was not altered by simvastatin treatment, thus suggesting that the sensitivity of smooth muscle to NO remained unchanged after the simvastatin treatment. The basal and stimulated production of NO was thus, suggested to be augmented in the lipophilic statin-treated strips. On the other hand, the inhibition of geranylgeranylation mimicked the effect of lipophilic statins. These observations thus suggest that the inhibition of protein geranylgeranylation played an important role in the enhancement of the endothelial NO production and attenuation of the vascular contractility seen with the lipophilic statins.

We next utilized the protein transduction technique\(^{12,13}\) to specifically inhibit the signal transduction mediated by Rho proteins in the intact arterial strips. The transduction of TATHA-RB but not TATHA-PBD attenuated the contractile responses in an endothelium-dependent, L-NAME–sensitive manner as observed with lipophilic statins. A pull-down assay demonstrated the specific interaction of TATHA-RB and TATHA-PBD to endogenous RhoA and Rac1. The specificity of the effect of TATHA-RB was also supported by an observation that the transduction of an unrelated protein, TATHA-protein A, had no effect on the contractility. Furthermore, RB had no effect without Tat PTD. We thereby provide direct evidence that long-term (≈24 to 30 hours) inhibition of RhoA activity in the endothelial cells removed the inhibition of NO production and thereby attenuated the contractile response in an intact artery. Both simvastatin and TATHA-RB upregulated the eNOS expression, thus suggesting that the NO-dependent attenuation of the contractile response was partly because of the upregulation of eNOS. The physiological relevance of these effects of statins and TATHA-RB seen with ex vivo treatment is supported by the observations with in vivo treatment.

The transduction of Tat PTD-tagged protein into the intact arterial strips was validated in the present study by the Western blot analysis as previously reported.\(^{19,27,28}\) TATHA-RB but not (His\(_n\))\(_8\)-RB was detected in the extracts of arterial strips as early as 15 minutes. TATHA-RB was also detected with negligible degradation even after 24-hours incubation. However, it is noteworthy that TATHA-RB was scarcely detected after 1-hour equilibration in physiological salt solution. This observation thus suggests that the Tat PTD–mediated protein transduction is reversible and no significant amount of Tat PTD–tagged proteins remained in the arterial strips when the contractile response was evaluated. This is consistent with our previous observation on the reversible effect seen with a dominant-negative fragment of the myosin phosphatase regulatory subunit MYPT1.\(^{19}\) It is thus indicated that the attenuation of the contractility seen with TATHA-RB was not because of its direct effect on either the smooth muscle contractile mechanism or the endothelial NO production.

The role of RhoA in the regulation of the endothelial production of NO has been demonstrated using cultured endothelial cells.\(^{5,6}\) The inhibition of RhoA by *Clostridium botulinum* C3 exoenzyme and overexpression of a dominant-negative mutant of RhoA were shown to increase eNOS expression by stabilizing mRNA, whereas the activation of RhoA by *Escherichia coli* cytotoxic necrotizing factor-1 downregulated it.\(^{5}\) RhoA and Rho-kinase were shown to not

**Figure 6.** Reversibility of the effect of simvastatin and TATHA-RB on the contractility. Concentration-response curves for the contraction induced by phenylephrine obtained just after 1-hour equilibration of the strips treated for 24 to 30 hours in DMEM without (control) and with 3×10\(^{-5}\) mol/L simvastatin or 1×10\(^{-6}\) mol/L TATHA-RB (first application). Contractile response was then sequentially evaluated with 1-hour interval of incubation in the resting buffer (second application). Second evaluation was thus performed 3 hours after terminating the treatment with simvastatin and TATHA-RB. Data are the mean±SEM (n=3). \(^{**}\)\(P<0.01; \ ^*\)\(P<0.05\) vs control. n.s. indicates not significantly different.
The attenuation of the contractile response seen with $1 \times 10^{-4}$ mol/L simvastatin was resistant to L-NAME and independent of the endothelial cells. This observation suggests that simvastatin had a direct effect on the smooth muscle cells at this high concentration. Furthermore, GGPP only partially prevented the attenuation of the contractile response, thus suggesting that only part of the effect of statin in situ endothelial cells of the artery. However, our observation on the effect of Y27632 suggests that Rho-kinase does not play a major role in the regulation of NO production in in situ endothelial cells. How RhoA regulates NO production thus remains to be elucidated.

In the present study, FPP as well as GGPP abolished the effect of lipophilic statins on vascular contractility. However, FTI did not show any effect, whereas GGTI mimicked the effect of lipophilic statin. Our findings thus suggested that geranyleranylation but not farnesylation plays a major role in the attenuation of the vascular contractility seen with lipophilic statins. We speculate that FPP did not directly have an effect on the lipophilic statin-induced attenuation of the vascular contractility. On the other hand, we speculate that FPP was converted to GGPP, thereby inhibiting the effect of statin. GGPP is synthesized by a single condensation of FPP and isopentenyl pyrophosphate.\textsuperscript{9,26} We suggest that the residual amount of isopentenyl pyrophosphate after the inhibition of HMG-CoA reductase by $3 \times 10^{-4}$ mol/L lipophilic statins was sufficient to convert the exogenously added FPP to GGPP in the statin-treated artery.

The present study thus provides the first evidence for the physiological role of RhoA in the regulation of NO production in in situ endothelial cells of the artery. However, our observation on the effect of Y27632 suggests that Rho-kinase does not play a major role in the regulation of NO production in in situ endothelial cells. How RhoA regulates NO production thus remains to be elucidated.

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The attenuation of the contractile response seen with $1 \times 10^{-4}$ mol/L simvastatin was resistant to L-NAME and independent of the endothelial cells. This observation suggests that simvastatin had a direct effect on the smooth muscle cells at this high concentration. Furthermore, GGPP only partially prevented the attenuation of the contractile response, thus suggesting that only part of the effect of statin only downregulate eNOS mRNA expression but also inhibit the phosphorylation of eNOS at Ser1177, thereby inhibiting NO production in cultured human endothelial cells.\textsuperscript{9} On the other hand, it has been well established that statins improve the endothelial function, especially by enhancing NO production in a manner not only dependent on but also independent of its cholesterol-lowering effect in humans and animal models.\textsuperscript{7–9} Statins were shown to upregulate the expression of eNOS mRNA and protein, enhance the basal and stimulated NO production and cause vasodilatation.\textsuperscript{11} The effects of statins were reported to be abolished by GGPP. All these observations strongly suggest that the inhibition of RhoA by statin plays a major role in the enhancement of NO production. However, it remains to be elucidated as to whether or not RhoA also plays a critical role in the regulation of NO production in situ endothelial cells of the vascular tissue. The present study thus provides the first evidence for the physiological role of RhoA in the regulation of NO produc-
seen at 1 × 10⁻⁴ mol/L was dependent on protein isoprenylation. The GGPP-resistant component thus could be non-specific effect. The effect seen with 1 × 10⁻⁴ mol/L, statins may also be related to the proapoptotic effect on the smooth muscle cells.³⁰–³²

In conclusion, we herein provide the first experimental evidence for the physiological role of RhoA in the regulation of the endothelial NO production and the contractility in the vascular tissues. The specific inhibition of the intracellular signaling mediated by RhoA enhanced the NO-dependent relaxation with no effect on the Ca²⁺ signaling in situ endothelial cells. RhoA is thus suggested to serve as a target molecule in the treatment of endothelial dysfunction associated with the vascular diseases such as arteriosclerosis and hypertension. The present study also suggests the cell-penetrating peptide-mediated protein transduction technique to be a powerful tool for investigating the role of the intracellular signaling molecule in the regulation of the cellular function in intact vascular tissue.

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Long-term inhibition of RhoA attenuates vascular contractility by enhancing endothelial NO production in an intact rabbit mesenteric artery

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Expanded Materials and Methods

Materials

Simvastatin, fluvastatin and pravastatin were supplied by Merck & Co. (Rahway, N.J., U.S.A.), Novartis Pharma Inc. (Tokyo, Japan) and Sankyo Co. (Tokyo, Japan), respectively. The plasmids pTATHA and pMALU5-ProteinA were kindly donated by Dr. Steven F. Dowdy (University of California, San Diego, CA, U.S.A.) and Dr. Masuo Aizawa (Tokyo Institute of Technology, Yokohama, Japan), respectively. Fura-PE3/AM, an acetoxymethyl ester from of fura-PE-3 was purchased from Texas Fluorescence Laboratory (Austin, TX, U.S.A.). Phenylephrine, ionomycin, Nω-nitro-L-arginine methyl ester (L-NAME), geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) were purchased from Sigma (St. Louis, MO, U.S.A.). The antibodies and their source were as follows: Anti-(His)6 antibody (Qiagen, Hilden, Germany), anti-RhoA antibody (sc-418; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), anti-Rac1 antibody (No.610650; BD Bioscience, San Jose, CA, U.S.A.), anti-Cdc42 antibody (sc-87; Santa Cruz Biotechnology), anti-eNOS antibody (No.610296; BD Bioscience), anti-tubulin antibody (LY1/2; Serotec, Kidlington, England, U.K.), horse radish peroxidase-conjugated anti-mouse IgG (Sigma), horse radish peroxidase-conjugated anti-rabbit IgG (Vector Laboratories, Burlingame, CA, U.S.A.) and horse radish peroxidase-conjugated anti-rat IgG (Serotec). Geranylgeranyl transferase I inhibitor (GGTI-298), farnesyl transferase inhibitor (FTI-276) and Y27632 were purchased from...
Calbiochem (San Diego, CA, U.S.A.). The composition of the physiological salt solution (PSS) was (in mmol/L): NaCl 123, KCl 4.7, NaHCO3 15.5, KH2PO4 1.2, MgCl2 1.2, CaCl2 1.25 and d-glucose 11.5. PSS containing 118 mmol/L K+ was prepared by replacing NaCl with equimolar KCl. PSS was aerated with 95% O2 and 5% CO2, with the resulting pH considered to be 7.4.

Tissue preparation

These protocols were approved by the Animal Care and the Committee of the Research Institute of Angiocardiology, Graduate School of Medical Sciences, Kyushu University. Japanese white male rabbits weighing 2.5 kg were used. The rabbits were sacrificed by intravenous injection of a lethal dose of sodium pentobarbital (83 mg/kg body weight) and following exsangination from carotid arteries. The mesenteric artery was immediately isolated, and the surrounding connective tissues were mechanically removed under a binocular microscope. The vessels were opened longitudinally, and cut into strips (4 mm long, 1 mm wide, 0.1 mm thick) in a circular direction. In some strips, the luminal surface was rubbed off with a cotton swab to remove endothelium. The aortic valves were excised, and three leaflets of the valve were used to monitor the changes in cytosolic Ca2+ concentration ([Ca2+]i) in situ endothelial cells, as previously described 3. The experimental number indicates the number of rabbits.

Preparation of recombinant proteins

A cDNA fragment containing a IgG binding region of amino acid residues 23-270 of Staphylococcal protein A 4 was amplified by PCR using pMALU5-Protein A as template, and subcloned into pTATHA to express recombinant protein A tagged with a (His)6 tag and a protein transduction domain (PTD) of the human immunodeficiency viral transactivation of transcription (Tat) protein and a hemagglutinin (HA) tag at its N-terminus. The cDNA
fragments encoding amino acid region 941-1075 of human Rho-kinase (Accession number; D87931) that contains a RhoA-binding domain (RB)\(^5\) and amino acid region 67-150 of human p21-activated protein kinase-1 (PAK1; Accession number; U24152) that contains a Rac1/Cdc42-binding domain (PBD)\(^6\) were obtained by PCR amplification directly from a cDNA library of human aorta (Clontech, Palo Alto, CA, U.S.A.). The fragments were subcloned into expression plasmid pQE30 (Qiagen, Hilden, Germany) to express recombinant proteins tagged with a (His)\(_6\) tag ((His)\(_6\)-RB, (His)\(_6\)-PBD) and pQE30TATHA to express recombinant proteins tagged with a (His)\(_6\) tag, Tat PTD and a HA tag (TATAHA-RB, TATHA-PBD). The preparation of pQE30TATHA was previously reported\(^7\). The recombinant proteins were expressed in appropriate bacterial strains and then affinity-purified through Ni\(^{2+}\)-loaded Hi-Trap chelating column on Akta Prime (Pharmacia Biotech, Tokyo, Japan), as previously described\(^7\). The protein concentration was determined with Coomassie protein assay kit (Pierce, Rockford, IL, U.S.A.). The purity of the proteins was higher than 90% as evaluated on SDS-PAGE (Fig.5 for an example).

*Long-term treatment of arterial and valvular strips*

The strips of mesenteric artery with and without endothelium, and aortic valves were treated for 24~30 h at 37 °C without (control) and with various reagents and recombinant proteins in the serum-free Dulbecco’s modified Eagle medium (DMEM) containing streptomycin and penicillin under a CO\(_2\) incubator, unless otherwise specified. The strips were then washed and equilibrated in PSS at least 1 h before evaluating the contractile responses to phenylephrine and high K\(^+\)-depolarization. To avoid the withdrawal effect of statins\(^8\), the evaluation of contractile response was completed within 2 h after terminating the treatment with statins.

*Measurement of force development*

Strips were mounted vertically in a quartz organ bath, which was warmed by circulating water
at 37 °C. One end of the strips was connected to a fixed hook, while the other end was connected to a strain gauge (TB-612-T, Nihon Koden, Japan). During the period of 1 h equilibration, the strips were stimulated with 118 mmol/L K+ depolarization every 15 min, and the resting force was elevated in a stepwise manner and finally adjusted to 250-300 mg, before starting to evaluate the contractile responses.

Cell culture

Bovine aortic endothelial cells (BAECs) were cultured in Dulbecco’s modified Eagle medium containing 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 50 µg/ml gentamycin as previously described 9. The cells were used for experiments at passages 11-18.

Front-surface fluorometry in valvular strips

The aortic valves were loaded with the Ca²⁺ indicator dye fura-PE3 during the last 2 h of statins treatment. Fura-PE3/AM and fetal bovine serum were added to DMEM at the final concentration of 5x10⁻⁵ mol/L and 5 %, respectively. The changes in the fluorescence intensity of Ca²⁺-fura-PE3 complex of the strips were monitored with a front-surface fluorometer as previously reported 10,11. The fluorescence intensities at 340 and 380 nm excitation were monitored and their ratio (F340/F380) were recorded as an indicator of [Ca²⁺], at 25 °C. At the end of the measurement, the response to 1x10⁻⁴ mol/L ionomycin was recorded as a reference response. The changes in the fluorescence ratio were expressed as a percentage, assigning the value obtained in the normal PSS and that obtained with ionomycin to be 0 % and 100 %, respectively.

Immunoblot detection of intracellular protein transduction in the intact strips of mesenteric artery
The strips were exposed to 1x10^{-6} mol/L recombinant proteins for 15 min and 24 h as described above. After thoroughly washing in PSS, the strips were snap frozen in liquid N\textsubscript{2}, and kept at -80 °C. Some strips were exposed to recombinant proteins for 24 h, washed in PSS, and equilibrated in PSS for 1 h as those used for force measurement, before freezing them in liquid N\textsubscript{2}. The strips were thawed on ice and homogenized in RIPA buffer (50 mmol/L Tris-HCl, pH 7.2, 1 % Triton X-100, 0.5 % sodium deoxycholate, 0.1 % SDS, 500 mmol/L NaCl, 10 mmol/L MgCl\textsubscript{2}, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µmol/L 4-aminidophenylmethane sulfonyl fluoride) by repeated freezing and thawing. The homogenates were clarified by centrifugation at 12,000 rpm, 15 min, 4 °C. Twenty µg of total protein was separated with SDS-PAGE on 12.5 % polyacrylamide gel, and transferred to polyvinylidene difluoride membrane. The recombinant proteins were detected by anti-(His)\textsubscript{6} antibody, followed by horseradish peroxidase-conjugated secondary antibody and an enhanced chemiluminescence technique (Amersham Pharmacia Biotech, Buckinghamshire, UK). The endogenous RhoA, Rac1 and Cdc42 in the homogenates were also detected by using the specific antibodies in a separate set of electrophoresis. Tubulin and actin were detected with immunoblot and naphthol blue black staining, respectively, to validate the equal loading of the protein.

*Immunoblot detection of endogenous eNOS in the intact the strips of mesenteric artery*

The strips were treated with 3x10^{-5} mol/L simvastatin or 1x10^{-6} mol/L TATHA-RB for 24 h. Some strips were then washed in PSS, and equilibrated in PSS for 3 h as those used for force measurement. The homogenates of the strips were prepared as described above. The equal amount of protein (125 µg) was subjected to an immunoblot analysis. The primary and secondary antibodies were diluted in Immunoreaction Enhancer Solution Can Get Signal\textsuperscript{TM} (Toyobo, Osaka, Japan). The chemiluminescence signal was detected using an image analysis system ChemiDoc XRS-J (BioRad, Tokyo, Japan). Tubulin was detected by immunoblotting,
to confirm the equal loading of the protein

Pull-down assay

The extract (1.5 mg total proteins obtained from 60 strips) of the arterial strips prepared in RIPA buffer was incubated with 85 µg TATHA-RB or TATHA-PBD for 30 min at 4 °C, as previously described 12,13. The mixture was then incubated with Ni²⁺-nitrilo acetate resin (Qiagen) that had been equilibrated in RIPA buffer for 1 h at 4 °C with agitation. The resin was thoroughly washed in the buffer (50 mmol/L Tris-HCl, 1% Triton X-100, 150 mmol/L NaCl, 10 mmol/L MgCl₂, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µmol/L 4-aminidophenylmethane sulfonyl fluoride, pH 7.2), and the bound protein was extracted in SDS-sample buffer (50 mmol/L Tri-HCl, pH 6.8, 2 % SDS, 5 % glycerol, 5% 2-mercaptoethanol, 0.01 % NaN₃, 0.01 % bromophenol blue) by heating at 100 °C for 5 min. The protein samples were subjected to immunoblot detection of RhoA and Rac1 as described above.

In vivo treatment with simvastatin and TATHA-RB

The rabbits were treated with intravenous injection of 0.33 mg/kg/day of simvastatin or 0.7 mg/kg/day of TATHA-RB for 3 days. The dose of simvastatin and TATHA-RB was consistent with that used described in previous reports 14-19. Next, the rabbits were sacrificed as described above. The mesenteric arteries were isolated, and the contractile response to phenylephrine and high K⁺ depolarization was examined in the presence and absence of 1x10⁻⁴ mol/L L-NAME.

Statistical analysis

The data are the mean±S.E.M. The unpaired Student’s t-test and an analysis of variance (ANOVA) evaluated statistical significance. P values of less than 0.05 were considered to be significant.
References for the Online Data Supplement


8. Vecchione C, Brandes RP. Withdrawal of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors elicits oxidative stress and induces endothelial dysfunction in mice.


17. Schwarze SR, Ho A, Vocero-Akbani A, Dowdy SF. In vivo protein transduction:


**Figure legends for the Online Data Supplement**

**Figure S1.** Effects of fluvastatin on the contractile responses to phenylephrine and 118 mmol/L K+ in the isolated rabbit mesenteric artery with an intact endothelium.

The concentration-response curves for the contractile response to phenylephrine in the absence and presence of 1x10^{-4} mol/L Nω-nitro-L-arginine methyl ester (L-NAME) in the strips treated for 24–30 h at 37 °C in DMEM without (control) and with fluvastatin. L-NAME was added 15 min before and during the evaluation of the contractile response.

The data are the mean±S.E.M. (n=4). *, P<0.05 vs. control; n.s., not significantly different from the control.

**Figure S2.** Immunoblot detection of transduction of TATHA-PBD and TATHA-proteinA. The strips were exposed to 3x10^{-6} mol/L TATHA-PBD, TATHA-proteinA for 15 min and 24 h, and then were thoroughly washed in PBS before extraction (15 min and 24 h). The purified proteins (50 ng) were loaded as a positive control. The arrows indicate the position of TATHA-PBD (19.6 kDa) and TATHA-proteinA (39.8 kDa).
Online Data supplement

Long-term inhibition of RhoA attenuates vascular contractility by enhancing endothelial NO production in an intact rabbit mesenteric artery

Noriko Shiga, Katsuya Hirano, Mayumi Hirano, Junji Nishimura, Hajime Nawata

and Hideo Kanaide

Expanded Materials and Methods

Materials

Simvastatin, fluvastatin and pravastatin were supplied by Merck & Co. (Rahway, N.J., U.S.A.), Novartis Pharma Inc. (Tokyo, Japan) and Sankyo Co. (Tokyo, Japan), respectively. The plasmids pTATHA and pMALU5-ProteinA were kindly donated by Dr. Steven F. Dowdy (University of California, San Diego, CA, U.S.A.) and Dr. Masuo Aizawa (Tokyo Institute of Technology, Yokohama, Japan), respectively. Fura-PE3/AM, an acetoxymethyl ester from of fura-PE-3 was purchased from Texas Fluorescence Laboratory (Austin, TX, U.S.A.). Phenylephrine, ionomycin, N\(^{o}\)-nitro-L-arginine methyl ester (L-NAME), geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) were purchased from Sigma (St. Louis, MO, U.S.A.). The antibodies and their source were as follows: Anti-(His)_6 antibody (Qiagen, Hilden, Germany), anti-RhoA antibody (sc-418; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), anti-Rac1 antibody (No.610650; BD Bioscience, San Jose, CA, U.S.A.), anti-Cdc42 antibody (sc-87; Santa Cruz Biotechnology), anti-eNOS antibody (No.610296; BD Bioscience), anti-tubulin antibody (LY1/2; Serotec, Kidlington, England, U.K.), horse radish peroxidase-conjugated anti-mouse IgG (Sigma), horse radish peroxidase-conjugated anti-rabbit IgG (Vector Laboratories, Burlingame, CA, U.S.A.) and horse radish peroxidase-conjugated anti-rat IgG (Serotec). Geranylgeranyl transferase I inhibitor (GGTI-298), farnesyl transferase inhibitor (FTI-276) and Y27632 were purchased from
Calbiochem (San Diego, CA, U.S.A.). The composition of the physiological salt solution (PSS) was (in mmol/L): NaCl 123, KCl 4.7, NaHCO3 15.5, KH2PO4 1.2, MgCl2 1.2, CaCl2 1.25 and D-glucose 11.5. PSS containing 118 mmol/L K+ was prepared by replacing NaCl with equimolar KCl. PSS was aerated with 95 % O2 and 5 % CO2, with the resulting pH considered to be 7.4.

**Tissue preparation**

These protocols were approved by the Animal Care and the Committee of the Research Institute of Angiocardiology, Graduate School of Medical Sciences, Kyushu University. Japanese white male rabbits weighing 2.5 kg were used. The rabbits were sacrificed by intravenous injection of a lethal dose of sodium pentobarbital (83 mg/kg body weight) and following exsangination from carotid arteries. The mesenteric artery was immediately isolated, and the surrounding connective tissues were mechanically removed under a binocular microscope. The vessels were opened longitudinally, and cut into strips (4 mm long, 1 mm wide, 0.1 mm thick) in a circular direction. In some strips, the luminal surface was rubbed off with a cotton swab to remove endothelium. The aortic valves were excised, and three leaflets of the valve were used to monitor the changes in cytosolic Ca2+ concentration ([Ca2+]i) in *in situ* endothelial cells, as previously described 3. The experimental number indicates the number of rabbits.

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A cDNA fragment containing a IgG binding region of amino acid residues 23-270 of Staphylococcal protein A 4 was amplified by PCR using pMALU5-Protein A as template, and subcloned into pTATHA to express recombinant protein A tagged with a (His)6 tag and a protein transduction domain (PTD) of the human immunodeficiency viral transactivation of transcription (Tat) protein and a hemagglutinin (HA) tag at its N-terminus. The cDNA
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The strips of mesenteric artery with and without endothelium, and aortic valves were treated for 24~30 h at 37 °C without (control) and with various reagents and recombinant proteins in the serum-free Dulbecco’s modified Eagle medium (DMEM) containing streptomycin and penicillin under a CO\(_2\) incubator, unless otherwise specified. The strips were then washed and equilibrated in PSS at least 1 h before evaluating the contractile responses to phenylephrine and high K\(^+\)-depolarization. To avoid the withdrawal effect of statins\(^8\), the evaluation of contractile response was completed within 2 h after terminating the treatment with statins.

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Strips were mounted vertically in a quartz organ bath, which was warmed by circulating water
at 37 °C. One end of the strips was connected to a fixed hook, while the other end was connected to a strain gauge (TB-612-T, Nihon Koden, Japan). During the period of 1 h equilibration, the strips were stimulated with 118 mmol/L K⁺ depolarization every 15 min, and the resting force was elevated in a stepwise manner and finally adjusted to 250-300 mg, before starting to evaluate the contractile responses.

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Bovine aortic endothelial cells (BAECs) were cultured in Dulbecco’s modified Eagle medium containing 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 50 µg/ml gentamycin as previously described ⁹. The cells were used for experiments at passages 11-18.

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The aortic valves were loaded with the Ca²⁺ indicator dye fura-PE3 during the last 2 h of statins treatment. Fura-PE3/AM and fetal bovine serum were added to DMEM at the final concentration of 5x10⁻⁵ mol/L and 5 %, respectively. The changes in the fluorescence intensity of Ca²⁺-fura-PE3 complex of the strips were monitored with a front-surface fluorometer as previously reported ¹⁰,¹¹. The fluorescence intensities at 340 and 380 nm excitation were monitored and their ratio (F340/F380) were recorded as an indicator of [Ca²⁺], at 25 °C. At the end of the measurement, the response to 1x10⁻⁴ mol/L ionomycin was recorded as a reference response. The changes in the fluorescence ratio were expressed as a percentage, assigning the value obtained in the normal PSS and that obtained with ionomycin to be 0 % and 100 %, respectively.

Immunoblot detection of intracellular protein transduction in the intact strips of mesenteric artery

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The strips were exposed to $1 \times 10^{-6}$ mol/L recombinant proteins for 15 min and 24 h as described above. After thoroughly washing in PSS, the strips were snap frozen in liquid N$_2$, and kept at -80°C. Some strips were exposed to recombinant proteins for 24 h, washed in PSS, and equilibrated in PSS for 1 h as those used for force measurement, before freezing them in liquid N$_2$. The strips were thawed on ice and homogenized in RIPA buffer (50 mmol/L Tris-HCl, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mmol/L NaCl, 10 mmol/L MgCl$_2$, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µmol/L 4-aminidophenylmethane sulfonyle fluoride) by repeated freezing and thawing. The homogenates were clarified by centrifugation at 12,000 rpm, 15 min, 4°C. Twenty µg of total protein was separated with SDS-PAGE on 12.5% polyacrylamide gel, and transferred to polyvinylidene difluoride membrane. The recombinant proteins were detected by anti-(His)$_6$ antibody, followed by horseradish peroxidase-conjugated secondary antibody and an enhanced chemiluminescence technique (Amersham Pharmacia Biotech, Buckinghamshire, UK). The endogenous RhoA, Rac1 and Cdc42 in the homogenates were also detected by using the specific antibodies in a separate set of electrophoresis. Tubulin and actin were detected with immunoblot and naphthol blue black staining, respectively, to validate the equal loading of the protein.

*Immunoblot detection of endogenous eNOS in the intact the strips of mesenteric artery*

The strips were treated with $3 \times 10^{-5}$ mol/L simvastatin or $1 \times 10^{-6}$ mol/L TATHA-RB for 24 h. Some strips were then washed in PSS, and equilibrated in PSS for 3 h as those used for force measurement. The homogenates of the strips were prepared as described above. The equal amount of protein (125 µg) was subjected to an immunoblot analysis. The primary and secondary antibodies were diluted in Immunoreaction Enhancer Solution Can Get Signal™ (Toyobo, Osaka, Japan). The chemiluminescence signal was detected using an image analysis system ChemiDoc XRS-J (BioRad, Tokyo, Japan). Tubulin was detected by immunoblotting,
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The extract (1.5 mg total proteins obtained from 60 strips) of the arterial strips prepared in RIPA buffer was incubated with 85 µg TATHA-RB or TATHA-PBD for 30 min at 4 °C, as previously described \(^{12,13}\). The mixture was then incubated with Ni\(^{2+}\)-nitrilo acetate resin (Qiagen) that had been equilibrated in RIPA buffer for 1 h at 4 °C with agitation. The resin was thoroughly washed in the buffer (50 mmol/L Tris-HCl, 1% Triton X-100, 150 mmol/L NaCl, 10 mmol/L MgCl\(_2\), 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µmol/L 4-aminidophenylmethane sulfonyl fluoride, pH 7.2), and the bound protein was extracted in SDS-sample buffer (50 mmol/L Tri-HCl, pH 6.8, 2 % SDS, 5 % glycerol, 5% 2-mercaptoethanol, 0.01 % NaN\(_3\), 0.01 % bromophenol blue) by heating at 100 °C for 5 min. The protein samples were subjected to immunoblot detection of RhoA and Rac1 as described above.

**In vivo treatment with simvastatin and TATHA-RB**

The rabbits were treated with intravenous injection of 0.33 mg/kg/day of simvastatin or 0.7 mg/kg/day of TATHA-RB for 3 days. The dose of simvastatin and TATHA-RB was consistent with that used described in previous reports \(^{14-19}\). Next, the rabbits were sacrificed as described above. The mesenteric arteries were isolated, and the contractile response to phenylephrine and high K\(^+\) depolarization was examined in the presence and absence of 1x10\(^{-4}\) mol/L L-NAME.

**Statistical analysis**

The data are the mean±S.E.M. The unpaired Student’s \(t\)-test and an analysis of variance (ANOVA) evaluated statistical significance. \(P\) values of less than 0.05 were considered to be significant.
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**Figure S1.** Effects of fluvastatin on the contractile responses to phenylephrine and 118 mmol/L K⁺ in the isolated rabbit mesenteric artery with an intact endothelium.

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