Circ J 2009; **73:** 361 – 370

Importance of Rac1 Signaling Pathway Inhibition in the Pleiotropic Effects of HMG-CoA Reductase Inhibitors

Mamunur Rashid, PhD; Shunsuke Tawara, PhD; Yoshihiro Fukumoto, MD; Minoru Seto, PhD*; Kazuo Yano, MS*; Hiroaki Shimokawa, MD

Background The pleiotropic effects of HMG-CoA reductase inhibitors (statins) are thought to be mediated through inhibition of small GTP-binding proteins; however, it remains to be examined whether clinical concentrations/doses of statins actually exert them.

Methods and Results In vitro studies with cultured human umbilical venous endothelial cells found that statins (atorvastatin, pitavastatin and pravastatin at 10 μmol/L) had no inhibitory effects on RhoA/Rho-kinase or Ras, but atorvastatin and pitavastatin inhibited membrane Rac1 expression. In animal studies of angiotensin II (AngII)-infused rats, atorvastatin showed only mild inhibitory effects on AngII-induced cardiovascular hypertrophy, whereas fasudil, a selective Rho-kinase inhibitor, significantly suppressed it. Statins had no inhibitory effects on RhoA/Rho-kinase, but inhibited both membrane and GTP-bound Rac1 in the heart, whereas fasudil only inhibited Rho-kinase activity. Furthermore, the combination of atorvastatin and fasudil showed more effective inhibitory effects than fasudil alone. Finally, in studies of normal healthy volunteers, clinical doses of pravastatin or atorvastatin (20 mg/day for 1 week) significantly inhibited Rac1, but not RhoA/Rho-kinase activity, in circulating leukocytes.

Conclusions The pleiotropic effects of statins, if any, at their clinical doses are mediated predominantly through inhibition of the Rac1 signaling pathway. (*Circ J* 2009; **73:** 361 – 370)

Key Words: Arteriosclerosis; Pleiotropic effects; Rho-kinase; Small GTPases; Statins

HMG-CoA reductase inhibitors (statins) are potent cholesterol-lowering drugs widely used in clinical practice for primary and secondary prevention of coronary artery disease[1]. Furthermore, their beneficial cardiovascular effects, beyond the lipid-lowering action, (the so-called pleiotropic effects) have attracted much attention[2–4]. The lipid-independent effects of statins could be mediated by reduced synthesis of the isoprenoids that are responsible for the post-translational modulation of intracellular proteins[1]. The pleiotropic effects of statins are thought to be mediated through inhibition of small GTP-binding proteins, such as Rho, Rac and Ras, whose correct membrane localization and GTPase activity are dependent on isoprenylation[5,6]. However, the specific cellular GTPase target protein(s) of statins still remains to be elucidated, especially at clinical doses.

Editorial p 231

Rho-kinase is one of the downstream effectors of the small G-protein, Rho. The Rho/Rho-kinase pathway has an important role in mediating various cellular functions, including contraction, actin cytoskeleton organization, cell adhesion and motility, proliferation, cytokinesis and gene expression, all of which are involved in the pathogenesis of cardiovascular diseases[7–9]. Previous studies suggest that the pleiotropic effects of statins are mediated through inhibition of the RhoA/Rho-kinase pathway; however, most of those studies were performed in vitro[3,5,10] and only a few were in vivo animal studies[1,11,12] and no human study has been performed. Moreover, supra-clinical concentrations/doses of statins cannot be tolerated by humans. Thus, in the present study, we examined whether clinical concentrations/doses of statins actually exert lipid-independent pleiotropic effects, if any, at all research levels, ranging from the enzymatic, cellular, animal, and human, and if so, what molecular mechanism(s) is involved.

(Received August 22, 2008; revised manuscript received September 13, 2008; accepted September 25, 2008; released online December 8, 2008)

*Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, Sendai, *Laboratory of Pharmacology, Asahi Kasei Pharma Co, Tokyo, Japan*

The Guest Editor for this article was Masafumi Kitakaze, MD.

Mailing address: Hiroaki Shimokawa, MD, Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan. E-mail: shimo@cardio.med.tohoku.ac.jp

Methods

ELISA Study

To examine the direct inhibitory effect of statins on Rho-kinase activity, we performed an ELISA assay on Rho-kinase activity using 10 μmol/L of lipophilic statins (atorvastatin and pitavastatin) and a hydrophilic statin (pravastatin), and as a positive control, the Rho-kinase inhibitors (fasudil, hydroxyfasudil and Y-27632)[13,14]. We evaluated Rho-kinase activity with an antibody to phosphorylated myosin binding subunit (p-MBS) of myosin phosphatase, a substrate of Rho-kinase[15] by an ELISA in vitro.

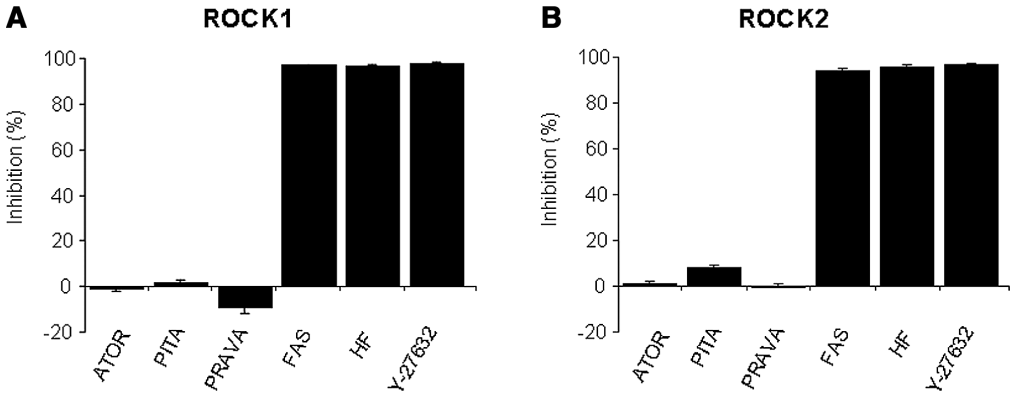

Fig 1. Statins have no direct inhibitory effects on Rho-kinase activity in vitro. None of the 3 statins (atorvastatin, ATOR; pitavastatin, PITA; and pravastatin, PRAVA, 10μmol/L each) directly inhibited the activity of the recombinant Rho-kinase isoforms, ROCK1 (**A**) or ROCK2 (**B**), whereas all Rho-kinase inhibitors (fasudil, FAS; hydroxyfasudil, HF; and Y-27632, 10μmol/L each) completely inhibited Rho-kinase activity. Results are expressed as mean±SEM (n=4 each).

Study With Cultured Cells and Drug Treatment

Human umbilical venous endothelial cells (HUVEC) (Takara Bio Inc, Otsu, Japan) were incubated at 37°C in 5% CO_2 in endothelial growth medium (EGM-2) for 1 day, and then the cells were treated with different concentrations (1–30μmol/L) of each statin (atorvastatin, pitavastatin and pravastatin) or Rho-kinase inhibitor (hydroxyfasudil) for 24 h. After treatment with each drug, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and sonicated after adding lysis buffer. For the subcellular fractionations, the cell samples were then ultra-centrifuged at 100,000 g at 4°C for 30 min.

Animal Studies

All protocols for the animal experiments were reviewed and approved by the Animal Care and Use Committee of the Tohoku University Graduate School of Medicine. We anesthetized male Wistar-Kyoto rats (14–17 weeks old, 300–350 g body weight) with intraperitoneal pentobarbital (50 mg/kg), and implanted an osmotic mini-pump (Alzet model 2ML4 or 2ML1, Durect, Cupertino, CA, USA) subcutaneously. The pumps contained angiotensin II (AngII, 0.75 $mg \cdot kg^{-1} \cdot day^{-1}$) dissolved in 0.15 mol/L NaCl containing 10 mmol/L acetic acid (AngII group) or vehicle alone (control) for 4 weeks (for the protocol of cardiovascular hypertrophy) or 1 week (for the protocol of RhoA, Rho-kinase and Rac1 activity in the heart). Both the AngII-infused and vehicle-treated rats were maintained with or without statin treatment (pitavastatin, atorvastatin and pravastatin at 1, 10 and 50 $mg \cdot kg^{-1} \cdot day^{-1}$, respectively)[16–18] or fasudil (100 $mg \cdot kg^{-1} \cdot day^{-1}$);[19] in their drinking water. In the combination treatment protocol, atorvastatin (10 $mg \cdot kg^{-1} \cdot day^{-1}$) and a low-dose of fasudil (10 $mg \cdot kg^{-1} \cdot day^{-1}$) were given to AngII-infused rats in their drinking water for 4 weeks.

Histological Analysis

Histological studies were performed for the determination of left ventricular (LV) weight, cardiomyocyte cross-sectional area, medial thickness (wall to lumen ratio) and perivascular fibrosis, as previously reported.[19]

Subcellular Fractionation

Preparation of whole cell lysates, membrane and cytosolic fraction were performed as previously described.[20]

Plasma Concentration of Drugs

We measured the plasma concentration of drugs after the 4-week treatment in rats that received a statin or fasudil in their drinking water, according to our previous report.[19] Human data were obtained from the literature provided by the manufacturer of each drug.

Western Blot Analysis

To quantify the activity of RhoA, Rho-kinase, Rac1 and Ras in the HUVEC and in the LV of the rat heart, an equal number of protein samples were loaded on SDS-PAGE gel and transferred into PVDF membranes (GE Healthcare, Buckinghamshire, UK). The membranes were immunoblotted with anti-phosphorylated ERM (ezrin, radixin, and moesin),[21] anti-MBS (Covance), and anti-phospho MBS (Thr696) (Upstate), anti-RhoA (Santa Cruz), anti-Rac1 (Upstate), anti-Ras (Upstate) and anti-β-actin (Sigma) and anti-α-tubulin (Molecular Probe). After incubating with horseradish-peroxidase-conjugated rabbit anti-mouse or goat anti-rabbit IgG antibody, blots were visualized by the enhanced chemiluminescence system (ECL Western Blotting Detection Kit, GE Healthcare). Densitometric analysis was performed by Scion Image Software.

Pull-Down Assay

Activities of RhoA and Rac1 were determined by the Rho binding domain of Rhotekin (Rhotekin RBD) and the p21-binding domain of the PAK1 (PAK-1 PBD) pull-down assay (Rho and Rac1 Activation Assay Kit, Upstate), respectively, according to the manufacturer's protocol.

Human Study

This protocol was approved by the Human Research Committee of the Tohoku University Graduate School of Medicine, and 10 normal healthy human volunteers (6 males, 4 females; mean age, 32.3±1.2 years; body weight, 64.9±5.2 kg; systolic blood pressure, 120±3 mmHg and diastolic blood pressure, 75±3 mmHg) participated after giving written consent. The exclusion criteria included abnormal serum lipid levels (total cholesterol level <180 mg/dl, HDL-cholesterol, LDL-cholesterol and triglycerides in the abnormal range), and liver or kidney dysfunction before and during the statin treatments. We examined the effects of both hydrophilic (pravastatin) and lipophilic (atorvastatin) statins that are clinically used in Japan. The volunteers received

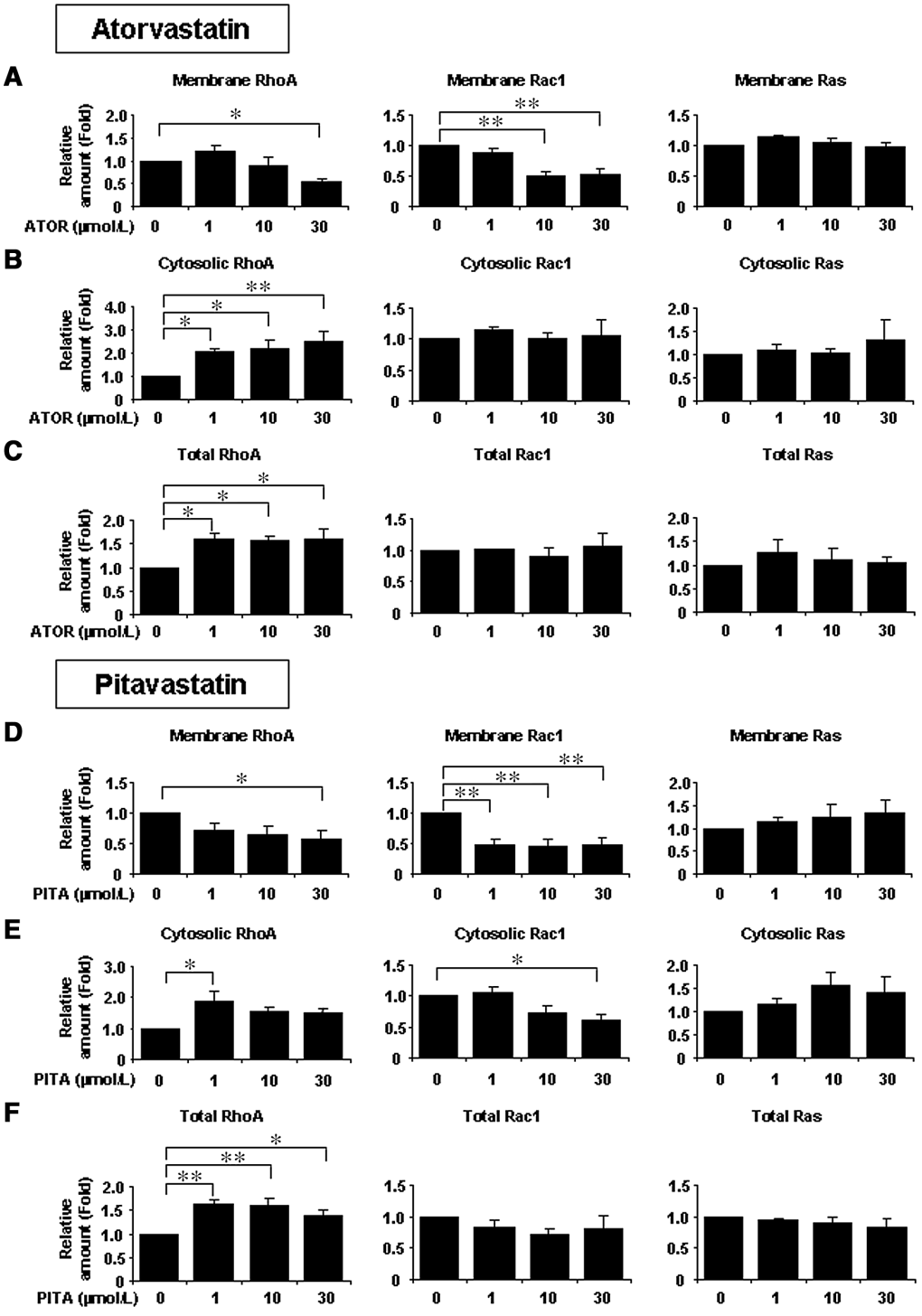

Fig 2. Different inhibitory effects of statins on small GTPase activity in human umbilical venous endothelial cells (HUVEC) in vitro. After incubation with HUVEC for 24 h, atorvastatin (ATOR) and pitavastatin (PITA) inhibited membrane Rac1 expression at both 10 and 30 μmol/L, and inhibited RhoA expression only at 30 μmol/L (**A**,**D**). Atorvastatin and pitavastatin increased both the cytosolic and total RhoA expression (**B**,**C**,**E**,**F**), whereas pitavastatin decreased cytosolic Rac1 (**E**). On the other hand, they had no inhibitory effects on Ras expression of the membrane, cytosol or whole cell lysates at 1–30 μmol/L (**A**–**F**). Results are expressed as mean ± SEM (n=3–4 each). *P<0.05, **P<0.01.

pravastatin (20 mg/day) or atorvastatin (20 mg/day) orally for 1 week in a randomized crossover manner with a 2-week washout interval. The dose used in this human study is the maximum dose for each statin approved in Japan. The 2-week interval is enough to restore lipid profiles to pre-statin treatment levels[22] Venous blood was collected under fasting conditions for the measurement of lipid profiles, activity of RhoA or Rho-kinase and Rac1, and drug safety, before the treatment and 2 h after the last intake of a statin.

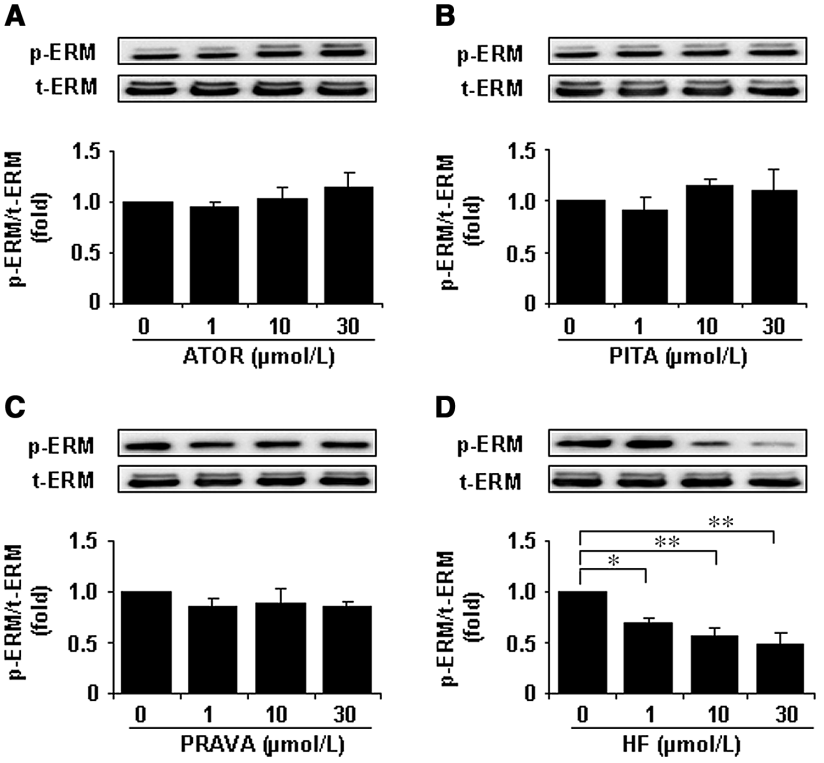

Fig 3. Effects of statins and a Rho-kinase inhibitor on Rho-kinase activity in human umbilical venous endothelial cells (HUVEC) in vitro. When Rho-kinase activity was evaluated by the ratio of phosphorylated ERM (p-ERM) and total ERM (t-ERM), none of the 3 statins (atorvastatin, ATOR, pitavastatin, PITA and pravastatin, PRAVA at 30μmol/L) had an inhibitory effect on Rho-kinase activity (**A**–**C**). By contrast, Rho-kinase activity was concentration-dependently inhibited by hydroxyfasudil (HF) (**D**). Results are expressed as mean±SEM (n=3–4 each). *P<0.05, **P<0.01.

Preparation of Leukocyte Extracts

Leukocytes were isolated according to the method previously described with a slight modification[23] Venous blood (20 ml) was collected in heparinized tubes from each volunteer after overnight fasting and 2 ml was collected without heparin for serum preparation and for baseline characteristics data. Polymorphonuclear leukocytes (PMNL) were isolated using the Monopoly Resolving Medium (DaiNippon Pharmaceutical Co Ltd, Tokyo, Japan) after centrifugation at 400 g for 30 min at room temperature. PMNL were first washed with saline alone and then washed again with saline containing 1 mmol/L EDTA at pH 8. The leukocyte pellets were suspended in 2 ml Hank's balanced salt solution (HBSS) and the cell yield determined using the trypan-blue exclusion test (usually 7–40×10^6 viable cells with a viability >96%) and then diluted with HBSS to obtain 2×10^6 cells/ml for the analysis of Rho-kinase activity. We then added fixative solution (50% trichloroacetic acid [Sigma]) and 0.5 mol/L dithiothreitol (Sigma) to the cell suspension and centrifuged at 20,630 g for 5 min at 4°C. The pellets were dissolved in extraction buffer (8 mol/L urea, 2% sodium dodecyl sulfate, 5% sucrose, and 5% 2-mercaptoethanol, 0.02% BPB dye) and the mixture was slowly rotated at 4°C overnight and was then stored at –80°C until use. Equal amounts of cell extract were loaded on 7.5% SDS-PAGE gel and immunoblotted for the detection of Rho-kinase activity, using rabbit polyclonal anti-phospho-myosin phosphatase (MYPT) 1 (Thr696) (Upstate) for p-MBS and mouse monoclonal anti-MYPT1 (BD Biosciences) for total MBS (t-MBS). Rho-kinase activity was expressed as the ratio of p-MBS and t-MBS in each sample. For the detection of active RhoA and Rac1, PMNL (5–20×10^6 cells) were pelleted rapidly from the cell suspension in HBSS and lysed by adding ice-cold magnesium lysis buffer. The protein concentration of the supernatant was determined by a bi-cinchoninic acid protein assay (Pierce). An equal amount of cell extract was used for the pull-down assay of RhoA and Rac1 activity, according to the manufacturer's protocol (Upstate).

Measurement of Lipid Profile

The effect of statins (pravastatin and atorvastatin) on the serum lipid profile (total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides), before and 1 week after statin treatment in the normal healthy volunteers, was measured by standard enzymatic methods.

Statistical Analysis

The results are expressed as mean±SEM. We performed statistical analysis by 1-way ANOVA followed by Dunnett's test and Tukey's test for multiple comparisons and paired and unpaired Student's t-test; P<0.05 was considered to be statistically significant.

Results

ELISA Assay

To exclude the possibility that statins have a non-specific direct inhibitory effect on enzymatic Rho-kinase activity, we first performed the ELISA assay experiments using recombinant Rho-kinase. None of the 3 statins (atorvastatin, pitavastatin, and pravastatin at 10μmol/L) had a direct inhibitory effect on ROCK1 or ROCK2, whereas the Rho-kinase inhibitors (fasudil, hydroxyfasudil and Y-27632) markedly inhibited Rho-kinase activity (**Figs 1A**,**B**).

Studies With Cultured Cells In Vitro

Statin Effects on RhoA, Rac1 and Ras Expression To clarify which small GTPases are actually inhibited by statins in vitro, we first examined the inhibitory effects of the 3 statins (atorvastatin, pitavastatin, and pravastatin at 1–30μmol/L) on the membrane protein expression of RhoA, Rac1 and Ras (ie, activity of small GTPases) in HUVEC

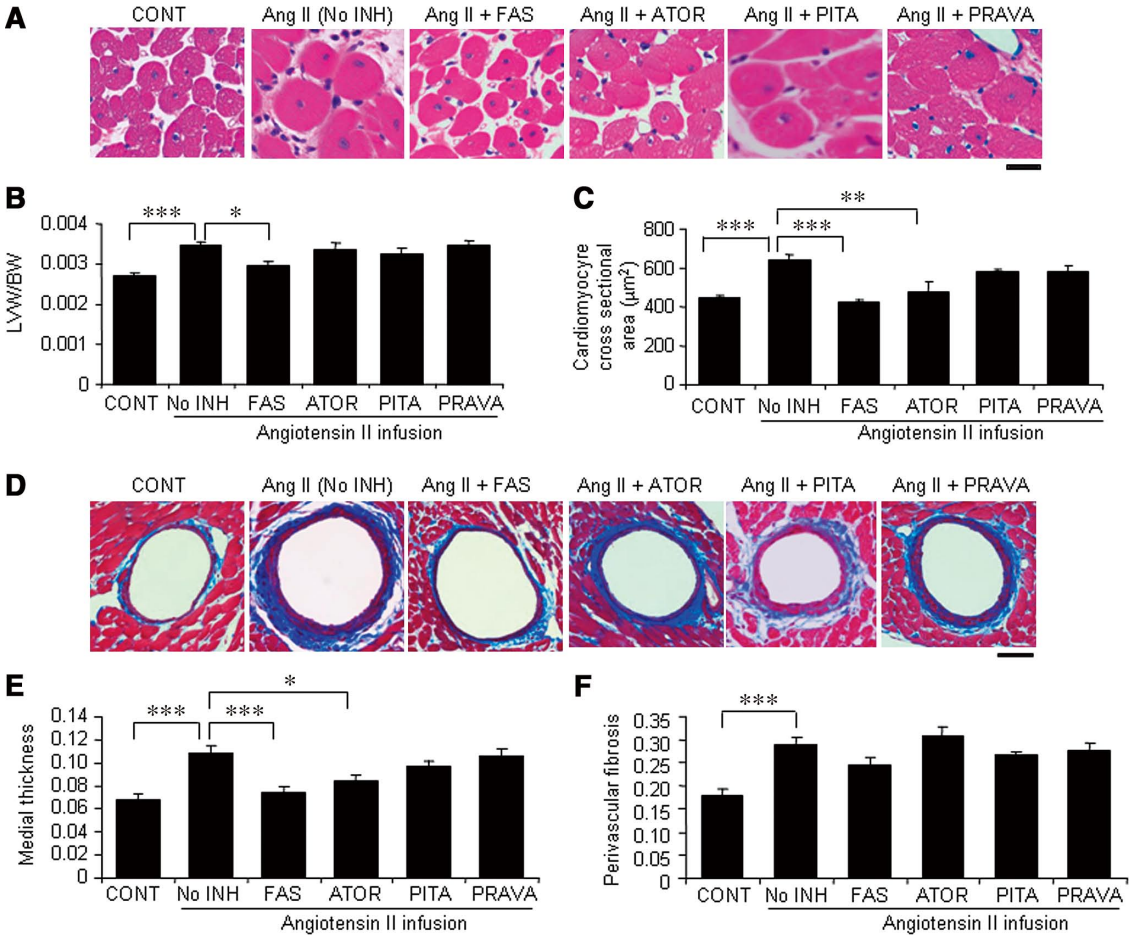

Fig 4. Inhibitory effects of long-term treatment with fasudil or a statin on angiotensin II-induced cardiovascular hypertrophy in rats. (**A**) Representative microphotographs of cardiomyocyte (H&E) in control (CONT) and angiotensin II (AngII)-infused rats (no inhibitor, No INH) and AngII-infused rats treated with either fasudil (FAS, 100 mg·kg^{-1}·day^{-1}), atorvastatin (ATOR, 10 mg·kg^{-1}·day^{-1}), pitavastatin (PITA, 1 mg·kg^{-1}·day^{-1}), or pravastatin (PRAVA, 50 mg·kg^{-1}·day^{-1}). Scale bar=20 μm. (**B**,**C**) AngII-induced increase in left ventricular weight was inhibited by fasudil, whereas AngII-induced cardiomyocyte hypertrophy was inhibited by fasudil and atorvastatin. (**D**) Representative microphotographs of small coronary arteries (Masson's trichrome) in control and AngII-infused rats and AngII-infused rats treated with either a statin or fasudil. Scale bar=50 μm. (**E**,**F**) Fasudil inhibited AngII-induced medial thickening and tended to inhibit perivascular fibrosis. Atorvastatin inhibited AngII-induced medial thickening, but not perivascular fibrosis, whereas the other statins had no inhibitory effects. Results are expressed as mean ± SEM (n=5–7 each). *P<0.05, **P<0.01, ***P<0.001.

after 24-h treatment. In the study using HUVEC, none of the statins inhibited the membrane expression of Ras (**Fig 2**), and pravastatin had no inhibitory effect on RhoA or Rac1 expression in any fractions of the cells (data not shown). Atorvastatin and pitavastatin inhibited membrane RhoA expression, but only at a higher concentration (30 μmol/L) (**Figs 2A**,**D**). By contrast, moderate and higher concentrations (10 and 30 μmol/L) of atorvastatin and pitavastatin inhibited membrane expression of Rac1 in those cells (**Figs 2A**,**D**). Furthermore, atorvastatin and pitavastatin increased both cytosolic and total RhoA expression (**Figs 2B**, **C**,**E**,**F**). By contrast, the 2 statins had no such enhancing effect on Rac1, rather pitavastatin decreased cytosolic Rac1 expression in HUVEC (**Fig 2E**).

Statin Effects on Rho-Kinase Activity We also examined the inhibitory effects of statins on Rho-kinase activity (as evaluated by the extent of phosphorylation of ERM, a target of Rho-kinase) in HUVEC. None of the statins had an inhibitory effect on Rho-kinase activity at 1–30 μmol/L (**Figs 3A**–**C**), but the Rho-kinase inhibitor (hydroxyfasudil) dose-dependently inhibited Rho-kinase activity (**Fig 3D**).

Animal Studies In Vivo

Next, we examined the mechanisms of the inhibitory effects of statins on AngII-induced cardiovascular hypertrophy in rats in vivo. At 4 weeks after treatment with a statin (atorvastatin, 10 mg·kg^{-1}·day^{-1}; pitavastatin, 1.0 mg·kg^{-1}·day^{-1}; pravastatin, 50 mg·kg^{-1}·day^{-1}) or a Rho-kinase inhibitor (fasudil, 100 mg·kg^{-1}·day^{-1}), we confirmed that the plasma concentration of the statins in rats were equivalent to their maximum plasma concentrations (C_{max} value) in humans (atorvastatin, 7.8±3.1 and 3.4±0.6 ng/ml; pitavastatin, 29.8±3.4 and 26.1±2.8 ng/ml; pravastatin, 11.9±4.9 and 16.5±2.1 ng/ml; fasudil, 139.6±49 and 256.7±17.4 ng/ml in rats and humans, respectively).

AngII infusion for 1 and 4 weeks significantly increased systolic blood pressure from 131±2 to 182±3 mmHg (n=6–10, P<0.001) and from 127±5 to 190±12 mmHg (n=6–10, P<0.01), respectively. Statins, fasudil or their combination did not alter AngII-induced hypertension (atorvastatin,

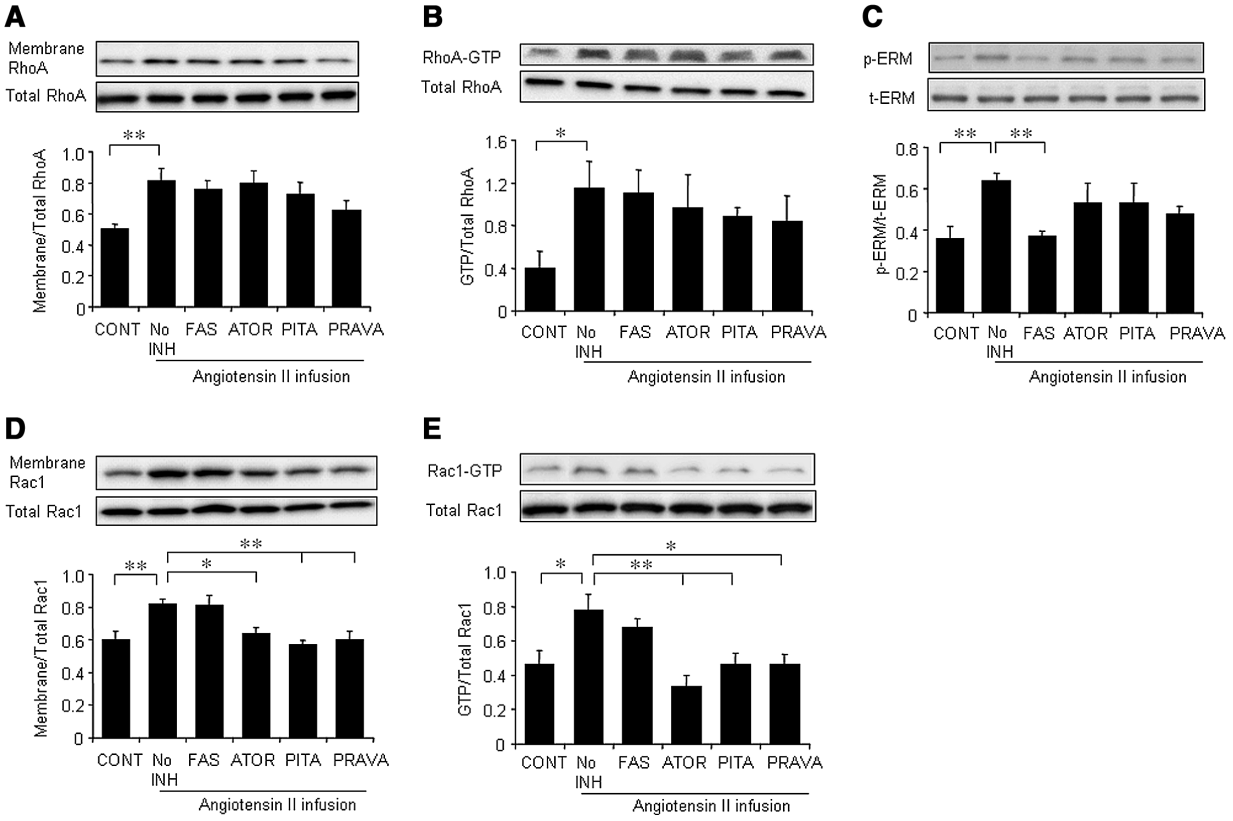

Fig 5. Effects of statins and fasudil on RhoA, Rac1 and Rho-kinase activities in angiotensin II-infused rats. Angiotensin II (AngII) infusion significantly increased both membrane RhoA and RhoA-GTP in the rat heart, for which statins (atorvastatin, ATOR, pitavastatin, PITA and pravastatin, PRAVA) and fasudil (FAS) had no inhibitory effect (**A**,**B**). None of the 3 statins was able to inhibit the AngII-induced increase in Rho-kinase activity (as expressed by the ratio of phosphorylated ERM and total ERM, p-ERM/t-ERM), whereas fasudil completely suppressed Rho-kinase activity (**C**). By contrast, the statins significantly inhibited the AngII-induced increase in membrane Rac1 and Rac1-GTP in the rat heart, whereas fasudil had no inhibitory effect (**D**,**E**). Results are expressed as mean ± SEM (n=6–10 each). *P<0.05, **P<0.01.

169±11 mmHg; pitavastatin, 168±10 mmHg; pravastatin, 191±9 mmHg; fasudil, 207±11 mmHg; atorvastatin + fasudil, 197±17 mmHg, n=6–10) or body weight (data not shown).

As we had previously demonstrated,[19] AngII infusion caused LV and cardiomyocyte hypertrophy in rats, which was significantly inhibited by simultaneous treatment with fasudil (**Figs 4A–C**). By contrast, none of the statins at clinical concentration inhibited LV hypertrophy, and only atorvastatin inhibited cardiomyocyte hypertrophy (**Figs 4A–C**). AngII significantly increased medial thickening and perivascular fibrosis of the coronary artery compared with sham-operated control animals, and simultaneous treatment with fasudil significantly inhibited medial thickening and tended to inhibit perivascular fibrosis (**Figs 4D–F**). Among the statins examined, only atorvastatin significantly inhibited medial thickening, but none of them inhibited perivascular fibrosis (**Figs 4D–F**).

We then examined whether statins actually inhibit RhoA, Rac1 and Rho-kinase activity in the rat heart in the AngII model. AngII infusion for 1 week significantly increased membrane RhoA and RhoA-GTP (active RhoA), as well as Rho-kinase activity, in the LV, for which statins had no inhibitory effects (**Figs 5A**,**B**), whereas fasudil completely inhibited Rho-kinase activity to the basal level (**Fig 5C**). By contrast, all the statins completely inhibited both membrane Rac1 and Rac1-GTP (active Rac1) in the LV, whereas fasudil had no effect (**Figs 5D**,**E**).

Because AngII activates both the Rac1 and RhoA/Rho-kinase pathways, we then examined whether combined therapy with atorvastatin and fasudil is more effective than monotherapy with fasudil. To test our hypothesis, we examined the inhibitory effect of the combination therapy (atorvastatin and a low-dose of fasudil) in the present AngII model. The combination therapy more effectively inhibited the AngII-induced cardiovascular hypertrophy than fasudil alone, including LV and cardiomyocyte hypertrophy (**Figs 6A–C**), medial thickness and perivascular fibrosis of the coronary artery (**Figs 6D–F**). However, the combination therapy had no inhibitory effect on RhoA activity, but significantly inhibited Rac1 activity in the rat heart (**Figs 6G**,**H**).

Human Studies

Finally, we examined the inhibitory effects of statins on RhoA and Rac1 signaling pathway in normal healthy volunteers, who received pravastatin and atorvastatin (20 mg/day each) orally for 1 week in a crossover manner with a 2-week washout. Both statins mildly but significantly reduced the serum levels of total cholesterol (199±5 and 202±6 mg/dl before and 168±4 and 148±5 mg/dl after the treatment with pravastatin and atorvastatin, respectively) and LDL-cholesterol (108±9 and 111±7 mg/dl before and 82±6 and 61±4 mg/dl after the treatment with pravastatin and atorvastatin, respectively) after 1-week treatment, even in normolipidemic volunteers, indicating the efficacy of the statin therapy.

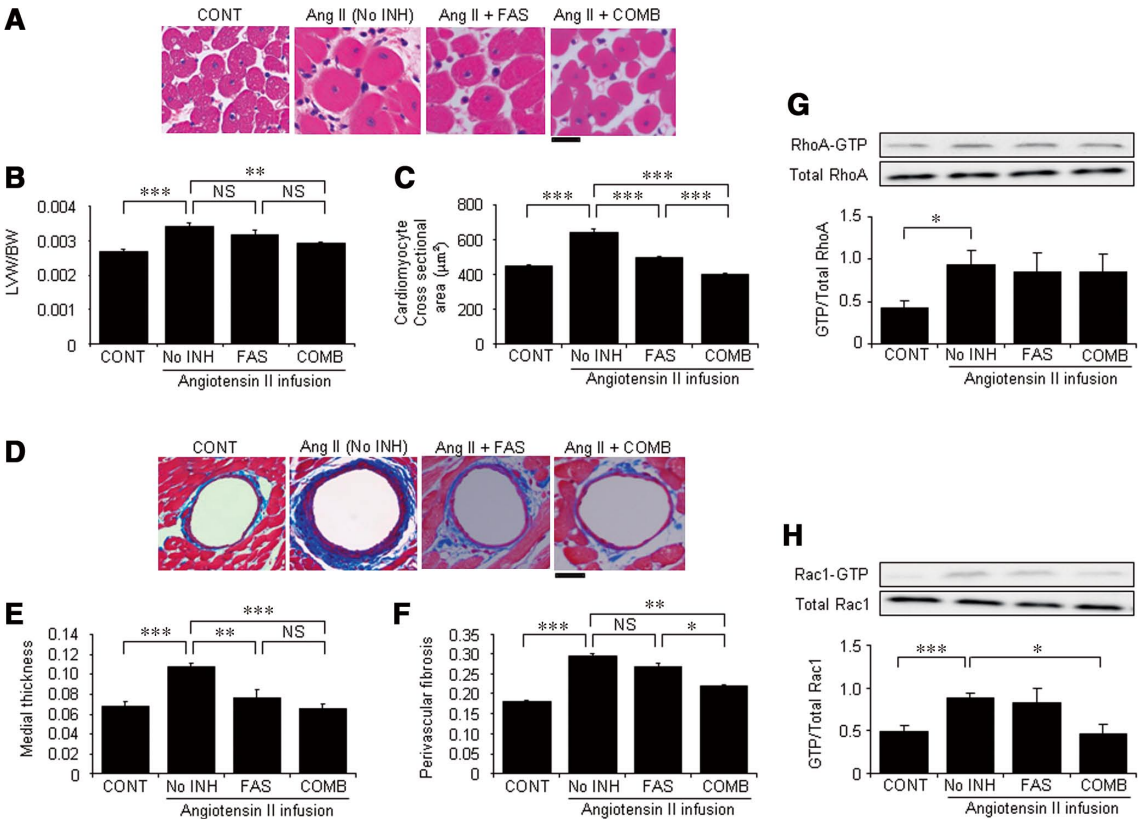

Fig 6. Inhibitory effects of the combination of fasudil and atorvastatin on angiotensin II-induced cardiovascular hypertrophy in rats. (**A**) Representative microphotographs of cardiomyocyte (H&E) in control (CONT) and angiotensin II (AngII)-infused rats (no inhibitor, No INH) and AngII-infused rats treated with either fasudil (FAS, 10 mg·kg^{-1}·day^{-1}) or the combination of fasudil and atorvastatin (COMB, 10 mg·kg^{-1}·day^{-1} each). Scale bar=20μm. (**B**,**C**) AngII-induced left ventricular hypertrophy and cardiomyocyte hypertrophy were more effectively inhibited by the combination therapy than by fasudil alone. (**D**) Representative microphotographs of small coronary arteries (Masson's trichrome). Scale bar=50μm. (**E**,**F**) The combination therapy more effectively inhibited AngII-induced medial thickening and perivascular fibrosis than fasudil alone. Results are expressed as mean±SEM (n=6–10 each). *P<0.05, **P<0.01, ***P<0.001. NS, not significant. (**G**,**H**) The combination therapy had no inhibitory effects on AngII-induced activation of RhoA in the rat heart (**G**), but significantly inhibited Rac1 activity (**H**). Results are expressed as mean±SEM (n=6–10 each). *P<0.05, **P<0.01.

RhoA-GTP and Rho-kinase activities in PMNL were unchanged after treatment with pravastatin or atorvastatin (**Figs 7A**,**B**), whereas both statins significantly inhibited Rac1-GTP (**Fig 7C**).

Discussion

In the present study, we examined the molecular mechanism for the pleiotropic effects of statins on small GTPases at their clinical concentrations/doses. We performed several lines of experiments at different levels, ranging from enzymatic, cellular, animal and human studies, in order to elucidate the specific molecular target(s) of statins. The present results indicate that the pleiotropic effects of statins are mediated predominantly though inhibition of the Rac1 signaling pathway, but not the RhoA/Rho-kinase or Ras signaling pathway, at the clinical and maximum doses used in Asian countries. To the best of our knowledge, this is the first study to demonstrate different inhibitory effects of clinical concentrations/doses of statins on GTPases at all research levels, including humans (**Fig 8**).

Mechanisms of the Effects of Statins In Vitro

There is growing interests in the pleiotropic effects of statins through inhibition of small GTP-binding proteins. To clarify exactly which small G-proteins are the targets of statins, we first examined their inhibitory effects on the membrane, cytosolic, and total expression of those proteins in HUVEC and our results demonstrate that statins more effectively inhibit Rac1 signaling than RhoA or Ras signaling in vitro. It has been recently reported that pitavastatin inhibits the proliferation and monocyte chemoattractant protein-1 gene expression in smooth muscle cells in response to lysophosphatidic acid through suppression of Rac1-mediated and NADPH oxidase-dependent generation of reactive oxygen species (ROS).[24] Although the detailed mechanisms of the different inhibitory effects of statins on RhoA and Rac1 remain to be fully elucidated in future studies, we were able to demonstrate in the present study that statins upregulate RhoA, but not Rac1, expression in HUVEC. Because RhoA is more abundant than Rac1 as a substrate of geranylgeranylation in the cytosol of the cell, these results suggest that RhoA is more susceptible to geranylgeranylation than Rac1. Therefore, this may be a possible reason why statins inhibit membrane Rac1, but not RhoA.

In the present study, none of the statins inhibited Rho-kinase activity in HUVEC, although atorvastatin and pitavastatin inhibited membrane RhoA expression at their higher

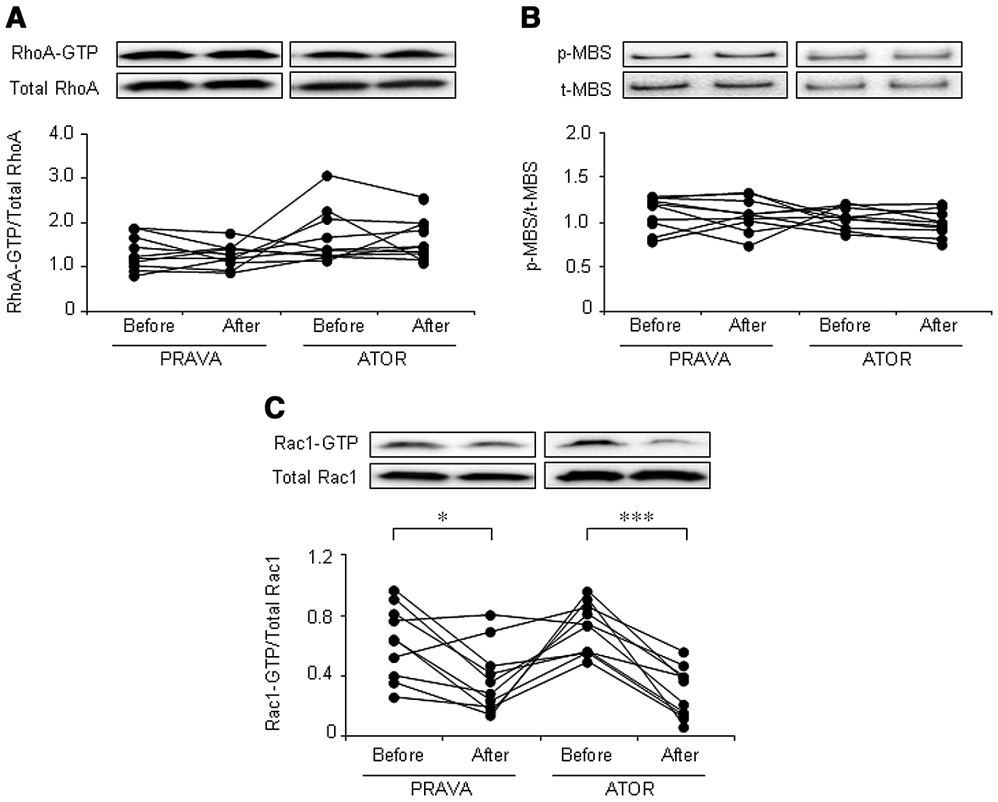

Fig 7. Effects of statins on the activity of RhoA, Rac1 and Rho-kinase in humans. (**A**,**B**) Cross-over oral treatment with pravastatin (PRAVA, 20 mg/day for 1 week) or atorvastatin (ATOR, 20 mg/day for 1 week) had no inhibitory effects on RhoA activity (ratio of RhoA-GTP/total RhoA) or Rho-kinase activity (as evaluated by the ratio of phosphorylated MBS, p-MBS and total MBS, t-MBS) in circulating human polymorphonuclear leukocytes (PMNL) (**A**,**B**). (**C**) By contrast, both statins significantly inhibited Rac1 activity (ratio of Rac1-GTP/total Rac1) in human PMNL. Results are plotted for each volunteer at 4 data points (before and after pravastatin; and before and after atorvastatin) (n=10). *P<0.05, ***P<0.0001.

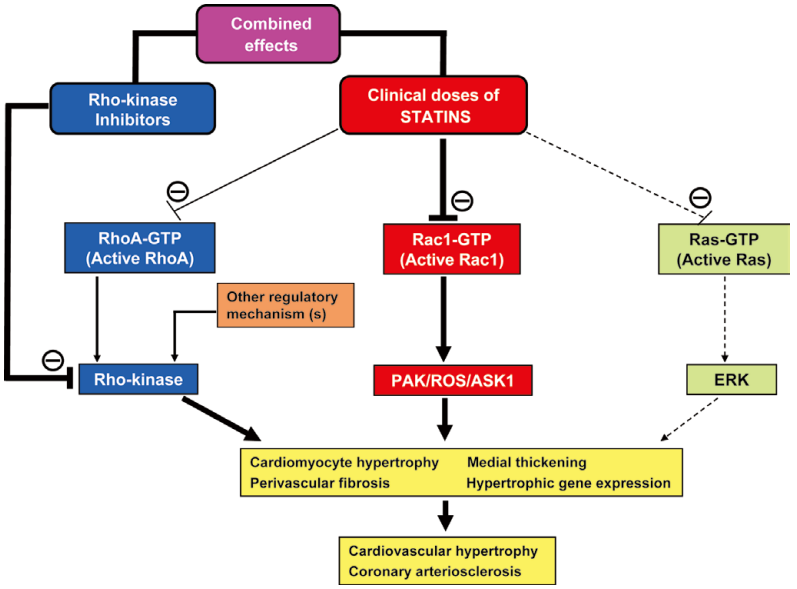

Fig 8. Different inhibitory effects of statins and Rho-kinase inhibitors on the RhoA, Rac1 and Ras pathways. Statins predominantly inhibit the Rac1 pathway at their Japanese clinical concentrations/doses, whereas they inhibit the RhoA and Ras pathways only at very high concentrations. By contrast, Rho-kinase inhibitors only inhibit the Rho-kinase pathway, which may be modulated by RhoA and other regulatory mechanism(s). The combination of a statin and a Rho-kinase inhibitor would exert more effective therapeutic effects than each monotherapy. PAK, p21-activated kinase; ASK1, apoptosis signal-regulating kinase 1; ROS, reactive oxygen species; ERK, extracellular signal-regulated kinase. Thicker lines, proven potent effect; thinner lines, proven weak effect; dashed lines, unproven effects.

concentration (30 μmol/L). In addition, ELISA assay showed that statins at 10 μmol/L do not directly inhibit Rho-kinase activity (ROCK1 and ROCK2). It was a somewhat unexpected finding that higher concentrations of statins failed to inhibit Rho-kinase, for which the enhanced and compensatory upregulation of RhoA by statins and other upstream mediators (eg, arachidonic acid)[25] may be involved. Further studies are needed to clarify the mechanisms involved. Taken together, the present results support the notion that statins at clinical concentrations mainly inhibit the Rac1

pathway, but their inhibitory effects on the RhoA/Rho-kinase pathway may not be evident (**Fig 8**).

Mechanisms of the Effects of Statins in Animals In Vivo

Several recent studies have shown that statins do not have a cholesterol-lowering effect in rats[11,18,26] Thus, the present rat model is suitable for isolating the pleiotropic effect of statins from their cholesterol-lowering effect. Most of the lipid-independent beneficial effects of statins are thought to be mediated by alterations in the RhoA and Rac1 signaling pathways, and both pathways are involved in AngII-induced cardiovascular hypertrophy in rats[11,12] We have recently demonstrated that fasudil inhibits AngII-induced cardiovascular hypertrophy in rats through inhibition of Rho-kinase[19] In the present in vitro study, we demonstrated that statins effectively inhibit Rac1 activity at lower concentrations, whereas they might inhibit RhoA, but not Ras activity, at higher clinical concentrations. Thus, we then focused on these 2 pathways (Rac1 vs RhoA) in terms of the molecular mechanisms of the pleiotropic effect of statins in rats in vivo.

In the present AngII infusion model, among the 3 statins examined, only atorvastatin partially inhibited cardiovascular hypertrophy and pitavastatin and pravastatin had no effect. By contrast, fasudil markedly suppressed the development of AngII-induced cardiovascular hypertrophy. AngII increases the membrane translocation of RhoA and Rac1 and their activity in the heart[27,28] Importantly, we were able to demonstrate that all the statins maintaining clinical concentrations inhibited Rac1, but not the RhoA/Rho-kinase pathway, whereas fasudil markedly suppressed Rho-kinase activation in vivo. These results suggest that statins predominantly inhibit the Rac1 pathway, but not the RhoA/Rho-kinase pathway in vivo and that RhoA/Rho-kinase is more important in the pathogenesis of AngII-induced cardiovascular hypertrophy in rats.

Previous reports showed that both the Rho/Rho-kinase and Rac1 pathway may be involved in the pathogenesis of cardiovascular diseases[11,12] In the present study, clinical concentration/dose of statins only inhibited Rac1 in vitro and in vivo, suggesting that combination therapy with statins and Rho-kinase inhibitors would be effective for cardiovascular diseases. Indeed, in the present study, combination therapy with atorvastatin and a low-dose of fasudil was more effective than fasudil alone for ameliorating AngII-induced cardiovascular hypertrophy in vivo. These findings suggest the therapeutic potential of the combination of a statin and a Rho-kinase inhibitor for the treatment of cardiovascular diseases.

Mechanisms of the Effects of Statins in Humans

Finally, we were able to demonstrate that clinical doses of statins inhibit Rac1 activity, but not RhoA or Rho-kinase activity, in circulating PMNL in humans. The present results are consistent with a previous report that statins inhibit ROS production through inhibition of Rac1 activity in the failing human heart[29] It has recently been shown that Rac1 also contributes to the pathogenesis of atrial fibrillation through activation of superoxide production by NAD(P)H oxidase, which is inhibited by statins through inhibition of Rac1 and NAD(P)H activation[30] It is interesting to note that both pravastatin (a hydrophilic statin) and atorvastatin (a lipophilic statin) exerted a comparable inhibitory effect on Rac1 activity in humans, suggesting a class effect of statins on the inhibition of Rac1 in humans.

Study Limitations

First, because the statins (atorvastatin and pravastatin) slightly but significantly reduced the serum levels of total cholesterol and LDL-cholesterol, even in normolipidemic volunteers, in agreement with a previous report[31] we cannot completely rule out the possible involvement of their cholesterol-lowering effect in the present results in humans. This point also remains to be examined in a future study, in which the effects of statins and those of cholesterol-lowering agents without the inhibitory effect on HMG-CoA reductase (eg, ezetimibe) will be compared. However, it is important to note that the statin treatments did not inhibit the RhoA/Rho-kinase pathway, even in the presence of a mild reduction in the serum cholesterol levels in humans, as well as in the rat model in which the statins do not reduce cholesterol levels. Second, the present findings with healthy volunteers should be confirmed in patients with cardiovascular diseases (eg, coronary artery disease, hypertension, and heart failure), in whom the RhoA/Rho-kinase pathway may be activated[7] Because Rac1 is also activated in various diseases in humans[6,29,30] it is important to note that statins mainly inhibited Rac1, but not RhoA/Rho-kinase, in the present AngII model in which both pathways were activated. Third, it remains to be examined in a future study whether treatment with higher doses of statins (>20 mg/day) and/or a longer period of treatment (>1 week) would inhibit the RhoA/Rho-kinase pathway in humans, as higher doses of statins (40–80 mg/day) are given to patients in the USA and other Western countries. In the present study, we used the maximum doses of pravastatin and atorvastatin (20 mg/day) allowed for clinical use in Japan, which are also the usual clinical doses in Asian countries.

Clinical Implications

The present study provides the first direct evidence that clinical concentrations/doses of statins predominantly inhibit Rac1, but not the RhoA/Rho-kinase pathway, at all research levels, ranging from the enzymatic, cellular, animal and human, regardless of their lipophilicity or hydrophilicity (**Fig 8**). Selective Rho-kinase inhibitors are currently under development and will be launched in the near future[8,9] The present findings also suggest that combination therapy with a statin and a Rho-kinase inhibitor could exert more effective cardiovascular protection than each monotherapy, validating the current development of selective Rho-kinase inhibitors for the treatment of cardiovascular diseases.

Acknowledgements

The present study was supported in part by the Global-COE Program and grants-in-aid from the Japanese Ministry of Education, Culture, Sports, Science and Technology, Tokyo, Japan, the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Pharmaceutical Safety and Research of Japan, and Technology Agency, CREST, Tokyo, Japan. The authors thank their co-workers at the Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine. The authors also thank Professor K. Kaibuchi and Dr M. Amano at the Department of Cell Pharmacology, Nagoya University and the Asahi Kasei Pharma for their cooperation in the present study. The authors would like to thank Pfizer, Kowa, Daiichi-Sankyo and Asahi Kasei Pharma Co, Japan, for providing atorvastatin, pitavastatin, pravastatin, and fasudil/hydroxyfasudil, respectively.

References

1. Schulz R. Pleiotropic effects of statins. *J Am Coll Cardiol* 2005; **45:** 1292–1294.
2. Davignon J. Beneficial cardiovascular pleiotropic effects of statins. *Circulation* 2004; **109:** 39–43.

3. Rikitake Y, Liao JK. Rho GTPases, statins and nitric oxide. *Circ Res* 2005; **97:** 1232–1235.
4. Nishikawa H, Miura S, Zhang B, Shimomura H, Arai H, Tsuchiya Y, et al. Statins induce the regression of left ventricular mass in patients with angina. *Circ J* 2004; **68:** 121–125.
5. Takemoto M, Liao JK. Pleiotropic effects of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors. *Arterioscler Thromb Vasc Biol* 2001; **21:** 1712–1719.
6. Brown JH, Del Re DP, Sussman MA. The Rac and Rho hall of fame: A decade of hypertrophic signaling hits. *Circ Res* 2006; **98:** 730–742.
7. Shimokawa H, Takeshita A. Rho-kinase is an important therapeutic target in cardiovascular medicine. *Arterioscler Thromb Vasc Biol* 2005; **25:** 1767–1775.
8. Shimokawa H, Rashid M. Development of Rho-kinase inhibitors for cardiovascular medicine. *Trends Pharmacol Sci* 2007; **28:** 296–302.
9. Liao JK, Seto M, Noma K. Rho-kinase (ROCK) inhibitors. *J Cardiovasc Pharmacol* 2007; **50:** 17–24.
10. Laufs U, Marra D, Node K, Liao JK. 3-Hydroxy-3-methylglutaryl-CoA reductase inhibitors attenuate vascular smooth muscle proliferation by preventing Rho GTPase-induced down-regulation of $p27^{kip1}$. *J Biol Chem* 1999; **274:** 21926–21931.
11. Takemoto M, Node K, Nakagami H, Liao Y, Grimm M, Takemoto Y, et al. Statins as antioxidant therapy for preventing cardiac myocyte hypertrophy. *J Clin Invest* 2001; **108:** 1429–1437.
12. Laufs U, Kilter H, Konkol C, Wassmann S, Bohm M, Nickenig G. Impact of HMG CoA reductase inhibition on small GTPases in the heart. *Cardiovasc Res* 2002; **53:** 911–920.
13. Shimokawa, H. Rho-kinase as a novel therapeutic target in treatment of cardiovascular diseases. *J Cardiovasc Pharmacol* 2002; **39:** 319–327.
14. Ishikura K, Yamada N, Ito M, Ota S, Nakamura M, Isaka N, et al. Beneficial acute effects of Rho-kinase inhibitor in patients with pulmonary arterial hypertension. *Circ J* 2006; **70:** 174–178.
15. Abe K, Morikawa K, Hizume T, Uwatoku T, Oi K, Seto M, et al. Prostacyclin does not inhibit Rho-kinase: An implication for the treatment of pulmonary hypertension. *J Cardiovasc Pharmacol* 2005; **45:** 120–124.
16. Fontaine D, Fontaine J, Dupont I, Dessy C, Piech A, Carpentier Y, et al. Chronic hydroxymethylglutaryl coenzyme A reductase inhibition and endothelial function of the normocholesterolemic rat: Comparison with angiotensin-converting enzyme inhibition. *J Cardiovasc Pharmacol* 2002; **40:** 172–180.
17. Aoki T, Yoshinaka Y, Yamazaki H, Suzuki H, Tamaki T, Sato F, et al. Triglyceride-lowering effect of pitavastatin in a rat model of postprandial lipemia. *Eur J Pharmacol* 2002; **444:** 107–113.
18. Fujioka T, Nara F, Tsujita Y, Fukushige J, Fukami M, Kuroda M. The mechanism of lack of hypocholesterolemic effects of pravastatin sodium, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, in rats. *Biochim Biophys Acta* 1995; **1254:** 7–12.
19. Higashi M, Shimokawa H, Hattori T, Hiroki J, Mukai Y, Morikawa K, et al. Long-term inhibition of Rho-kinase suppresses angiotensin II-induced cardiovascular hypertrophy in rats in vivo: Effect on endothelial NAD(P)H oxidase system. *Circ Res* 2003; **93:** 767–775.
20. Wei L, Imanaka-Yoshida K, Wang L, Zhan S, Schneider MD, DeMayo FJ, et al. Inhibition of Rho family GTPases by Rho GDP dissociation inhibitor disrupts cardiac morphogenesis and inhibits cardiomyocyte proliferation. *Development* 2002; **129:** 1705–1714.
21. Hattori T, Shimokawa H, Higashi M, Hiroki J, Mukai Y, Tsutsui H, et al. Long-term inhibition of Rho-kinase suppresses left ventricular remodeling after myocardial infarction in mice. *Circulation* 2004; **109:** 2234–2239.
22. Stroes ES, Koomans HA, de Bruin TW, Rabelink TJ. Vascular function in the forearm of hypercholesterolaemic patients off and on lipid-lowering medication. *Lancet* 1995; **346:** 467–471.
23. Liu PY, Chen JH, Lin LJ, Liao JK. Increased Rho kinase activity in a Taiwanese population with metabolic syndrome. *J Am Coll Cardiol* 2007; **49:** 1619–1624.
24. Kaneyuki U, Ueda S, Yamagishi S, Kato S, Fujimura T, Shibata R, et al. Pitavastatin inhibits lysophosphatidic acid-induced proliferation and monocyte chemoattractant protein-1 expression in aortic smooth muscle cells by suppressing Rac-1-mediated reactive oxygen species generation. *Vasc Pharmacol* 2007; **46:** 286–292.
25. Araki S, Ito M, Kureishi Y, Feng J, Machida H, Isaka N, et al. Arachidonic acid-induced Ca^{2+} sensitization of smooth muscle contraction through activation of Rho-kinase. *Pflugers Arch* 2001; **441:** 596–603.
26. Saka M, Obata K, Ichihara S, Cheng XW, Kimata H, Nishizawa T, et al. Pitavastatin improves cardiac function and survival in association with suppression of the myocardial endothelin system in a rat model of hypertensive heart failure. *J Cardiovasc Pharmacol* 2006; **47:** 770–779.
27. Aoki H, Izumo S, Sadoshima J. Angiotensin II activates RhoA in cardiac myocytes: A critical role of RhoA in angiotensin II-induced premyofibril formation. *Circ Res* 1998; **82:** 666–676.
28. Satoh M, Ogita H, Takeshita K, Mukai Y, Kwiatkowski DJ, Liao JK. Requirement of Rac1 in the development of cardiac hypertrophy. *Proc Natl Acad Sci USA* 2006; **103:** 7432–7437.
29. Maack C, Kartes T, Kilter H, Schafers HJ, Nickenig G, Bohm M, et al. Oxygen free radical release in human failing myocardium is associated with increased activity of Rac1-GTPase and represents a target for statin treatment. *Circulation* 2003; **108:** 1567–1574.
30. Adam O, Frost G, Custodis F, Sussman MA, Schafers HJ, Bohm M, et al. Role of Rac1 GTPase activation in atrial fibrillation. *J Am Coll Cardiol* 2007; **50:** 359–367.
31. Sakamoto T, Kojima S, Ogawa H, Shimomura H, Kimura K, Ogata Y, et al. Usefulness of hydrophilic vs lipophilic statins after acute myocardial infarction: Subanalysis of MUSASHI-AMI. *Circ J* 2007; **71:** 1348–1353.