Inhibition of Rab1 GTPase and Endoplasmic Reticulum-to-Golgi Trafficking Underlies Statin’s Toxicity in Rat Skeletal Myofibers

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ABSTRACT

HMG-CoA reductase inhibitor statins are used for the treatment of hypercholesterolemia. However, statins have adverse effects on skeletal muscles with unknown mechanism. We have reported previously that fluvastatin induced vacuolation and cell death in rat skeletal myofibers by depleting geranylgeranylpyrophosphate (GGPP) and suppressing small GTPases, particularly Rab (FASEB J 21:4087–4094, 2007). Rab1 is one of the most susceptible Rab isoforms to GGPP depletion and is essential for endoplasmic reticulum (ER)-to-Golgi trafficking. Here, we explored whether Rab1 and ER-to-Golgi vesicle trafficking were affected by statins in cultured single myofibers isolated from flexor digitorum brevis muscles of adult rats. Western blot analysis revealed that Rab1A protein resided predominantly in membrane but not in cytosol in control myofibers, whereas it was opposite in fluvastatin-treated myofibers, indicating that fluvastatin inhibited Rab1A translocation from cytosol to membrane. GGPP supplementation prevented the effect of fluvastatin on Rab1A translocation. Brefeldin A, a specific suppressor of ER-to-Golgi trafficking, induced vacuolation and cell death in myofibers in a manner similar to that of fluvastatin. Although ER-to-Golgi traffic suppression induces unfolded protein response (UPR) and cell death in some cell types, neither fluvastatin nor brefeldin A up-regulated UPR in myofibers. Immunofluorescence study revealed that the distribution of an ER marker, calnexin, was restricted to the region around nucleus with fluvastatin, suggesting the inhibition of ER membrane traffic by fluvastatin. We conclude that suppression of Rab1 GTPase and the subsequent inhibition of ER-to-Golgi traffic are involved in statin-induced skeletal myotoxicity.

Introduction

Statins, or HMG-CoA reductase inhibitors, are commonly prescribed drugs for hypercholesterolemia (Endo, 1992). However, statins have adverse effects on skeletal muscles, ranging from slight myalgia to severe rhabdomyolysis (Arora et al., 2006). Among skeletal muscles, statins selectively injure fast-twitch glycolytic type II skeletal muscles (Waclawik et al., 1993; Westwood et al., 2005). Vacuolation of myofibers is a typical morphological feature of statin-induced myotoxicity (Waclawik et al., 1993; Westwood et al., 2005). Because millions of people in the world are taking statins every day (Dobson, 2008), it is an urgent task to elucidate the mechanism of the adverse effect of statins for its prevention and treatment.

A primary target of statins is an inhibition of mevalonate synthesis. Mevalonate is a precursor not only for cholesterol but also for isoprenoids such as geranylgeranylpyrophosphate (GGPP) or farnesylpyrophosphate (FPP). Prenylation with GGPP or FPP is essential for activation of small GTPases, including Rab, Ras, Rac, Raf, and Rho families (Zhang and Casey, 1996). By suppression of mevalonate synthesis, statins could deplete GGPP or FPP to the extent of preventing small GTPases from prenylation. Accumulating evidence suggests that depletion of isoprenoids, particularly GGPP, is critical for statin-induced myopathy (Flint et al., 1997; Sakamoto et al., 2007), whereas depletions of chole-

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ABBREVIATIONS: GGPP, geranylgeranylpyrophosphate; Arf, ADP ribosylation factor; BFA, brefeldin A; CBB, Coomassie Brilliant blue; ER, endoplasmic reticulum; Flv, fluvastatin; FPP, farnesylpyrophosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GRP, glucose-regulated protein; Oatp, organic anion transporting polypeptide; PBS, phosphate-buffered saline; Tum, tunicamycin; UPR, unfolded protein response.
terol and ubiquinone are not responsible (Mullen et al., 2010).

Intracellular vesicle trafficking is essential for cell survival. When proteins are newly synthesized in endoplasmic reticulum (ER), they are packed in small vesicles and transported to Golgi apparatus via the ER-Golgi intermediate compartment (Marie et al., 2008). The proteins are matured in Golgi apparatus and redistributed to their destinations in the cell (Emr et al., 2009). Rab GTPases regulate intracellular vesicle trafficking events, such as vesicle budding, delivery, tethering, and fusion. More than 60 isoforms of Rab GTPases have been found in mammalian cells. Each Rab isoform has distinct target membranes (reviewed in Stenmark, 2009). Although it is not clear how it could determine specific membrane targeting, GGPP has a role in hydrophobicity and membrane association of Rab.

We found that statins, including fluvastatin (Flv), pravastatin, and simvastatin, induce vacuolation and cell death in primary cultured rat skeletal myofibers by inhibiting Rab GTPases (Sakamoto et al., 2007). However, it is unknown which Rab isoforms and vesicle trafficking pathways are affected by statins. In this study, we focused on Rab1 and ER-to-Golgi trafficking, because Rab1 is responsible for ER-to-Golgi trafficking, which is the most fundamental intracellular membrane trafficking system (Stenmark, 2009). Furthermore, Rab1B is the most susceptible to GGPP depletion among Rab subfamily investigated with a low concentration (200 nM) of simvastatin in mouse N2a neuroblastoma cells (Ostrowski et al., 2007). Rab1 has two isoforms, Rab1A and Rab1B. Whereas Rab1A is dominant in human skeletal muscles (Bao et al., 1998), both Rab1A and Rab1B are expressed in rat skeletal muscles with 92% amino acid sequence similarity (Vieth et al., 1989). In this study, we analyzed the effects of fluvastatin on Rab1A distribution and ER-to-Golgi trafficking in rat myofibers.

Materials and Methods

Isolation of Myofibers. All the protocols used met the National Institutes of Health animal experiment guidelines and were approved by the Animal Research Committee of Fukushima Medical University. The isolation of single skeletal myofibers was performed as described previously (Sakamoto et al., 2007, 2008; Tanaka et al., 2010) with some modifications. Male Wistar rats (8–16 weeks old) were anesthetized with ether and exsanguinated. The total number of rats used in this study was 16. The body weight of the rats ranged from 160 to 210 g. For anesthesia, an airtight glass container (2 liters in volume) was filled with evaporated ether (from approximately 5 ml of liquid ether absorbed in cotton), in which a rat was placed. Flexor digitorum brevis muscles were extracted from both soles and the connective tissue was removed from the muscles with scissors under a binocular microscope (Nikon, Tokyo, Japan). Flexor digitorum brevis muscles were cleaned and incubated at 37°C for 2.5 to 3.0 h in Ringer’s solution (2.7 mM KCl, 1.2 mM KH₂PO₄, 0.5 mM MgCl₂, 137 mM NaCl, 8.1 mM NaHCO₃, 1 mM CaCl₂, 10 mM glucose) containing 0.3% collagenase (Wako Pure Chemicals, Tokyo, Japan) and 0.001% penicillin-G (Banyu Pharmaceutical, Tokyo, Japan) and 0.001% streptomycin (Meiji Seika, Tokyo, Japan). After incubation, the muscles were washed three times with Ringer’s solution, and the fibers were dispersed in Dulbecco’s modified essential medium containing 10% fetal bovine serum, 0.001% penicillin-G, 0.001% streptomycin, and 10 μM cytarabine.

Culture of Myofibers. Cultured myofibers were separated from fibroblasts by the following way. To minimize the number of fibroblasts, which would otherwise eventually overgrow myofibers during culture (Sakamoto et al., 2007, 2009), the preparation was allowed to settle for 10 min. The sediments were transferred to a 60-mm dish and cultured overnight. By then, almost all fibroblasts had adhered to the bottom of the dish, while myofibers were still floating. After gentle agitation, supernatants were harvested and cultured in Dulbecco’s modified essential medium containing 10% fetal bovine serum, 0.001% penicillin-G, 0.001% streptomycin, and 10 μM cytarabine.

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Analysis. Each experiment was repeated three to 10 times. The data were expressed as the means ± S.E.M. Statistical significance between two groups or among multiple groups was evaluated using Student’s t test, Scheffé’s test after the F-test, or one-way analysis of variance.

Results

Effect of Fluvastatin on Rab1A Protein Distribution in Cultured Myofibers. First, we examined whether fluvastatin inhibited Rab1A activation in cultured skeletal myofibers. To do so, we analyzed the distribution of Rab1A protein between membrane and cytosol of rat myofibers. It was expected that an active form of Rab1A was in the membrane, whereas an inactive form was in the cytosol. After incubation with fluvastatin (1 μM) for 4 days, myofibers were ultracentrifuged and fractionated into membrane and cytosol. Each fraction was subjected to the Western blot by using antibody for Rab1A. In control myofibers, Rab1A was abundant in membrane (Fig. 1, A and C), but scarce in cytosol (Fig. 1, B and D). In contrast, in Flv-treated myofibers, Rab1A signal was weak in membrane (Fig. 1, A and C), but robust in cytosol (Fig. 1, B and D). Supplementation of GGPP, but not FPP, reversed the effect of fluvastatin (Fig. 1).

The effect of fluvastatin was time-dependent. Even on day 1, Rab1A was recognizable in the cytosol of fluvastatin-treated myofibers (Fig. 2A). With fluvastatin, the cytosolic fraction of Rab1A progressively increased, whereas the membrane fraction of Rab1A progressively decreased and the significant change was observed from 2 days of fluvastatin treatment (Fig. 2B). Without fluvastatin, this change was never observed (Figs. 1A and 2A). This indicated that fluvastatin inhibited translocation of Rab1A from cytosol to membrane by depleting GGPP and preventing geranylgeranylation of Rab1A. This is a direct evidence for statin-induced inhibition of membrane translocation of Rab1 in skeletal myofibers.

Effect of Brefeldin A on Skeletal Myofibers. Next, we attempted to knock down Rab1A expression in skeletal myofibers. However, floating cultured myofibers did not allow us to introduce small interference RNA intracellularly. Alternatively, we treated myofibers with brefeldin A, a specific inhibitor of ER-to-Golgi trafficking, which is a pathway mediated by Rab1. Brefeldin A is an agent that specifically binds and inhibits BIG (Brefeldin A-inhibited guanine nucleotide exchange protein), which is an activator of Arf1 (ADP ribosylation factor 1). Arf1 is also a small GTPase, which mediates ER-to-Golgi trafficking together with Rab1A. If brefeldin A inactivates BIG, Arf1 is inactivated, and as a result, ER-to-Golgi trafficking should be selectively inhibited.

Figure 3A compares myofibers cultured for 4 days in the absence (control; Fig. 3Aa) or presence of 1 μM fluvastatin.
Both fluvastatin (1 μM) and brefeldin A (higher than 10 μM) induced numerous vacuoles. The average number of vacuoles was increased by both drugs in a time-dependent (Fig. 3B) and concentration-dependent (Fig. 3C) manner. The EC50 values of fluvastatin and brefeldin A for inducing vacuolation were 0.3 and 18.5 μM, respectively.

On day 6 with fluvastatin or brefeldin A, both agents increased the number of trypan blue-stained myofibers, whereas the control fibers were mostly unstained. Numerous blebs were observed on the surface of myofibers treated with fluvastatin as well as brefeldin A, which were stained with trypan blue, indicating cell death (Fig. 4A, b and c). The concentration-viability relationships of the drugs were obtained on day 6 (Fig. 4B). The median lethal concentrations (LC50) of fluvastatin and brefeldin A were 0.3 and 8.6 μM, respectively (Fig. 4B). Because morphological changes induced by fluvastatin and brefeldin A were similar, we conclude that both fluvastatin and brefeldin A suppressed ER-to-Golgi trafficking and induced vacuolation and cell death in skeletal myofibers.

Effects of GGPP or FPP Supplementation on Fluvastatin- or Brefeldin A-Induced Vacuolation and Cell Death. Fluvastatin and brefeldin A induced similar morphological changes in myofibers. However, if brefeldin A could also inhibit HMG-CoA reductase, the effect should be prevented by GGPP but not by FPP in a manner similar to fluvastatin.

As we have shown previously (Sakamoto et al., 2007), fluvastatin at 10 μM induced vacuolation and cell death in myofibers after 4 and 6 days of treatment, respectively (Fig. 5). Supplementation of GGPP, but not FPP, prevented both of these effects of fluvastatin (Fig. 5). The same protocol was performed with brefeldin A instead of fluvastatin (Fig. 6). As with supplementation experiments, myofibers were pre-treated with 3 μM GGPP or FPP for 1 h before adding 30 μM brefeldin A. On day 4, the number of vacuoles induced by brefeldin A was 115.7 ± 32.6 (n = 4). Neither GGPP nor FPP suppressed brefeldin A-induced vacuolation (Fig. 6A) and cell death (Fig. 6B) in myofibers. This result indicated that molecular targets of fluvastatin and brefeldin A were distinct.

(Fig. 3Ab) or 30 μM brefeldin A (Fig. 3Ac). Both fluvastatin (1 μM) and brefeldin A (higher than 10 μM) induced numerous vacuoles. The average number of vacuoles was increased by both drugs in a time-dependent (Fig. 3B) and concentration-dependent (Fig. 3C) manner. The EC50 values of fluvastatin and brefeldin A for inducing vacuolation were 0.3 and 18.5 μM, respectively.

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and assured that the effects of brefeldin A were not caused by HMG-CoA reductase inhibition.

**Effects of Fluvastatin and Brefeldin A on ER Stress.**

Perturbations in ER homeostasis are termed “ER stress” and lead to unfolded protein response (UPR) (Kaufman, 1999). Excessive UPR induces activation of caspases and consequently apoptosis (Kim et al., 2006). Suppression of ER-to-Golgi trafficking induces UPR followed by activation of caspases in some types of cells (Carew et al., 2006; Cooper et al., 2006; Citterio et al., 2008). Therefore, we investigated in myofibers whether fluvastatin or brefeldin A induce UPR, using GRP78 up-regulation as an indicator of UPR.

Myofibers were treated for 4 days with fluvastatin (1 μM), brefeldin A (30 μM), or tunicamycin (3 μM). Tunicamycin is a well documented inducer of UPR. GRP78 expression was analyzed by Western blotting (Fig. 7, A).GRP78 expression was represented in A. C, quantified data of myofiber death under the conditions described above. ++, $p < 0.01$. Scheffe’s test was used as post hoc test.

Next, we compared the manners of cell death induced by these three drugs (Fig. 7C). After 4 days of treatment, the numbers of trypan blue-positive myofibers were 17.3 ± 1.5% in control, 85.8 ± 1.5% with fluvastatin, 78.8 ± 1.7% with brefeldin A, and 53.7 ± 2.9% with tunicamycin (Fig. 7C). There was little correlation between the expression levels of GRP78 protein and the percentage of cell death. This suggests that UPR was not a main factor of myofiber death induced by fluvastatin or brefeldin A.

**Effect of Fluvastatin on ER Marker Calnexin Distribution in Myofibers.** To justify our conclusion that fluvastatin inhibits ER-Golgi transport, we performed an experiment that actually assayed ER-Golgi trafficking. Among a number of established and standard protocols to measure whether ER-Golgi trafficking is intact in cells, we used an immunofluorescent method of calnexin as a marker of ER. Calnexin is an ER chaperone protein, which is located specifically in ER membrane and functions to maintain correct folding of glycoprotein (Ruddock and Molinari, 2006). We stained myofibers with an antibody against calnexin to highlight ER in myofibers. Figure 8A shows that calnexin is distributed diffusely throughout the cytosol of a control myofiber. In contrast, in a myofiber treated with 1 μM fluvastatin for 4 days, calnexin accumulated distinctly in the region around the nucleus (Fig. 8B). Similar micrographs were obtained in three control myofibers and four fluvastatin-treated...
myofibers. This indicates that fluvastatin inhibited ER-Golgi membrane traffic and prevented calnexin distribution throughout the cytosol, confirming that fluvastatin disturbed intracellular ER membrane trafficking in myofibers.

**Discussion**

In the present study, we demonstrated that fluvastatin inhibited the translocation of Rab1A from cytosol to membrane, and this inhibition was prevented by supplementation of GGPP, but not FPP. This indicates that fluvastatin inhibited HMG-CoA reductase and mevalonate synthesis and depleted GGPP, and as a result, prevented geranylgeranylation of Rab1A. Rab1 is isoprenylated only by GGPP, but not by FPP, for its activation (Khosravi-Far et al., 1991). Therefore, fluvastatin consequently inhibited membrane association of Rab1A and thereby inactivated Rab1A.

Our present results suggest that inhibition of Rab1A is responsible for and precedes statin-induced vacuolation and cell death in skeletal muscles. As shown in Fig. 1, the loss of Rab1 localization in membrane occurs on day 4 of 1 μM fluvastatin treatment, by which time vacuolation is already well advanced but cell death is not (Fig. 3). However, as demonstrated in Fig. 2, as early as on days 1 and 2 fluvastatin inhibited translocation of Rab1, whereas vacuolation was not yet significant (Fig. 3). This indicates that inhibition of Rab1 translocation precedes and causes the subsequent vacuolation and even later loss of viability in myofibers.

Using transmitted electron microscopy, we previously demonstrated that statins induced vacuolation and ER expansion in myofibers (Sakamoto et al., 2007). Similar ER expansion was also observed in skeletal muscle biopsy samples from statin-administered rodents (Waclawik et al., 1993; Westwood et al., 2005). Vacuolation was also observed in Ypt1-deficient yeast Saccharomyces cerevisiae (Becker et al., 1991). Rab1 is an ortholog of Ypt1, which regulates ER-to-Golgi trafficking in yeast (Segev et al., 1988). Abnormal intracellular vesicle accumulation was reported to derive from ER (Becker et al., 1991). Brefeldin A is an inhibitor of ER-to-Golgi traffic, which dilated ER in chronic lymphocytic leukemia cells (Carew et al., 2006). Similar morphological changes among statin treatment, Rab1/Ypt1 deficiency, and brefeldin A treatment support our conclusion that statin-induced vacuolation in myofibers is caused by the inhibition of Rab1A.

Our conclusion was further supported by our calnexin result. Treatment of 1 μM fluvastatin for 4 days, which did not yet cause loss of cell viability, dramatically reduced calnexin distribution throughout a myofiber, but instead accumulated calnexin around the nucleus. We previously reported that a higher concentration of 10 μM fluvastatin induced significant loss of viability only after 5 days, but not after 4 days of treatment (see figure 1D of Sakamoto et al., 2007). In the present study, myofibers were treated with 1 μM fluvastatin for 4 days. Therefore, it is clear that the loss of calnexin distribution occurs before the loss of viability. Thus we conclude that the effect on calnexin distribution is a cause of general loss of cell viability. Calnexin is a chaperon protein distinctly localized in ER membrane. If ER membrane traffic is inhibited, calnexin remains in the perinucleolar area, where it is originally synthesized by gene translation. Rab1A is an important molecule for the maintenance of intracellular tubular network structure (Marie et al., 2009). Therefore, our immunofluorescence data of calnexin directly and strongly support our conclusion that fluvastatin inactivated Rab1A and disturbed ER membrane traffic, and this must be a cause of vacuolation and subsequent cell death in myofibers.

There seems to be a discrepancy in the reported relationships of Rab1 inhibition and cell death between yeasts and mammalian cells. Gene disruption of Ypt1 was lethal for yeasts (Segev and Botstein, 1987). However, gene knockdown of Rab1B or dominant negative Rab1A did not impair viability of mammalian cells (Filipeanu et al., 2006; Sklan et al., 2007). This discrepancy may be caused by the different Rab1 isoforms expressed in each cell type. Two isoforms of Rab1 (Rab1A and Rab1B) have been found in mammalian cells, whereas only one isoform was found in yeasts (Vielh et al., 1989). In mammalian cells, one isoform might have compensated for another, but this was not possible in yeast. Because statins inhibit both Rab1A and Rab1B (Ostrowski et al., 2007), the toxicity of statins may be more prominent than that of gene interference of Rab1 in mammalian cells.

There is a possibility that reduced GGPP by statins affected not only Rab1 but also various other small GTPases, including Rac, RhoA, and RhoB (Maeda et al., 2005; Greenwood et al., 2006). However, Ostrowski et al. (2007) reported that reduced GGPP by statins inhibited a selective subset of small GTPases. Simvastatin at a low concentration (200 nM) decreased the membrane association of Rac1 and Rab1, but not that of Rac4 and Rab5. On the other hand, a higher
conductance regulator (Shen et al., 1995) and suppressed translocation of cystic fibrosis transmembrane protein (Dugan et al., 1995). It is noteworthy that statins are intrinsic factors required for geranylgeranylation, which distinguish different Rab isoforms.

Suppression of Rab1 attenuates translocation to membrane of various proteins, including angiotensin II receptor (Yoo et al., 2002), and are involved in the most fundamental ER-to-Golgi membrane trafficking, its turnover may be more rapid than that of other Rab isoforms and thus are more susceptible to GGPP depletion. Another possibility is that there are intrinsic factors required for geranylgeranylation, which distinguish different Rab isoforms.

If Rab1 inhibition is the main cause of statin toxicity, why does it preferentially appear in skeletal muscles? We found that this is because of an interplay of protein transport and prenylation. In statins, Oatp2b1 and Oatp1a4 are expressed in rat skeletal myofibers, but not in fibroblasts or L6 cells (Sakamoto et al., 2008), so that the influx of statins is accelerated in myofibers. Because of the transporters, the median lethal concentration of fluvastatin is 30 times lower in myofibers than in fibroblasts isolated from skeletal muscles or L6 myoblasts (Sakamoto et al., 2008). More recently, Knauer et al. (2010) confirmed the expression and function of human OATP2B1 as a statin uptake transporter in human skeletal muscles.

In the present study, neither fluvastatin nor brefeldin A induced UPR in skeletal myofibers. It is surprising and interesting, because not only statins but also brefeldin A induced UPR may be different between skeletal myofibers and fibroblasts isolated from skeletal muscles or L6 myoblasts (Sakamoto et al., 2008). More recently, Knauer et al. (2010) confirmed the expression and function of human OATP2B1 as a statin uptake transporter in human skeletal muscles.

At least part of the mechanism for statin-induced skeletal myotoxicity.

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Authorship Contributions

Participated in research design: Sakamoto and Kimura.

Conducted experiments: Sakamoto and Wada.

Performed data analysis: Sakamoto and Wada.

Wrote or contributed to the writing of the manuscript: Sakamoto, Wada, and Kimura.

Other: Sakamoto and Kimura acquired funding for the research.

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