SmgGDS as a Crucial Mediator of the Inhibitory Effects of Statins on Cardiac Hypertrophy and Fibrosis

Novel Mechanism of the Pleiotropic Effects of Statins

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Abstract—The detailed molecular mechanisms of the pleiotropic effects of statins remain to be fully elucidated. Here, we hypothesized that cardioprotective effects of statins are mediated by small GTP-binding protein GDP dissociation stimulator (SmgGDS). SmgGDS+/− and wild-type (WT) mice were treated with continuous infusion of angiotensin II (Ang II) for 2 weeks with and without oral treatment with atorvastatin or pravastatin. At 2 weeks, the extents of Ang II–induced cardiac hypertrophy and fibrosis were comparable between the 2 genotypes. However, statins significantly attenuated cardiomyocyte hypertrophy and fibrosis in WT mice, but not in SmgGDS+/− mice. In SmgGDS+/− cardiac fibroblasts (CFs), Rac1 expression, extracellular signal–regulated kinases 1/2 activity, Rho-kinase activity, and inflammatory cytokines secretion in response to Ang II were significantly increased when compared with WT CFs. Atorvastatin significantly reduced Rac1 expression and oxidative stress in WT CFs, but not in SmgGDS+/− CFs. Furthermore, Bio-plex analysis revealed significant upregulations of inflammatory cytokines/chemokines and growth factors in SmgGDS+/− CFs when compared with WT CFs. Importantly, conditioned medium from SmgGDS+/− CFs increased B-type natriuretic peptide expression in rat cardiomyocytes to a greater extent than that from WT CFs. Furthermore, atorvastatin significantly increased SmgGDS secretion from mouse CFs. Finally, treatment with recombinant SmgGDS significantly reduced Rac1 expression in SmgGDS+/− CFs. These results indicate that both intracellular and extracellular SmgGDS play crucial roles in the inhibitory effects of statins on cardiac hypertrophy and fibrosis, partly through inhibition of Rac1, Rho kinase, and extracellular signal–regulated kinase 1/2 pathways, demonstrating the novel mechanism of the pleiotropic effects of statins. (Hypertension. 2016;67:878-889. DOI: 10.1161/HYPERTENSIONAHA.115.07089.)

Key Words: angiotensin II ■ chemokines ■ oxidative stress ■ pravastatin ■ statin
region. Rac1, as a component of NADPH oxidases that produce reactive oxygen species (ROS), contributes to the development of angiotensin II (Ang II)–induced cardiac hypertrophy in mice. In this study, we hypothesized that cardioprotective effects of statins are mediated by SmgGDS. Thus, we examined whether SmgGDS is involved in the inhibitory effects of statins on Ang II–induced cardiac hypertrophy and fibrosis in mice in vivo, and if so, what molecular mechanisms are involved.

Materials and Methods

Ang II–Induced Cardiac Hypertrophy

We used 10-week-old male SmgGDS hetero-deficient (SmgGDS−/−) mice to evaluate cardiac hypertrophy and fibrosis as previously reported because SmgGDS−− mice are embryo-lethal. Age- and sex-matched wild-type (WT) littermate mice were used as controls. Mice were subcutaneously and continuously infused with Ang II (2.0 mg/kg per day; WAKO, Tokyo, Japan) or saline for 2 weeks by Alzet osmotic pumps (model 2002, Durect Corporation, CA), and they were orally administered either atorvastatin (10 mg/kg per day, Pfizer), pravastatin (50 mg/kg per day, Daiichi Sankyo, Tokyo, Japan), or placebo for 2 weeks. Systolic blood pressure was measured using a noninvasive tail-cuff system (BP-2000, Visitech Systems, Inc, NC) before and 2 weeks after pump implantation without anesthesia.

Transverse Aortic Constriction

Transverse aortic constriction was performed as previously described with slight modification. Mice at 8 to 10 weeks of age weighing 24.9±0.6 g were anesthetized with isoflurane (1.5%–2.0%). Between the right innominate and left carotid arteries, the transverse aorta was constricted with a 7-0 silk suture against a 27-gauge blunted needle, which was removed immediately after the ligation to yield a constriction of 0.51 mm in diameter. Sham-operated mice were subjected to the same procedure without transverse aortic constriction. After the operations, the mice were followed up for 4 weeks to evaluate cardiac fibrosis.

Echocardiography

Echocardiography was performed using the Vevo 2100 (Visualsonics, Toronto, Canada) under anesthesia with inhaled isoflurane (0.5%–1.0% v/v). Between the right innominate and left carotid arteries, the transverse aorta was constricted with a 7-0 silk suture against a 27-gauge blunted needle, which was removed immediately after the ligation to yield a constriction of 0.51 mm in diameter. Sham-operated mice were subjected to the same procedure without transverse aortic constriction. After the operations, the mice were followed up for 4 weeks to evaluate cardiac fibrosis.

Histological Analysis

The mouse heart was fixed with 10% formalin and embedded in paraffin. Myocardial cross-sectional area and interstitial fibrosis area were analyzed by Image J Software (NIH, Bethesda, MD). For immunostaining, the heart sections were embedded in paraffin and stained with SmgGDS antibody (100:1; BD Biosciences, 612511, NJ) and were then counter stained with hematoxylin–eosin as previously described. LV diastolic function was assessed by the measurement of LV transmitral early peak flow velocity (E) to LV transmitral late peak flow velocity (A) wave ratio (E/A ratio).

Isolation of Cardiac Fibroblasts

Isolation of cardiac fibroblasts (CFs) from mouse ventricular tissue was performed as previously described. The heart tissue was enzymatically digested by collagenase type II (Worthington, NJ) and was then placed in a dish filled with Dulbecco modified Eagle’s medium (DMEM) containing 4.5-g/L glucose supplemented with 20% fetal bovine serum (FBS) and 1× penicillin/streptomycin. After removal of the atria, both ventricles were teased apart and pipetted into small pieces. The pellet containing cardiomyocytes was discarded and the supernatant, containing mostly CFs, was centrifuged and resuspended in medium. The CFs were plated and cultured on a 10-cm dish with DMEM with 10% FBS at 37°C in a humidified atmosphere of 5% CO2.

Cell Culture and Drug Treatment

CFs were starved and pretreated with atorvastatin (10 μmol/L) for 24 hours, followed by Ang II (1 μmol/L) stimulation for 24 hours. CFs were treated with recombinant SmgGDS protein (1 μg/mL; Abnova, Taipei, Taiwan) for 24 hours. After the treatment with each drug, the cells were washed with ice-cold phosphate-buffered saline and added 10% trichloroacetic acid. Then, the cells were sonicated after adding lysis buffer (8 mol/L urea, 62.5 mmol/L Tris, and 2% SDS).

Detection of ROS

We treated CFs with Ang II (1 μmol/L) with and without atorvastatin (10 μmol/L) for 6 hours at 37°C in 5% CO2, washed them with PBS, and loaded them with 2,7-dichlorofluorescein diacetate (5 μmol/L; Molecular Probes, Eugene, OR) for 30 minutes at 37°C. Presence of ROS was detected as green fluorescence (488 nm) by fluorescence microscopy (BIOREVO, Keyence). The relative fluorescence intensity was measured by BZ-II analyzer (Keyence) software.

Cell Proliferation Assay

Mouse CFs were seeded in 96-well plates (2×103 cells/well) in 100-μL DMEM with 10% FBS. On the next day, CFs were stimulated with DMEM containing 0% or 5% FBS for ≤3 days. Cells were counted on days 1 and 3 using Cell Titer 96 MTT assay method (Promega, Fitchburg, WI).

Preparation of Conditioned Medium

CFs were starved and stimulated with Ang II (1 μmol/L) with or without atorvastatin (10 μmol/L) in 100-mm dish with 10-mL DMEM. After 24-hour stimulation, the conditioned medium (CM) was collected, filtered to remove cell debris, and concentrated with an Amicon Ultra-15 centrifugal filter (Millipore Corp, MA) to yield concentrated CM. Cytokine/chemokine and growth factors in CM were measured with the Bio-Plex multiplex mouse cytokine assay kit (Bio-Rad, Hercules, CA) according to manufacturer recommendation. Each experiment was performed in duplicate.

Treatment of Neonatal Rat Cardiomyocytes With CM

Isolation of neonatal rat cardiomyocytes (NRCMs) was performed as previously described. The heart from 1- to 3-day-old Wistar rat (Japan SLC, Shizuoka, Japan) was minced and dissociated with collagenase type II (Worthington, NJ). After dispersed, cells were incubated on 10-cm dishes for 30 minutes at 37°C in 5% CO2, incubator twice. Nonattached viable cells in supernatant were collected and supplemented with 10-μmol/L cytosine β-D-arabinofuranoside (Sigma-Aldrich, Tokyo, Japan) to prevent growth of nonmyocytes. Then, NRCMs were seeded into 12-well gelatin-coated dishes (4×104 cells/well; Asahi Glass, Tokyo, Japan) with DMEM containing 1-g/L glucose and pyruvate with 10% FBS at 37°C in a humidified atmosphere of 5% CO2. Cells were starved for 12 hours, then treated with DMEM containing 10% CM from CFs for 24 hours.

Western Blot Analysis

Protein samples were extracted from CFs. An equal amount of protein samples were loaded on SDS–PAGE gel and transferred to
polyvinylidene difluoride membranes (GE Healthcare, United Kingdom). The primary antibodies were those to Rac1 (1000:1, Millipore, 05-389), SmgGDS (500:1, BD Biosciences, 612511), α-tubulin (2000:1, Sigma, T9026), total myosin phosphatase targeting protein (1000:1, BD Biosciences, 612165), phosphorylated myosin phosphatase targeting protein (1000:1, Millipore, ABS45), phosphorylated extracellular signal-regulated kinases (ERK1/2; 1000:1, Cell Signaling, no. 9101), and total-ERK1/2 (1000:1, Cell Signaling, no. 4695). The regions containing proteins were visualized by the enhanced chemiluminescence system (ECL Prime Western Blotting Detection Reagent; GE Healthcare, Buckinghamshire, United Kingdom). Densitometric analysis was performed by the Image J Software.

Real-Time Polymerase Chain Reaction
Total RNA was converted to cDNA using PrimeScript RT Master Mix (Takara Bio; Shiga, Japan). Quantitative real-time polymerase chain reaction on the CFX 96 Real-Time PCR Detection System (Bio-Rad) was performed using SYBR Premix ExTaQ Probes (Takara Bio). The Ct value determined by the CFX Manager Software (version 2.0, Bio-Rad) for all samples was normalized to housekeeping gene Gapdh, and the relative fold change was computed by the comparative Ct (ΔΔCt) method.

Statistical Analysis
Statistical analysis was performed with JMP pro 11 (SAS Institute Inc; Cary, NC). Results are shown as means±SEM. Comparisons of parameters were performed with the unpaired Student’s t test or Tukey test for multiple comparisons. P<0.05 was considered to be statistically significant.

Study Approval
All protocols involving animals and humans in this study were approved by the Institutional Review Board of Tohoku University.

Results
Lack of Inhibitory Effects of Statins on Cardiac Hypertrophy and Fibrosis in SmgGDS+/– Mice
We first examined whether SmgGDS contributes to the inhibitory effects of statins on Ang II–induced cardiac hypertrophy and fibrosis in vivo. Ang II significantly increased heart weight but to a similar extent in both WT and SmgGDS+/– mice (Figure 1A). Tail-cuff system and telemetry recording of blood pressure showed that systolic blood pressure was lower in SmgGDS+/– mice compared with WT mice without significant difference (Figure 1B; Figure S1 in the online-only Data Supplement). Ang II significantly and equally increased cardiomyocyte size and interstitial fibrosis in both the genotypes (Figure 1C–1E). Importantly, both statins significantly reduced Ang II–induced cardiac hypertrophy and fibrosis in WT mice, which inhibitory effects by statins were absent in SmgGDS+/– mice (Figure 1C–1E). We also used transverse aortic constriction model to evaluate the effect of pressure-overload on cardiac fibrosis with and without atorvastatin treatment. Interestingly, SmgGDS+/– mice showed tendency but no significant change in the statin-mediated reduction rate of fibrotic area (%) compared with WT mice after transverse aortic constriction (Figure S2), suggesting the crucial role of SmgGDS in Ang II signaling. Echocardiography showed that both statins significantly inhibited Ang II–induced increase in LV wall thickness in WT mice, which effects of statins were again absent in SmgGDS+/– mice (Figure 2A, 2C, and 2D). We further examined LV functions, systolic function by LV ejection fraction and diastolic function by E/A ratio, in both the genotypes. Although Ang II showed no effects on LV ejection fraction (Figure 2E), it markedly reduced E/A ratio in both the genotypes to a similar extent, which was significantly inhibited by statins in WT mice, but were again absent in SmgGDS+/– mice (Figure 2F).

To further elucidate the cell types that participate in the SmgGDS-mediated protective effects of statins, we examined the localization of SmgGDS-expressing cells in the heart sections of Ang II–infused mice (Figure 3). Immunostaining revealed that the number of SmgGDS-positive cells was increased in fibrotic area in response to Ang II in WT mice (Figure 3A). In contrast, the Ang II–induced upregulation of SmgGDS in the fibrotic area was markedly reduced in SmgGDS+/– mice (Figure 3B). Immunofluorescence staining demonstrated that SmgGDS was strongly expressed in thrombospondin–1–positive CFs in the heart of WT mice (Figure S3). Thus, we hypothesized that CFs mainly contribute to the SmgGDS-mediated protective effects by statins.

Role of SmgGDS in the Inhibitory Effects of Statins on Rac1 in CFs
Previous studies have demonstrated that SmgGDS promotes Rac1 degradation by transporting it to the nucleus.15,16,27 We also have recently demonstrated that statins selectively enhance the degradation of Rac1 via SmgGDS in ECs.11 Thus, we hypothesized that SmgGDS regulates Rac1-signaling pathway in CFs as well. SmgGDS expression was significantly decreased (by approximately half), and Rac1 expression was significantly increased (by 1.5-fold) in SmgGDS+/– CFs compared with WT CFs at baseline (Figure 4A). We next stimulated CFs with Ang II with or without atorvastatin. Notably, atorvastatin significantly inhibited Rac1 expression by 40% in WT CFs but had no effect in SmgGDS+/– CFs (Figure 4B). Proliferation of CFs in response to 5% FBS was enhanced in SmgGDS+/– CFs compared with WT CFs (Figure 4C), which is consistent with the enhanced fibrosis in SmgGDS+/– heart. Because it is widely accepted that Rac1 accelerates ROS production,28 we evaluated ROS levels by 2,7-dichlorofluorescein staining (Figure 4D), which showed that elevated ROS levels in SmgGDS+/– CFs was not significantly changed by atorvastatin treatment, which implicates the involvement of SmgGDS in Ang II signaling. Because Rac1 accelerates hypertrophy and inflammation,29 we further examined hypertrophic and inflammatory intracellular signaling in CFs. Because ERK1/2 signaling is involved in the mechanism of cardiac hypertrophy and is regulated by downstream signals of Rac1,30 we examined ERK1/2 activation in CFs. Similar to Rac1 expression, we found significant increase in ERK1/2 activities in SmgGDS+/– compared with WT CFs (Figure 5A and 5B). Furthermore, we examined Rho-kinase activity by measuring phosphorylated/total myosin phosphatase targeting protein in CFs.31,32 Interestingly, Rho-kinase activity was also elevated in SmgGDS+/– compared with WT CFs (Figure 5A and 5C). These results indicate the enhancement of hypertrophic and inflammatory signaling in SmgGDS+/– CFs. We next stimulated CFs by Ang II with or without atorvastatin treatment. Although Ang II did not alter ERK and Rho-kinase activity in WT CFs, Ang II increased these activities in SmgGDS+/– CFs (Figure 5D–5F; Figure S4). Importantly, atorvastatin inhibited Ang II–induced Rho-kinase activation in WT CFs, but not in...
SmgGDS+/− CFs (Figure 5D and 5F). We further examined Ang II receptor type 1 (AT1R) mRNA in CFs and hearts in WT and SmgGDS+/−. Interestingly, the AT1R mRNA level was significantly upregulated in CFs and hearts of SmgGDS+/− mice compared with those of WT (Figure S5A and S5B). These results suggest that SmgGDS is profoundly involved in the regulatory mechanism of Ang II–AT1R signaling. Furthermore, we confirmed the relationship between SmgGDS and other guanine nucleotide exchange factor that downregulates Rac1 activity, such as Tiam1. However, the baseline expression of Tiam-1 was almost equal between WT and SmgGDS+/− CFs (Figure S6). In addition, the expression of Tiam-1 was significantly increased by statin treatment in WT CFs, which increase was relatively less in SmgGDS+/− CFs. These results implicate the Tiam-1–independent downregulation of Rac1 by SmgGDS.

**Possible Role of Extracellular SmgGDS for the Inhibitory Effects of Statins**

The interaction between CFs and cardiomyocytes is partly mediated by secreted cytokines/chemokines and growth factors in an autocrine/paracrine manner. To elucidate whether SmgGDS is involved in this interaction, we prepared CM from WT and SmgGDS+/− CFs in response to Ang II with or without atorvastatin treatment. We first evaluated the profiles of cytokines/chemokines and growth factors in CM. Interestingly, CM from SmgGDS+/− CFs revealed significant increases in the inflammatory cytokines and growth factors compared with those from WT CFs (Figure 6A). The secretion of macrophage colony-stimulating factor, chemokine (C–X–C motif) ligand 2, and eotaxin, all of which promote inflammatory cell migration and inflammation, were significantly increased in SmgGDS+/− CFs compared with WT CFs. Furthermore, interleukin (IL)-6 and IL-17, which have been described to be involved in cardiac hypertrophy, were also increased in SmgGDS+/− CFs (Figure 6A). In addition, many inflammatory cytokines/chemokines and growth factors were elevated in CM from SmgGDS+/− CFs compared with WT CFs (Figure S7). These results suggest the potential role of SmgGDS in the regulation of cytokines secretion and statin-mediated inhibitory effects on cardiac hypertrophy and fibrosis.

Thus, we next examined whether CM from CFs directly affects cardiomyocytes. We prepared primary culture of
NRCMs and stimulated them with CM from WT and SmgGDS−/− CFs (Figure 6B). CM from WT CFs treated with Ang II and atorvastatin reduced B-type natriuretic peptide expression in NRCMs compared with CM from WT CFs treated with Ang II alone (Figure 6B). Importantly, these effects of atorvastatin were not observed in NRCMs stimulated with CM from SmgGDS+/− CFs (Figure 6C). These results suggest that both intracellular and extracellular SmgGDS contribute to the inhibitory effects of statins on Ang II–mediated hypertrophic signals in CFs and cardiomyocytes. Finally, we found significant increase in the SmgGDS secretion from WT CFs in response to atorvastatin (Figure 7A and 7B). In contrast, SmgGDS expression in total cell lysates was unaltered by Ang II and atorvastatin (Figure 7A and 7B). Thus, we next examined the effects of extracellular SmgGDS. CFs were treated with GST-tagged recombinant SmgGDS protein (GST-SmgGDS, 1 μg/mL) for 24 hours. After 24-hour treatment with GST-SmgGDS, we found both intrinsic and GST-tagged SmgGDS in CFs, suggesting an uptake of extracellular GST-SmgGDS into intracellular space (Figure 7C and 7D). ERK activity did not change by treatment with GST-SmgGDS. However, extracellular GST-SmgGDS significantly reduced

Figure 2. SmgGDS exerts protective effect of statins in angiotensin II (Ang II)–induced cardiac hypertrophy and left ventricular (LV) diastolic dysfunction. Echocardiographic analyses of Ang II–infused wild-type (WT) and SmgGDS−/− mice treated with statins (atorvastatin 10 mg/kg per day or pravastatin 50 mg/kg per day) or vehicle for 2 weeks. A and B, Representative images of M-mode echocardiography (A), and pulse wave Doppler in LV inflow (B) in each treatment group. C and D, LV anterior wall thickness (C), LV posterior wall thickness (D), LV ejection fraction (E), and E/A ratio (F) were measured. Results are expressed as mean±SEM (n=7–10 in each group). *P<0.05. AT indicates atorvastatin; LVAWd, diastolic LV anterior wall diameter; LVEF, LV ejection fraction; LVPWd, diastolic LV posterior wall diameter; and PR, pravastatin.

Figure 3. Expression of SmgGDS in cardiac fibroblasts. Representative photomicrographs of Masson trichrome staining (left) and SmgGDS immunostaining (middle) of the heart from wild-type (WT) and SmgGDS−/− mice treated with angiotensin II (Ang II) or vehicle. Scale bars, 100 μm. Right, High magnification photomicrographs of the box area in the middle column. SmgGDS was highly expressed in cardiac fibroblasts in WT mice (arrows). Scale bars, 50 μm.
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Rac1 expression in SmgGDS+/− CFs (Figure 7C and 7D), suggesting that extracellular SmgGDS blocks Rac1 expression. In addition, we used siRNA to knockdown SmgGDS in CFs to confirm the role of SmgGDS for Rac1 expression and ERK activity. Importantly, SmgGDS siRNA reduced expression of SmgGDS ≈40% and tended to increase Rac1 expression and ERK activity in CFs, which were consistent with the data of SmgGDS+/− CFs (Figure S8).

Intracellular and Extracellular SmgGDS in ECs and Cardiac Myocytes

To test whether extracellular SmgGDS can be detected in the culture medium of other cardiac cell components, such as ECs and cardiomyocytes, we used human umbilical endothelial cells and NRCMs, which were treated with atorvastatin. Interestingly, we found that extracellular SmgGDS was detected in the culture medium of human umbilical endothelial cells, which was significantly increased by statin treatment (Figure S9A). In contrast, we found that intracellular SmgGDS only tended to be increased in NRCMs by statin treatment. However, we did not detect secretion of SmgGDS from NRCMs at baseline or by statin treatment (Figure S9B).

Discussion

The novel findings of this study are that (1) SmgGDS plays a central role for the inhibitory effects of statins on cardiac hypertrophy and fibrosis induced by Ang II in mice in vivo, (2) the main cell type of SmgGDS expression in response to Ang II is CFs, (3) the SmgGDS-mediated beneficial effects of statins are achieved through inhibition of Rac1, ERK1/2, and Rho-kinase pathway, and (4) both intracellular and extracellular SmgGDS work as a key molecule for the beneficial effects of statins. To the best of our knowledge, this is the first study that demonstrates that SmgGDS is an important molecular switch that mediates the pleiotropic effects of statins through its intracellular and extracellular effects (Figure 8).

Importance of Rac1 Inhibition in the Pleiotropic Effects of Statins

In this study, we were able to demonstrate that SmgGDS is necessary for the cardioprotective effects of statins against Ang II–induced cardiac hypertrophy and fibrosis in vivo (Figure 1). Several studies have shown that Rac1 plays a critical role in the development of cardiovascular diseases.18,38 It has been demonstrated that Ang II–induced cardiac hypertrophy and
ROS formation were significantly reduced in cardiomyocyte-specific Rac1−/− mice.\textsuperscript{18} In patients with end-stage heart failure, Rac1 activities were elevated in the LV and were suppressed by statins,\textsuperscript{38} which is consistent with the present findings. Thus, inhibition of Rac1 plays a crucial role for the inhibitory effects of statins on cardiac hypertrophy and fibrosis.

SmgGDS inhibits Rac1 by promoting nuclear translocation and degradation by proteasome system.\textsuperscript{16,27} We have also demonstrated that SmgGDS promotes Rac1 degradation by statins\textsuperscript{11} in vitro. Thus, it is possible that SmgGDS inhibits Rac1 degradation by statins in vivo.\textsuperscript{13} Thus, inhibition of Rac1 plays a crucial role for the inhibitory effects of statins on cardiac hypertrophy and fibrosis.

**SmgGDS Inhibits Rho Kinase and Rac1 in CFs**

Rho kinase contributes to the pathogenesis of several cardiovascular diseases.\textsuperscript{20,39–41} In this study, we found parallel increase in Rho kinase activity and Rac1 in SmgGDS−/− CFs compared with WT CFs. Previous studies have demonstrated that SmgGDS activates RhoA,\textsuperscript{42,43} which is the upstream activator of Rho kinase.\textsuperscript{44} However, in this study, Rho kinase activity was significantly increased in SmgGDS−/− CFs. Because Rho kinase regulates NADPH oxidases that produce ROS,\textsuperscript{45–47} activated Rho kinase activity and Rac1 induces excessive ROS in SmgGDS−/− CFs. Excessive ROS (oxidative stress) promotes inflammation and proliferation of CFs. Thus, significant increase in ERK1/2 activity in SmgGDS−/− CFs is consistent with increased Rho-kinase and Rac1 activities. Conversely, Rho-family small GTPases, such as Rac1 and Cdc42, activate mitogen-activated protein kinase pathway including ERK1/2.\textsuperscript{30,48} Rac1 activation by hypertrophic stimuli is crucial for ERK1/2 activation in cardiomyocytes.\textsuperscript{49} Thus, Rac1 upregulation in SmgGDS−/− CFs leads to Rho-kinase and ERK1/2 activation, in part, by oxidative stress. In contrast, SmgGDS inhibits Rho kinase and Rac1, resulting in reduced ROS generation. Intracellular ROS level is regulated not only by Rac1 expression but also by its activation and other many regulatory molecules, such as xanthine oxidase, nuclear respiratory factor-2, and heme oxygenase-1.\textsuperscript{50–52} Thus, we need to clearly elucidate precise mechanisms for the SmgGDS-mediated regulation of intracellular ROS levels. When we consider the role of ROS in intracellular signaling, we need to consider the levels of ROS and its compensation systems, which are composed of complex interactions among multiple intracellular signaling and cell surface receptors. In this study, mRNA expression of AT1R was significantly upregulated in

Figure 5. Increased activities of extracellular signal–regulated kinases (ERK) 1/2 and Rho kinase in SmgGDS−/− cardiac fibroblasts (CFs). A-C. Western blot analyses of ERK1/2 activity (phosphorylated/total-ERK1/2) (B) and Rho-kinase activity (phosphorylated/total myosin phosphatase targeting protein [MYPT]) (C) in wild-type (WT) and SmgGDS−/− CFs. D and E, Western blot analysis of ERK1/2 activity (E) and Rho-kinase activity (F) in WT and SmgGDS−/− CFs stimulated by angiotensin II (Ang II, 1 μmol/L) with or without atorvastatin (ATOR, 10 μmol/L) for 24 hours. Results are expressed as mean±SEM (n=3). *P<0.05, †P<0.05 vs WT CFs without simulation.
CFs and hearts of SmgGDS−/− mice compared with WT. These results suggest the involvement of SmgGDS in statin-mediated regulation of the Ang II–AT1R system (Figure 8).

**Novel Role of Extracellular SmgGDS in the Beneficial Effects of Statins**

Previous studies have demonstrated the close interaction between CFs and cardiomyocytes in the development of cardiac hypertrophy and fibrosis. In this study, we demonstrated that many cytokines/chemokines and growth factors, such as IL-17, macrophage colony-stimulating factor, and chemokine (C–X–C motif) ligand 2, were significantly increased in CM from SmgGDS−/− CFs, which may partly explain the augmented hypertrophy and fibrosis in SmgGDS−/− mice in vivo. In addition, the secretion of monocyte chemoattractant protein-1 was significantly reduced by statin treatment in WT CFs, but not in SmgGDS−/− CFs. Because these cytokines accelerate cardiac hypertrophy and fibrosis, the present results may partially explain the different responses to statin treatment between WT and SmgGDS−/− mice. Namely, statins may require SmgGDS to block the secretion of IL-6 and IL-17 from CFs, which contributes to their beneficial effects on cardiomyocytes hypertrophy. Taken together, extracellular SmgGDS plays a crucial role in the regulation of statin-mediated cytokines secretion and inflammatory signaling.

One of the novel findings of this study is that SmgGDS is secreted from CFs in response to statin treatment. Furthermore, treatment with recombinant SmgGDS reduced Rac1 expression in SmgGDS−/− CFs to the equivalent level of WT CFs, with no change in ERK1/2 activity. This suggests that extracellular SmgGDS also regulates intracellular Rac1 expression in an ERK/mitogen-activated protein kinase-independent manner. Moreover, both Western blotting and fluorescent immunostaining revealed the direct uptake of recombinant SmgGDS into mouse CFs. Thus, it is conceivable that statin-induced SmgGDS secretion results in uptake of extracellular SmgGDS into CFs and reduces Rac1 expression in an autocrine/paracrine manner. Consistently, conditioned media prepared from

Figure 6. SmgGDS contributes to the secretion of inflammatory cytokines from cardiac fibroblasts (CFs) and mediates cardiomyocytes hypertrophy. A, Quantitative analysis of cytokine levels in conditioned medium (CM) from CFs stimulated by angiotensin II (Ang II, 1 μmol/L) with or without atorvastatin (ATOR, 10 μmol/L) for 24 hours. B, Scheme of an experimental protocol. Neonatal rat cardiomyocytes (NRCMs) were treated with CM (10%) prepared from wild-type (WT) and SmgGDS−/− CFs for 24 hours. C, Relative mRNA expressions of B-type natriuretic peptide (BNP) in NRCMs treated with CM prepared from WT and SmgGDS−/− CFs. Results are adjusted by GAPDH. Results are expressed as mean±SEM (n=4 in each group). *P<0.05, #P<0.05 vs CM from WT CFs in each treatment group.
CFs induced hypertrophic signal in NRCMs, which was significantly accelerated in those from SmgGDS+/– CFs. This implicates the potential role of extracellular SmgGDS for the inhibitory effects of statins on cardiac hypertrophy and fibrosis. Further mechanistic studies are warranted for the insight of extracellular SmgGDS. These results implicate that intracellular and extracellular SmgGDS work together to regulate Rac1 and ROS levels, which may partly explain the complete lack of statin-mediated effects in SmgGDS+/– mice.

Study Limitations
Several limitations should be mentioned for this study. First, we have used high dose of Ang II (2.0 mg/kg per day) in this study. Second, although the levels of pERK1/2 were significantly elevated in SmgGDS+/– CFs compared with WT CFs, the expression of pERK1/2 in cardiac tissues was comparable with or without atorvastatin treatment. Third, the roles of intracellular SmgGDS in cardiomyocytes remain to be elucidated. Cardiomyocyte-specific deletion of SmgGDS may provide information for this issue. However, immunostaining demonstrated limited expression of SmgGDS in cardiomyocytes in vivo. Fourth, in contrast to the increased expression of SmgGDS in ECs, the expression of SmgGDS did not increase in CFs in response to statin treatment. However, the statin treatment significantly increased the secretion of SmgGDS from CFs, which mechanisms need to be precisely explored in future studies. Furthermore, we need to elucidate the mechanisms for the SmgGDS-mediated inhibition of Rac1 in CFs. Here, it has been reported that SmgGDS binds to C-terminal polybasic region of Rac1 and transports it to the nucleus, where it is degraded in various cells such as ovarian and ECs.11,16,27 Thus, it is possible that the same mechanism is also involved in CFs. Finally, the precise mechanisms for the statin-mediated secretion of SmgGDS and its role for the inhibition of Ang II signaling remain to be elucidated. The development of neutralizing antibody against SmgGDS may help us elucidate the effects of extracellular SmgGDS in the future. Further mechanistic experiments will reveal the novel role of extracellular SmgGDS in the regulation of inflammation and hypertrophy in cardiac tissue.

Clinical Implications
It is widely known that statins exert beneficial effects in patients with cardiovascular diseases independent of their cholesterol-lowering effects.2,55,56 This study demonstrates the novel mechanism of the pleiotropic effects of statins with intracellular and extracellular SmgGDS as a key molecular switch. Thus, it is conceivable that the beneficial effects of
statins could be mediated not only by their cholesterol-lowering and anti-inflammatory effects but also their paracrine effects by secreted SmgGDS. Importantly, SmgGDS secretion from CFs was increased by low concentration of atorvastatin. These results indicate that intracellular and extracellular SmgGDS are clinically effective in inhibition of cardiovascular disease. The novel aspects of the pleiotropic effects of statins remain to be examined in future clinical studies, which will provide clues to develop novel therapeutic strategies in patients with cardiovascular diseases.

In conclusions, we were able to demonstrate that SmgGDS plays a crucial role in the inhibitory effects of statins on Ang II–induced cardiac hypertrophy and fibrosis in mice in vivo. SmgGDS could be a novel mediator of the pleiotropic effects of statins as an intracellular molecular switch and an extracellular circulating player in our body.

Perspectives
The pleiotropic effects of statins may be exerted by SmgGDS, which is one of the guanine nucleotide exchange factors. We have elucidated that SmgGDS attenuates hypertrophic/fibrotic signals such as Rac1, ERK1/2, and Rho kinase in mice. Furthermore, SmgGDS was secreted by statins, which may affect surrounding cells extracellularly. All these intracellular and extracellular effects by SmgGDS may contribute to the pleiotropic effects of statins. We need further studies for the mechanisms of the statin-mediated secretion of SmgGDS and its role for the inhibition of Ang II signaling. Further analyses in human will establish a concept on the role of SmgGDS in statin treatment, which is useful for the development of novel therapeutic strategy for treatment of cardiac hypertrophy and fibrosis.

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Disclosures
None.

References


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SmgGDS and Cardioprotective Effects of Statins

What Is New?

- SmgGDS plays a central role for the inhibitory effects of statins on cardiac hypertrophy and fibrosis induced by angiotensin II.
- These effects are achieved through inhibition of Rac1, extracellular signal–regulated kinases 1/2, and Rho-kinase pathway.
- Both intracellular and extracellular SmgGDS work for cardioprotective effects.

What Is Relevant?

- Angiotensin II promotes hypertension and oxidative stress, which promotes hypertensive heart disease and left ventricle diastolic disorder.
- SmgGDS could be a therapeutic target of hypertensive heart disease through statin’s pleiotropic effects.

Summary

SmgGDS is an important molecular switch that mediates the pleiotropic effects of statins through its intracellular and extracellular effects.

Novelty and Significance


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Supplemental Material

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- Novel Mechanism of the Pleiotropic Effects of Statins -

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Supplemental Figures
Figure Legends
Legends to Supplemental Figures

Figure S1. Blood Pressure Monitoring of Mice
Telemetry blood pressure monitoring was performed in wild-type (WT) and SmgGDS+/– mice for 11 days. Representative monitoring is shown. Both mice were implanted with osmotic pump at day 6, and subcutaneously infused with AngII (2.0 mg/kg/day).

Figure S2. Fibrosis of Heart in Pressure Overload Model Mice
A. Representative sections of Masson’s trichrome staining of heart tissues from WT and SmgGDS+/– mice operated transverse aortic constriction (TAC) and treated with or without atorvastatin. Scale bars, 100 μm. B. Fibrosis area in heart from WT and SmgGDS+/– mice operated TAC and treated with or without atorvastatin. C, D. Change ratio of fibrosis area in heart by atorvastatin in (C) TAC-operated and (D) AngII-infused WT and SmgGDS+/– mice. Results are expressed as mean ± SEM. TAC; transverse aortic constriction. ATOR; atorvastatin. *P<0.05.

Figure S3. Expression of SmgGDS in Thrombospondin 1-Positive Cardiac Fibroblasts
Representative confocal image slicing of the heart from (A) WT and (B) SmgGDS+/– mice treated with AngII (2.0 mg/kg/day) for 1 week. SmgGDS (Alexa Flour-488, green), thrombospondin 1 (TSP-1, Alexa Fluor-563, red), DAPI (nucleus, blue). Scale Bar=50 μm.

Figure S4. Short-term Activation of ERK1/2 and Rho-kinase in Cardiac Fibroblasts
Western blot analyses of ERK1/2 activity (phosphorylated/total-ERK1/2) and Rho-kinase activity (phosphorylated/total-MYPT) in wild-type (WT) and SmgGDS+/– CFs treated with AngII (1 μmol/L) with or without atorvastatin (ATOR, 10 μmol/L) for 1 hours. Results are expressed as mean ± SEM (n = 3). *P<0.05.

Figure S5. Increased AT1R mRNA in SmgGDS+/– CFs
A. Relative mRNA expressions of Angiotensin II Type I Receptor (AT1R) in WT and SmgGDS+/– CFs treated with AngII with or without atorvastatin for 24 hours. B. Relative mRNA expressions of AT1R in left ventricular free wall from WT and SmgGDS+/– mice. Results are adjusted by GAPDH. Results are expressed as mean ± SEM (n = 3). *P<0.05.
Figure S6. **SmgGDS Deletion Does Not Affect Tiam-1 expression**
Western blot analyses of expression of Tiam-1, one of the GEF which downregulates Rac1 activity in WT and SmgGDS+/– CFs treated with AngII (1 µmol/L) with or without atorvastatin (ATOR, 10 µmol/L) for 24 hours. Results are expressed as mean ± SEM (n = 3). *P<0.05.

Figure S7. **The Profiles of Cytokines/Chemokines and Growth Factors in Conditioned Medium from Cardiac Fibroblasts**
Quantitative analysis of cytokine levels in conditioned medium from CFs stimulated by AngII (1 µmol/L) with or without atorvastatin (ATOR, 10 µmol/L) for 24 hours. Results are expressed as mean ± SEM (n = 4 in each group). N.D.; not detected. *P<0.05. #P<0.05 vs. CM from WT CFs in each treatment group.

Figure S8. **The effects of SmgGDS Knock-down by siRNA for Rac1 Expression and ERK Activation**
Western blot analyses of SmgGDS expression, Rac1 expression, and ERK1/2 activity (phosphorylated/total-ERK1/2) in WT CFs. CFs were transfected with mock-siRNA or SmgGDS-siRNA for 72 hours, then treated with or without GST-tagged recombinant SmgGDS protein (GST-SmgGDS, 1µg/ml) for 24 hours. Results are expressed as mean ± SEM (n = 3). *P<0.05.

Figure S9. **Intracellular and Extracellular SmgGDS in Endothelial cells and Cardiac Myocytes**
Western blot analysis of conditioned medium (CM) and total cell lysates (TCL) prepared from (A) human umbilical endothelial cells (HUVECs) and (B) neonatal rat cardiomyocytes (NRCMs) stimulated by AngII (1 µmol/L) with or without atorvastatin (ATOR, 10 µmol/L) for 24 h (n = 3 in each group). Extracellular SmgGDS was not detected in the conditioned medium from NRCMs. Results are expressed as mean ± SEM. ATOR; atorvastatin. TCL; total cell lysates. CM; conditioned medium. *P<0.05.

Figure S10. **AT1R protein expression in SmgGDS+/– CFs**
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