Statins up-regulate SmgGDS through β1-integrin/Akt1 pathway in endothelial cells

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1. Introduction

HMG-CoA reductase inhibitors (statins) are potent cholesterol-lowering drugs widely used in clinical practice for primary and secondary prevention of coronary artery disease.1,2 Furthermore, the beneficial cardiovascular effects of statins, beyond their cholesterol-lowering action, the so-called pleiotropic effects, have attracted much attention.3,4 The pleiotropic effects of statins could be mediated by reduced synthesis of isoprenoids that are responsible for the post-translational modulation of intracellular proteins.1 Since membrane localization of and GTPase activity of small GTP-binding proteins (e.g. Rho, Rac, and Ras) are dependent on isoprenylation, the pleiotropic effects of statins have been considered to be mediated by inhibition of those small GTP-binding proteins.5,6

However, we have previously demonstrated that regular-doses of statins (atorvastatin and pravastatin, 20 mg/day for 1 week) significantly inhibit Rac1 in animals and humans.7 Rac1 plays a crucial role in generating reactive oxygen species (ROS) and is an important mediator of cardiovascular hypertrophy.8 Simvastatin inhibits Rac1-mediated ROS production in the heart and vascular smooth muscle cells (VSMC) in mouse models of cardiovascular hypertrophy induced by angiotensin II (AngII) or pressure overload.9,10 In addition, simvastatin inhibits AngII-induced hypertension and hydrogen peroxide production in the aorta in rats.11 and atorvastatin inhibits Rac1-mediated ROS production in the aorta of spontaneously hypertensive rats.12 These findings are further supported by the analysis of failing human heart tissues where increased ROS generation is associated with increased Rac1 activity, both of which are attenuated by statins.13 We have recently demonstrated that the cardiovascular protective effects of statins are partly mediated by small GTP-binding protein dissociation stimulator (SmgGDS) with a resultant Rac1 degradation and reduced oxidative stress.14 However, it remains to be elucidated what molecular

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mechanisms are involved. Since the SmgGDS-mediated beneficial effects of statins represents the third mechanism of action of statins, in addition to their inhibition of cholesterol synthesis in the liver and that of small GTP-binding proteins, it is important to characterize the novel SmgGDS-mediated beneficial effects of statins.

β1-Integrin plays an important role in angiogenesis through several cellular responses. β1-Integrin is a direct target of miR-223 as its down-regulation by miR-223 causes VEGF- and basic fibroblast growth factor-induced angiogenesis through phosphorylation of Akt and that activation of β1-integrin conversely enhances Akt phosphorylation. In the present study, we thus aimed to elucidate the molecular mechanisms of SmgGDS up-regulation by statins with a special reference to the β1-integrin/Akt pathway in endothelial cells.

2. Methods

2.1 Animal study

We conducted all mouse experiments in accordance with experimental protocols that were approved by the Animal Care and Use Committee of the Tohoku University Graduate School of Medicine (2013-461), which was granted by the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health. C57/BL6 mice were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). C57/BL6 mice were administrated either atorvastatin (10 mg/kg per day), pravastatin (50 mg/kg per day), or placebo by gavage every day for 1 week. After the 1-week treatment, the animals were anaesthetized with isoflurane and perfused with ice-cold PBS. Immediately after dissection, the aorta, heart, liver, and skeletal muscles were frozen by liquid nitric oxide. The frozen tissues were homogenized in CelLytic™ MT cell lysis reagent (Sigma, C3228) by Precellys 24 beads homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). The homogenates were centrifuged and the supernatants were used for western blot analysis.

2.2 Cell culture and drug treatment

Human umbilical venous endothelial cells (HUVEC) and human aortic endothelial cells (HAoEC) (Takara Bio, Inc., Otsu, Japan) were incubated in smooth muscle cell growth media (Takara Bio, Inc., Otsu, Japan) and human aortic smooth muscle cells (HAoSMC) (Takara Bio, Inc., Otsu, Japan) were incubated in smooth muscle cell growth medium (SmGM-2, Lonza) under standard conditions. H9c2 cells, neonatal rat cardiac cells (European Collection of Cell Cultures, Salisbury, UK) were incubated in DMEM supplemented with 10% fetal bovine serum (FBS) and 2 mmol/L glutamine under the standard conditions. H9c2 cells were induced to differentiate towards the cardiac-like phenotype as described previously. Differentiation was induced by culturing myoblasts in DMEM supplemented with 1% FBS and 10 mmol/L all-trans-retinoic acid (RA) (Sigma) with the medium being replaced every 2 days. HepG2 human liver carcinoma cells (Cellular Engineering Technologies, Inc., IA, USA) were incubated in Hepatocellular Carcinoma Expansion Media (Cellular Engineering Technologies, Inc.) supplemented with 10% FBS under the standard conditions. C2C12 mouse myoblast cell line (European Collection of Cell Cultures) were incubated in DMEM supplemented with 10% fetal bovine serum and 4.5 g/L glucose, and differentiated in DMEM containing 2% horse serum under the standard conditions. Before treatment with atorvastatin, the cells were starved in serum-free media for 24 h, and were then treated with different concentrations of atorvastatin (10 and 30 µmol/L) (Pfizer) for 24 h. After the treatment with atorvastatin, the cells were washed twice with ice-cold PBS and sonicated after adding lysis buffer. The lysates were centrifuged and the supernatants were used for western blot analysis. Cell used in the experiments was the same batch at different passage (passage 4–7).

2.3 Transfection of HUVEC with siRNA

Multiple siRNA duplexes for Akt1, Akt2, Akt3, GSK-3β, β1-integrin, VEGF-R2, and caveolin-1 were purchased from Qiagen (Hilden, Germany). A functional non-targeting siRNA that was bioinformatically designed by Qiagen was used as a mock control. HUVEC were transfected with HiPerfect Transfection Reagent (Qiagen) with either 10 nmol/L mock control siRNA or 10 nmol/L siRNA specific for target proteins as described previously. After 72-h post-transfection, the cells were analysed by western blot. In the case that HUVEC transfected siRNA was treated with atorvastatin, atorvastatin was treated in last 24 h.

2.4 Western blot analysis

To quantify the expression levels of several proteins in cultured cells and mouse tissues, the same amount of protein sample was separated by SDS–PAGE and transferred to PVDF membranes (GE Healthcare, WI, USA). The membranes were immunoblotted with the primary antibodies, including anti-SmgGDS (BD transduction Lab., CA, USA), anti-phospho-Akt (Ser473) (Cell Signaling), anti-Akt (pan) (Cell Signaling), anti-GSK-3α/β (Cell Signaling), anti-phospho-GSK-3β (Ser9) (Cell Signaling), anti-Akt1 (Cell Signaling), anti-Akt2 (Cell Signaling), anti-Akt3 (Cell Signaling), anti-β1-integrin (Cell Signaling), anti-VEGF-R2 (Cell Signaling), anti-caveolin-1 (Cell Signaling), anti-β-actin (Abcam), and anti-a-tubulin (Sigma). After incubating with horseradish-peroxidase-conjugated rabbit anti-mouse, goat anti-rabbit antibody, blots were visualized by the enhanced chemiluminescence system (ECL Western Blotting Detection Kit, GE Healthcare). Densitometric analysis was performed by Image J (NIH) software.

2.5 Statistical analysis

Results are expressed as mean ± S.E.M. for all studies. Comparisons of means between two groups were performed by the unpaired Student’s t-test. A two-way ANOVA was performed with Tukey’s HSD (honestly significant difference) post hoc test for the data contained two variables (Figures 5 and 6) and see Supplementary material online, Figure S2. All other experiments were analysed with a one-way ANOVA with Dunnett’s post hoc test. Statistical significance was evaluated with JMP 8 (SAS Institute). All reported P-values are two-tailed, with a P-value of <0.05 indicating statistical significance.

3. Results

3.1 Statins up-regulate SmgGDS in endothelial cells

To examine the up-regulation of SmgGDS in vivo, we first examined the effects of oral treatment with statins, atorvastatin (10 mg/kg per day), and pravastatin (50 mg/kg per day) for 1 week, in mice. The statins significantly increased SmgGDS protein expression especially in the aorta (Figure 1A–D). In addition, IF staining demonstrated that the expression of SmgGDS was increased in aortic endothelium in response to a lower dose of atorvastatin (Figure 2A). Also, western blotting demonstrated that the lower dose of statin significantly increases SmgGDS expression in the aortic tissue (Figure 2B). Then, to separate the effects of statins on the endothelium and VSMC in blood vessels, we examined the effects of atorvastatin on SmgGDS expression in cultured human umbilical endothelial cells (HUVEC), human aortic endothelial cells (HAoEC) and human aortic smooth muscle cells (HAoVSMC) in vitro. Atorvastatin significantly increased SmgGDS expression in a concentration-dependent manner in HUVEC (Figure 3A) and in HAoEC (Figure 3C) but not in HAoVSMC (Figure 3B). Interestingly, atorvastatin did not have effects in cultured cardiomyocytes (differentiated H9c2 cells).
hepatocytes (HepG2 cells) or skeletal muscle cells (differentiated C2C12 cells) \textit{in vitro} (see Supplementary material online, Figure S1). Furthermore, IF staining demonstrated that atorvastatin increased the expression of SmgGDS especially in hepatic sinusoidal endothelial cells (see Supplementary material online, Figure S2), but not in skeletal muscle cells (see Supplementary material online, Figure S3). In addition, western blotting demonstrated that atorvastatin did not up-regulate SmgGDS protein expression in the mouse aortic tissue after removal of the endothelium (see Supplementary material online, Figure S4). These results indicate that statins up-regulate SmgGDS in endothelial cells.

### 3.2 Atorvastatin induces phosphorylation of Akt in endothelial cells

Next, we examined the molecular mechanisms for the up-regulation of SmgGDS by atorvastatin. In the previous study, we showed that SmgGDS up-regulation by statins is inhibited by PI3K or Akt inhibitors, and is enhanced by GSK-3β selective inhibitor, whereas we did not observe GSK-3β phosphorylation by atorvastatin.\textsuperscript{14} Accordingly, we examined whether atorvastatin enhances Akt phosphorylation in HUVEC and HAOVSMC. As expected, Akt phosphorylation was transiently increased by atorvastatin at 10 min in HUVEC but not in HAOVSMC (Figure 4A and B). In the present study, KLF2 expression was significantly increased in HUVECs by statin treatment (see Supplementary material online, Figure S5). Thus, when we consider the endothelial-specific roles of KLF2, statin-mediated KLF2 up-regulation in ECs may involve the mechanism that delineates endothelial cells and VSMCs in response to statins. However, further mechanistic experiment is indispensable for the clarification. In contrast, atorvastatin did not enhance GSK-3β phosphorylation in HUVEC (see Supplementary material online, Figure S6). Moreover, inhibition of GSK-3β by siRNA had no effects on SmgGDS expression (see Supplementary material online).
Importantly, atorvastatin did not increase SmgGDS mRNA expression (see Supplementary material online, Figure S8). These results suggest that the atorvastatin-mediated increase in the SmgGDS expression is partly due to the post-translational modification.

### 3.3 Akt1 mediates atorvastatin-induced SmgGDS expression

We next examined which Akt isoform is responsible for the SmgGDS induction by atorvastatin among the three isoforms. Akt genes are widely expressed in various tissues, but Akt1 is mostly abundant in the brain, heart, and lung, Akt2 in the skeletal muscle, and Akt3 in the brain and kidney. The experiments with three siRNAs to each isoform of Akt showed that Akt1, but not Akt2 or Akt3, mediates the effects of atorvastatin to up-regulate SmgGDS (Figure 5). Furthermore, treatment with Akt activator (SC79) significantly up-regulated SmgGDS, indicating the important role of Akt in the SmgGDS expression (see Supplementary material online, Figure S9).

### 3.4 β1-Integrin mediates the atorvastatin-induced increase of SmgGDS

Finally, we examined the upstream mediator(s) of Akt1 for up-regulation of SmgGDS by atorvastatin. It was reported that hydrophilic
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stain, pravastatin, is incorporated into human hepatocytes but not into HUVEC, whereas lipophilic stain, simvastatin, is incorporated into human hepatocytes and HUVEC. Since SmgGDS expression is increased by both types of stains (Figure 1A), cell membrane proteins could be important for up-regulation of SmgGDS. Here, we focused on three cell membrane proteins, including β1-integrin, VEGF receptor-2 (VEGF-R2), and caveolin-1, all of which have been reported to associate with Akt in endothelial cells. Importantly, siRNA to β1-integrin abolished the effects of atorvastatin to up-regulate SmgGDS (Figure 6A) and β1-integrin siRNA reduced Akt phosphorylation (see Supplementary material online, Figure S10), indicating that atorvastatin increases SmgGDS expression partly through β1-integrin signalling. Although VEGF-R2 expression was also increased by atorvastatin, siRNA to VEGF-R2 had no inhibitory effects on the up-regulation of SmgGDS by atorvastatin (Figure 6B). Since we previously demonstrated that VEGF is up-regulated by statins, these

Figure 3 Atorvastatin up-regulates SmgGDS in endothelial cells but not in HAoVSMC in vitro. Atorvastatin (ATOR) concentration-dependently up-regulated SmgGDS protein expression in HUVEC (A) but not in HAoVSMC (B) after 24-h treatment (n = 3 each). Results are expressed as mean ± SEM. (C) In the HAoEC, SmgGDS was up-regulated after stimulation with atorvastatin (n = 3 each). Results are expressed as mean ± SEM.

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results suggest that the VEGF/VEGF-R2 pathway is parallel to the SmgGDS up-regulation by atorvastatin without significant interactions. Finally, siRNA to caveolin-1 had no inhibitory effects on the SmgGDS up-regulation by atorvastatin (Figure 6C).

4. Discussion

To the best of our knowledge, the present study provides the first direct evidence that statins increase intracellular SmgGDS in the endothelium through the β1-integrin/Akt1 pathway (Figure 7), demonstrating the molecular mechanisms of the third mechanism of cardiovascular protective effects of statins,14 in addition to their inhibitory effects on cholesterol synthesis in the liver and on small GTP-binding proteins.1–6

4.1 Mechanisms for the increase of intracellular SmgGDS by statins

The present study demonstrates that multiple processes may be involved in the up-regulation of SmgGDS by statins. Statins activate β1-integrin with a resultant phosphorylation and activation of Akt1, which increases intracellular SmgGDS possibly through post-translational pathway (Figure 7). As we have previously reported, increased intracellular SmgGDS facilitates Rac1 degradation and reduces ROS production, exerting cardiovascular protective effects, independent of cholesterol levels, which are the pleiotropic effects of statins.14 In contrast to their inhibitory effects on cholesterol synthesis in the liver and on small GTP-binding proteins.

4.2 Role of the β1-integrin/Akt pathway in the statin-mediated SmgGDS up-regulation

In the present study, we showed that Akt1 is important for up-regulation of SmgGDS expression by atorvastatin. Akt plays a crucial role for vascular homeostasis and angiogenesis,23 and regulates many aspects of cellular function, including cell survival,15 glucose metabolism,33,34 cell cycle,35,36 and protein synthesis.37,38 In endothelial cells, the three caveolin isoforms in mammalian cells are 22–24 kDa integral membrane proteins; caveolin-1,29 and caveolin-3,30 as the upstream candidate mediator(s) of Akt1 for up-regulation of SmgGDS by atorvastatin. Furthermore, down-regulation of GSK-3β by its siRNAs did not influence SmgGDS expression, suggesting that GSK-3β does not modulate SmgGDS expression. Since mRNA expression of SmgGDS was not increased by atorvastatin, it is highly possible that SmgGDS expression is regulated by post-translational modifications. However, it remains to be examined whether or not intracellular SmgGDS levels are directly modulated by Akt. Further studies are needed to elucidate the mechanisms for statin-mediated increase of intracellular SmgGDS.

In the present study, we examined three cell membrane proteins, including β1-integrin,17,18 VEGF-R2,29 and caveolin-1,30 as the upstream candidate mediator(s) of Akt1 for up-regulation of SmgGDS by atorvastatin. β1-Integrin plays important roles in cell adhesion, migration, survival, angiogenesis,15 and establishment of endothelial cell polarity and lumen formation.16 It was previously demonstrated that VEGF-induced phosphorylation of VEGF-R2 and Akt in endothelial cells17 and that activation of β1-integrin by an activating β1-integrin antibody enhances Akt phosphorylation.18 VEGF-R2 is one of the main receptors of VEGF in endothelial cells and VEGF exhibits multiple biological activities in endothelial cells, including enhancement of endothelial cell survival.23,39 The effects of on cell survival have been shown to be mediated through VEGF-R2/Pi3K/Akt pathway.23,29,40 Caveolin is a component protein of caveolae that are specialized plasmalemmal microdomains.30 The three caveolin isoforms in mammalian cells are 22–24 kDa integral membrane proteins; caveolin-1...
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4.3 Endothelium-specific up-regulation of SmgGDS by statins

In the present study, we showed that atorvastatin up-regulates SmgGDS protein expression in endothelial cells, but not in the other tissues and cells (Figures 1, 2 and 3, and also see Supplementary material online, Figures S1, S2, S3 and S4). We focused on Kruppel-like factor 2 (KLF2) to explain the specificity for endothelial cells because previous reports had shown EC-specific relationship between KLF2 and statins. It was reported that KLF2 is a transcriptional regulator of statin-mediated effects in ECs and that statin-mediated up-regulations of eNOS and thrombomodulin require KLF2. It was previously shown that knockdown of caveolin-1 by its antisense oligonucleotides prevented mechanosensitive Akt phosphorylation in VSMC, whereas in endothelial cells, Akt phosphorylation is increased by knockdown of caveolin-1 by its siRNA. In the present study, up-regulation of SmgGDS by atorvastatin was inhibited by β1-integrin siRNA, but was not influenced by VEGF-R2 or caveolin-1 siRNA. In addition, basal SmgGDS expression was not influenced by β1-integrin siRNA, while β1-integrin expression was unaltered by atorvastatin. Furthermore, in the present and previous studies, we demonstrated that VEGF-R2 and VEGF are up-regulated by statins. These findings suggest that statin-induced SmgGDS up-regulation is mainly through the β1-integrin/Akt1 pathway but also could be through VEGF/VEGF-R2/Akt pathway mediated by β1-integrin. Thus, the present study identifies β1-integrin/Akt1 signalling as a novel therapeutic target for the cardiovascular protective effects of statins (Figure 7). To further confirm this notion, further studies with β1-integrin-deficient mice and Akt1-deficient mice are needed.
A close link between KLF2 and heme oxygenase-1 (HO-1) in human vascular ECs, demonstrating that atorvastatin-mediated up-regulation of HO-1 with a resultant antioxidant effect is KLF2-dependent. Finally, it was further demonstrated that statin has a strong anti-inflammatory effect on human peripheral blood monocytes, including up-regulation of the anti-atherogenic factor KLF-2. Importantly, it has also been demonstrated that the expression of HuR in ECs is regulated by shear stress and statin treatment and HuR regulated other stress-sensitive genes, including KLF2, endothelial nitric oxide synthase (eNOS), and BMP 4. Indeed, in the present study, KLF2 expression was significantly increased in HUVECs by statin treatment (see Supplementary material online, Figure S5). Thus, when we consider the endothelial-specific roles of KLF2, statin-mediated KLF2 up-regulation in ECs may also be involved in the mechanisms that delineates ECs and other cell types in response to statins. However, further mechanistic experiment is indispensable for the clarification.

4.4 Study limitations
Several limitations should be mentioned for the present study. First, although we examined SmgGDS expression in the aorta, heart, liver, and skeletal muscles in the present study, we did not examine that in other tissues. Second, in the present study, we examined only the effects of statins in normal mice. Thus, it remains to be examined whether the present findings could be observed in disease conditions, such as hypertension and diabetes mellitus. Third, the obvious limitation of the present study is that the present findings need to be confirmed in humans. Fourth, the doses of statins used in the present in vivo study were higher than clinical doses in humans. Fifth, the present results do not explain the mechanism by which statins interact with β1-integrin. Finally, the present results do not explain how phosphorylated Akt mediates the post-translational modification. Further mechanistic experiments are necessary to demonstrate the mechanisms by which statins up-regulate SmgGDS through the β1-integrin/Akt1 pathway.

Figure 6 Atorvastatin up-regulates SmgGDS through β1-integrin in HUVEC. Western blot analysis of total cell lysate of HUVEC after 72-h treatment with mock or β1-integrin (A), VEGF-R2 (B), or caveolin-1 (C) siRNAs in HUVEC (n = 3 each). Atorvastatin (10 μmol/L) was added during the last 24 h. (A) siRNA to β1-integrin abolished the effects of atorvastatin to up-regulate SmgGDS in HUVEC. (B) Although VEGF-R2 expression was also increased by atorvastatin, siRNA to VEGF-R2 had no inhibitory effects on the up-regulation of SmgGDS by atorvastatin. (C) siRNA to caveolin-1 had no inhibitory effects on the SmgGDS up-regulation by atorvastatin. Results are expressed as mean ± SEM.
4.5 Perspectives

It has been clearly demonstrated the potential interactions with nitric oxide (NO) production and availability, which may be a key beneficial pleiotropic action of statins. Notably, Akt is a known activator of eNOS, and elevated ROS combine with NO to form damaging peroxynitrite and reducing bioavailability of NO. Next, we have recently demonstrated with SmgGDS-deficient mice that the cardioprotective effects of statins are partly mediated by SmgGDS up-regulation. Thus, it is possible that SmgGDS up-regulation could be a new therapeutic target for cardiovascular diseases. In the present report, we demonstrated that \( \beta_1 \)-integrin/Akt pathway has a crucial role in up-regulation of SmgGDS by statins. In addition, it was previously reported that an activating \( \beta_1 \)-integrin antibody enhances Akt phosphorylation. These results suggest that \( \beta_1 \)-integrin could be a pharmacological target for SmgGDS up-regulation. Integrins exert transducer functions for mechanical stress in endothelial cells, and \( \beta_1 \)-integrin plays an important role in VEGF-R2 activation by mechanical stress. We have developed non-invasive angiogenic therapies with shock wave and ultrasound, in which VEGF expression is up-regulated by the mechanical stimuli. In addition, integrins mediate the mechano-transduction pathway of low-intensity pulsed ultrasound in osteoblasts and chondrocytes. Thus, it is conceivable that both pharmacological and mechanical stimulation of \( \beta_1 \)-integrin could be a new therapeutic strategy for cardiovascular diseases with a special reference to SmgGDS.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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