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Full Paper

Mechanism of Statin-Induced Contractile Dysfunction in Rat Cultured Skeletal Myofibers

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Abstract. An adverse effect of statins, cholesterol-lowering drugs, is contractile dysfunction of skeletal muscles. We investigated the mechanism underlying this effect in cultured myofibers isolated from rats. Fluvastatin (Flv) for 72 h decreased caffeine- and ionomycin-induced contraction of myofibers and Ca^{2+} release from sarcoplasmic reticulum (SR). Ca^{2+}-shortening curves measured in skinned myofibers indicated that myofibrillar Ca^{2+} sensitivity was unaffected by Flv. A luciferin–luciferase assay revealed less ATP contents in Flv-treated myofibers. Among mevalonate metabolites, including geranylgeranylpyrophosphate (GGPP), farnesylpyrophosphate (FPP), coenzyme Q9, and coenzyme Q10, only GGPP prevented Flv-induced ATP reduction. A selective Rab geranylgeranyltransferase (GG transferase) inhibitor, perillyl alcohol (POH), and a specific GG transferase-I inhibitor, GGTI-298, both mimicked Flv in decreasing ATP and contraction. Mitochondrial membrane potential was decreased by Flv, and this effect was rescued by GGPP and mimicked by POH and GGTI-298. An endoplasmic reticulum (ER)-to-Golgi traffic inhibitor, brefeldin A, and a Rho inhibitor, membrane permeable exoenzyme C3 transferase, both decreased ATP. We conclude that statin-induced contractile dysfunction is due to reduced Ca^{2+} release from SR and reduced ATP levels in myofibers with damaged mitochondria. GGPP depletion and subsequent inactivation of Rab1, possibly along with Rho, may underlie the mitochondrial damage by Flv.

Keywords: statin, skeletal muscle, prenylation, mitochondria, autophagy

Introduction

Statins decrease serum cholesterol by inhibiting HMG-CoA reductase (1) and effectively prevent cardiovascular events (2). Thus, statins are widely used for the treatment of hypercholesterolemia. However, 0.5% – 15% of statin recipients developed adverse effects on skeletal muscles, ranging from slight myalgia to severe rhabdomyolysis (3), including the reports on reduction of muscle contractility in humans (4) and rats (5). The mechanism underlying these adverse effects remained unclear.

Recently, we found that fluvastatin (Flv) and pravastatin induced vacuolar degeneration and cell death in cultured single skeletal myofibers from rat flexor digitorum brevis (FDB) muscles (6). These morphological changes induced by Flv were prevented by the supplementation of geranylgeranylpyrophosphate (GGPP), a mevalonate metabolite, indicating an involvement of small GTPases isoprenylated with GGPP.

We further performed investigations to identify small GTPases involved in the Flv effect. Various small GTPases require geranylgeranylation for activation, including Rho, Rac, Cdc, and Rab. There are two geranylgeranyltransferases (GG transferases): One is GG transferase-I, which mediates geranylgeranylation of small GTPases except Rab, including Rho, Rac, and Cdc. The other is Rab GG transferase (also called GG transferase-II), which prenylates exclusively Rab GTPases. Perillyl alcohol (POH) is an inhibitor of both Rab GG transferase and GG transferase-I, but it is twice as potent in inhibiting Rab GG transferase as GG transferase-I (7). GGTI-298 is a specific inhibitor of GG transferase-I (8).

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Our previous study revealed that inactivation of Rab was responsible for the Flv effects of vacuolation and cell death in myofibers because only POH mimicked Flv, but not GGTI-298 (6). Since there are more than 60 isoforms of Rab, it is a challenge, which we took, to identify the major Rab isoform responsible for the Flv effect. It had been reported that one of the most susceptible isoforms to GGPP depletion was Rab1, which is essential for vesicular transport from the endoplasmic reticulum (ER) to the Golgi apparatus (ER-to-Golgi traffic) (9). We found in myofibers that Rab1 was inactivated by Flv and that the effect of Flv was reproduced by brefeldin A (BFA), a specific inhibitor of ER-to-Golgi traffic. These results suggested that depletion of GGPP and subsequent inactivation of Rab1 lead to inhibition of ER-to-Golgi membrane traffic and this is one of the main causes of statin-induced vacuolation and cell death in the skeletal myofibers (10).

During the course of this study, we also found that caffeine-induced contraction of myofibers was suppressed by statins (6). However, the underlying mechanism of contractile suppression remains unknown. In this study, we investigated the mechanism of statin-induced contractile dysfunction in cultured single skeletal myofibers from rats.

Materials and Methods

Ethical approval

All experiments were performed in accordance with the regulations of the Animal Research Committee of Fukushima Medical University.

Isolation of myofibers

We isolated single skeletal myofibers as described previously (6, 11) with some modifications. In brief, male Wistar rats (9 – 24-week-old) were anesthetized with ether and exsanguinated. Rat FDB muscles were incubated for 3 h at 37°C in 0.3% collagenase (Wako, Tokyo)–containing Ringer's solution, with 2.7 mM KCl, 1.2 mM KH_2PO_4, 0.5 mM $MgCl_2$, 137 mM NaCl, 8.1 mM $NaHCO_3$, 1 mM $CaCl_2$, 10 mM glucose, 0.001% penicillin-G (Banyu Pharmaceutical, Tokyo), and 0.001% streptomycin (Meiji Seika, Tokyo). Then myofibers were dispersed by agitation using a wide mouth Pasteur pipette in 10% FBS / Dulbecco modified Eagle's medium (DMEM) (Wako Pure Chemical Industries, Osaka) and maintained at 37°C in a humidified atmosphere of 5% CO_2 / 95% air. To minimize contamination with tendon fibroblasts, we cultured the isolated myofibers overnight, so that almost all the fibroblasts adhered to the bottom of the culture dish but not the myofibers (12). After that, we harvested the myofiber-rich medium and seeded it on the dishes coated with laminin (Sigma-Aldrich, St. Louis, MO, USA). Finally, 10 μM cytarabine (AraC) was added to the medium to prevent the proliferation of fibroblasts.

Contraction assay

To quantify the contraction of myofibers, we measured their length in the absence and presence of caffeine. The images of myofibers were obtained under a microscopy with a digital camera system (DP40; Olympus, Tokyo). The lengths of 10 – 30 single skeletal myofibers were measured by Image J Version 1.40G (US National Institutes of Health), and then the average length was calculated by the following equation:

$$\text{Contraction (\%)} = (ML1 - ML2) / ML1 \times 100$$

, where ML1 and ML2 mean average lengths of myofibers before and after caffeine treatment, respectively.

Skinned fibers

We made skinned myofibers by the technique developed by Natori (13) and examined the effect of Flv on Ca^{2+} sensitivity of myofibrils. We removed sarcolemma by immersing the myofibers for 30 min in 0.1% saponin-containing high K^+ solution, with 94.7 mM KOH, 20 mM PIPES, 5.3 mM Mg-methanesulphonate (Ms), 0.1 mM CaMs, 10 mM NaN_3, 5.1 mM ATP, and 10 mM EGTA (pH 7.2 with MS). To obtain the relationship between Ca^{2+} concentration and shortening of myofibers, myofibers were incubated for 1 h in saponin-free high-K^+ solution with various concentrations of CaMs. The contraction of skinned fibers was measured using the same equation described above in the *Contraction assay*.

ATP measurement in myofibers

The skeletal myofibers cultured in non-coated dishes were centrifuged (500 rpm, for 1 min) in 1.5-ml Eppendorf tubes, and the medium was discarded. A 50-μl aliquot of extraction solution (10% trichloroacetic acid (TCA) / 4 mM EDTA / PBS) was added to the sedimented myofibers. Then the cell membrane was lysed and ATP was extracted from myofibers. After centrifugation (500 rpm, for 1 min), we sampled the supernatant (20 μl) and diluted it 50-fold. Using this sample, we measured the amount of ATP by the luciferin–luciferase assay (AB-2300; ATTO, Tokyo). Simultaneously, we measured the amount of protein from sedimented skeletal muscle remnants by adopting the DC protein assay kit based on Lowry's method (Bio-Rad, Hercules, CA, USA). The data were expressed as the ratio of the amounts of ATP/protein.

Measurement of mitochondrial membrane potential ($\Delta\Psi m$)

Myofibers were plated on glass-bottomed dishes (Matsunami, Osaka) in DMEM with 10% FBS and 10 μM Ara-C. We measured $\Delta\Psi$m with the JC-1 Mitochondrial Membrane Potential Assay Kit (Cayman Chemical, Ann Arbor, MI, USA). Fluorescence of JC-1 is red in cells with polarized mitochondria, while it is green in cells with depolarized mitochondria. Measurement of JC-1 fluorescence was monitored by a fluorescent microscope TE-2000 (Nikon, Tokyo) with the Aqua Cosmos imaging system (Hamamatsu Photonics, Hamamatsu). The data are expressed as the percentage of cells with red fluorescence.

Measurement of $[Ca^{2+}]_i$

After 3 days of culture with or without 10 μM Flv, we removed the medium and then myofibers were preloaded with 3 μM Fura-2AM (Dojindo, Kumamoto), a ratiometric Ca^{2+} fluorescence indicator, for 30 min at 37°C in Tyrode's solution. Tyrode solution contained 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 0.33 mM NaH_2PO_4, 5.6 mM glucose, and 5 mM HEPES (pH 7.4 with 2.4 mM NaOH). We used this solution with 100 μM *N*-benzyl-*p*-toluene sulfonamide (BTS, a myosin II inhibitor) to inhibit contraction of myofibers during fluorescence measurement (14). Measurement of Fura-2 fluorescence was performed with the Aqua Cosmos imaging system. The data are expressed as the ratio of fluorescence intensity at 510 nm excited at 340 and 380 nm (F340/F380). Image acquisition was maintained at a frequency of one image every 15 s.

Electron microscopy

Skeletal myofibers were attached to the laminin-coated coverslips and cultured as described above. They were fixed and processed for a conventional transmission electron microscopy as described recently (15).

Drugs

Most of the pharmacological agents without description were purchased from Sigma-Aldrich. Fluvastatin (Flv) (Novartis, Basel, Switzerland) and simvastatin (Smv) (Wako Pure Chemical Industries) are used in a series of experiments in our continuing study (6, 11). Statins and BFA were dissolved in dimethylsulphoxide (DMSO), and the final DMSO concentration was 0.1%. DMSO at a concentration of 0.1% was applied to the control preparations in every experiments using statins or BFA. CoQ9 and CoQ10 were dissolved in special detergent for ubiquinones (E0216-Z151; Eisai Co., Ltd., Osaka) and kept as 10 mM stock solutions. Membrane-permeable exoenzyme C3 transferase (mpC3) was purchased from Cytoskeleton, Inc. (Denver, CO, USA).

Analysis

Each experiment was repeated 3 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, and Dunnett's test after the F test or by one-way analysis of variants (ANOVA).

Results

Effects of Flv and Smv on caffeine-induced contraction in myofibers

We first examined the effects of Flv and Smv on contraction of myofibers by measuring the myofiber lengths before and after application of 30 mM caffeine for 1 h. The rate of contraction was calculated as described in the methods. Flv (10 μM) treatment for 24 and 48 h did not reduce the contraction rate significantly (Fig. 1A). How-

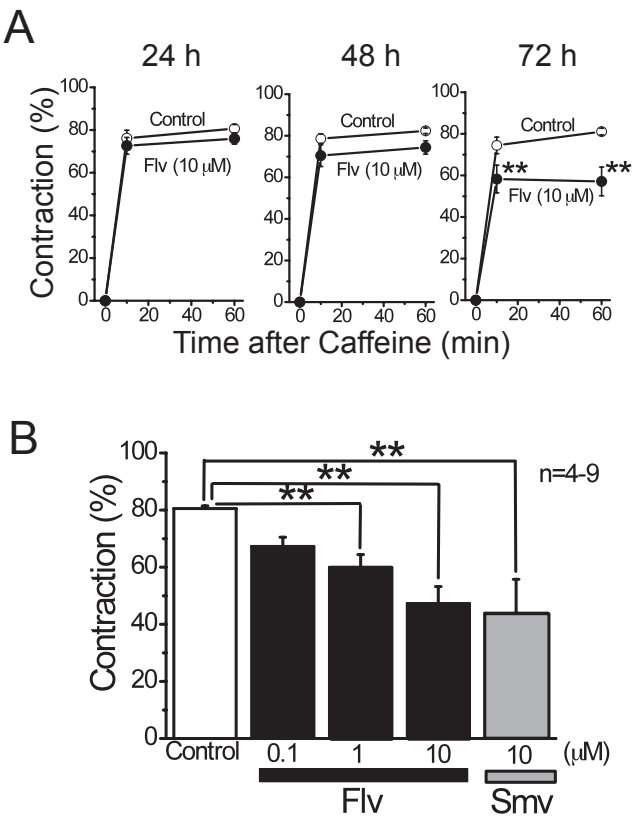

Fig. 1. Effect of Flv and Smv on caffeine-induced contraction in myofibers. A: Myofibers were cultured in the absence or presence of 10 μM Flv for 24, 48, and 72 h. At 10 and 60 min with caffeine, we took pictures of myofibers in culture dishes and measured the lengths to calculate muscle contraction rates. $^{**}P < 0.01$ vs. Control. B: Summary of caffeine-induced contraction of myofibers treated with Flv or Smv at the indicated concentrations for 72 h. $^{**}P < 0.01$ vs. Control.

ever, 72-h treatment reduced the rate from 81.1 ± 1.6% to 57.1 ± 6.9% ($P < 0.01$, n = 5, Fig. 1A). This suggested that the inhibitory effect of Flv on myofiber contraction was time-dependent.

Next, we examined the effect of various concentrations of Flv on caffeine-induced contraction. Flv significantly inhibited contraction at a concentration range higher than 1 μM, and the effect was concentration-dependent (Fig. 1B). Smv at 10 μM also suppressed the contraction in a similar manner to Flv. Thus, the inhibition of myofiber contraction by statins was both time- and concentration-dependent.

Effect of Flv on Ca^{2+} release from sarcoplasmic reticulum (SR) in myofibers

As for the possible mechanisms of decreased contractility in skeletal myofibers, three factors should be considered: intracellular Ca^{2+} concentration, Ca^{2+} sensitivity of myofibrils, and ATP content. We first suspected the possibility of decreased cytosolic Ca^{2+} concentration in Flv-treated myofibers, and examined caffeine-induced Ca^{2+} release by preloading Fura-2 in myofibers. Figure 2A shows traces of averaged Fura-2 ratios from control myofibers (n = 5) and Flv-treated myofibers (n = 10). Caffeine at 10 mM raised Fura-2 ratio (F340/F380) significantly in the control, while it was markedly suppressed in Flv-treated myofibers. The resting Fura-2 ratios of control and Flv-treated myofibers were not significantly different (Fig. 2B). The peak difference ratio was obtained from each myofiber by subtracting the basal ratio form the peak and averaged ratios are shown in Fig. 2C. The significant statistical difference in the peak ratios indicates that Flv impaired Ca^{2+} release from SR in myofibers.

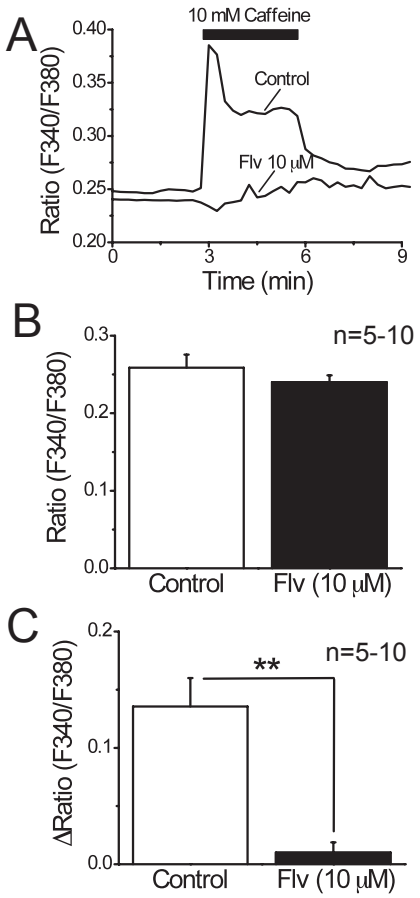

Fig. 2. Flv reduces caffeine-induced Ca^{2+} release. A: Ratio of Fura-2 fluorescence intensity (F340/F380) showing caffeine-induced cytosolic $[Ca^{2+}]_i$ changes in skeletal myofibers. Myofibers were treated with 0.1% DMSO (Control) or 10 μM Flv for 72 h. B: Ratio of Fura-2 at resting conditions just before the application of caffeine. Data were summarized from experiments represented in panel A. C: Averaged difference of ratios at the maximum during caffeine-treatment and that before caffeine. Data were obtained from 20 cells. $^{**}P < 0.01$ vs. Control.

Effect of Flv on ionomycin-induced contraction of myofibers

Next we tested whether decreased Ca^{2+} release from SR was the sole reason for the impaired contraction in Flv-treated myofibers by supplying ample Ca^{2+} to the cytosol with the Ca^{2+}-ionophore ionomycin. After 72 h with or without 10 μM Flv, 10 μM ionomycin was introduced to myofibers for 60 min (Fig. 3A). The contraction by ionomycin in Flv-treated myofibers was significantly less than that in the control (n = 5, $P < 0.01$). This suggests that statin-induced contractile dysfunction is due not only to reduced Ca^{2+} release from SR, but also to other mechanisms such as abnormal contractile proteins and/or diminished ATP contents.

Effect of Flv on myofibrillar Ca^{2+} sensitivity

To examine whether Flv affected contractile proteins, we made skinned myofibers to check their Ca^{2+} sensitivity. Skinned myofibers were treated for 72 h with or without 10 μM Flv, and the relationships between Ca^{2+} concentrations and contractile shortening were obtained (Fig. 3B). The maximum rates of contraction in control and Flv-treated myofibers were similar ($P > 0.05$, n = 3). The EC_{50} values of the Ca^{2+}-contraction curves were 48.2 ± 4.5 and 44.2 ± 7.6 nM in the control and Flv-treated myofibers, respectively ($P > 0.05$, n = 3). This indicates that Flv does not affect contractile proteins.

Effect of Flv on ATP contents in myofibers

Since ATP is essential for muscle contraction, ATP may be decreased in Flv-treated myofibers in which contraction is suppressed. We measured the amount of ATP contents in single cultured myofibers in the control and after Flv treatment. The ATP content was 28.7 ± 24.7

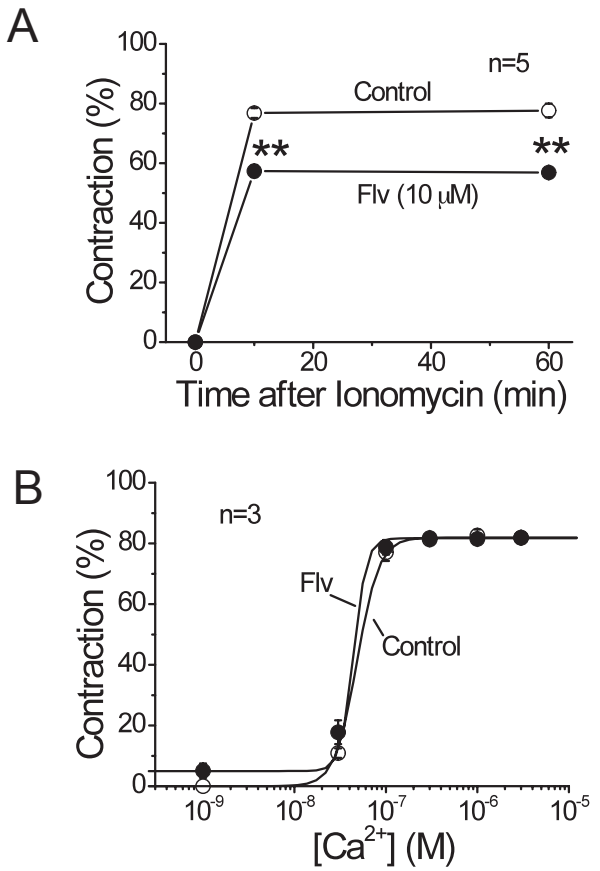

Fig. 3. Flv reduced ionomycin-induced contraction without affecting Ca^{2+} sensitivity. A: Myofibers were cultured in the absence or presence of 10 μM Flv for 72 h. After 10- and 60-min ionomycin stimulation, we took pictures of myofibers and calculated contraction rates. $^{**}P < 0.01$ vs. Control. Open circles indicate control and filled circles with Flv. B: Ca^{2+}-concentration–contraction relationships of skinned myofibers treated with or without 10 μM Flv for 72 h. Open circles indicate control and filled circles with Flv.

nmol/mg (n = 5) in the control, and this value was used as 100% for normalization. Figure 4A shows that Flv decreased ATP contents to 24.4 ± 5.4% (n = 5) of the control myofibers. This indicated that decreased ATP is another reason of contractile dysfunction and that mitochondria are likely affected in Flv-treated myofibers.

Effects of isoprenoids on diminished ATP and contraction by Flv

GGPP and farnesylpyrophosphate (FPP) are isoprenoid metabolites of mevalonate. Statins inhibit mevalonate synthesis, resulting in depletion of both GGPP and FPP. We examined the effect of GGPP or FPP supplementation on ATP and contractile reduction by Flv (Fig. 4A). ATP was measured in myofibers after 72-h Flv-treatment with or without GGPP (3 μM) or FPP (3 μM). In the presence of GGPP, Flv reduced ATP to only 72.9 ± 8.0% (n = 3, $P < 0.05$) of the control, indicating that GGPP prevented ATP reduction. FPP did not have this effect (32.6 ± 7.9%, n = 3, $P > 0.05$). GGPP, but not FPP, also prevented contractile inhibition by Flv (Fig. 4B). These indicate that GGPP depletion is responsible for the effects of Flv on ATP and contractile impairment in myofibers.

Effect of GG transferase inhibitors on ATP content.

To elucidate which small GTPases are involved in the maintenance of intracellular ATP contents, we tested inhibitors of two GG transferases, Rab GG transferase and GG transferase-I, on ATP content in myofibers. In our previous study, we found that both POH, a non-selective inhibitor of Rab GG transferase, and GGTI-298, a specific inhibitor of GG transferase-I, reduced the myofiber viability within 10 h (6). Therefore, 4 h was employed in this study. Both POH (Fig. 4C) and GGTI-298 (Fig. 4D) significantly attenuated ATP contents of myofibers. This indicated that Rab is involved, but in addition some other small GTPases may also be involved in the mechanism of ATP reduction by Flv in myofibers.

Effect of Flv on mitochondria in myofibers

Because ATP was reduced in Flv-treated myofibers, we hypothesized that Flv may damage the mitochondria and disturb ATP synthesis. In electronmicrographs of myofibers treated with 1 μM Flv for 96 h, there were many swollen mitochondria, which were apparently included in autophagosomes (Fig. 5B). This suggested that Flv affected mitochondria. We further investigated the effect of Flv on $\Delta\Psi$m, by using JC-1, a $\Delta\Psi$m indicator. JC-1 staining revealed that $\Delta\Psi$m was depolarized in Flv-treated myofibers compared to that in control myofibers (Fig. 5C), indicating abnormal mitochondria. This effect depended on the concentration of Flv (Fig. 5D). This suggests that mitochondrial abnormality underlies the ATP decrease in Flv-treated myofibers.

GGPP depletion is responsible for Flv-induced loss of $\Delta\Psi$m

We further investigated the mechanism of loss of $\Delta\Psi$m. GGPP prevented Flv-induced $\Delta\Psi$m reduction (Fig. 6A). Furthermore, $\Delta\Psi$m loss was induced by both POH (1 mM) and GGTI-298 (10 μM) (Fig. 6B). This suggests that inhibition of both Rab GG transferase as well as GG transferase-I could reproduce mitochondrial abnormality, and thus Rab and possibly some other small GTPase are involved in Flv-induced loss of $\Delta\Psi$m. These are similar to the results obtained in ATP reduction by Flv, further suggesting that mitochondrial dysfunction with loss of $\Delta\Psi$m is responsible for ATP decrease and subsequent contractile dysfunction in Flv-treated myofibers.

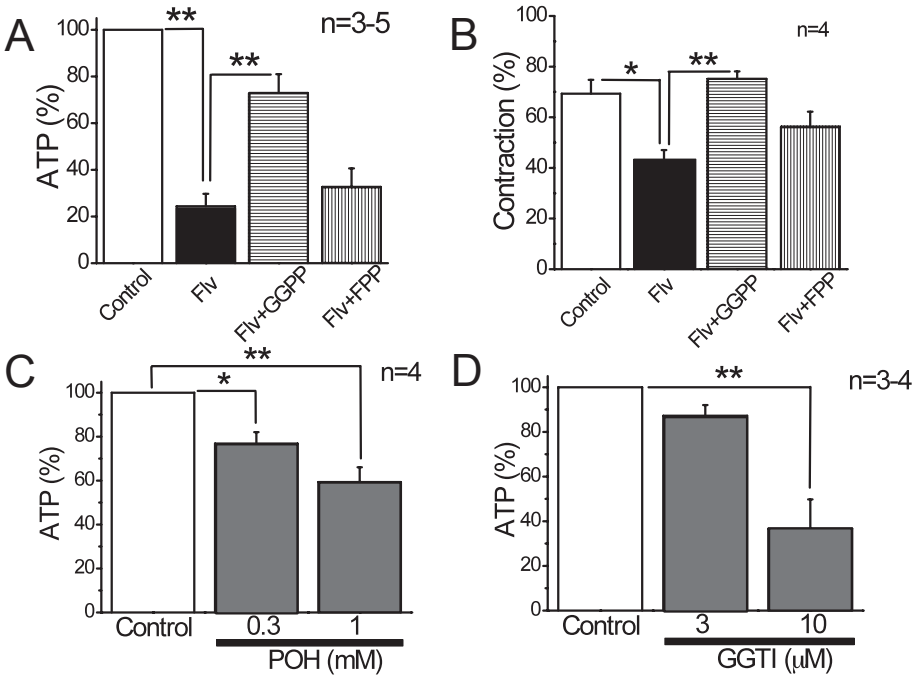

Fig. 4. Effects of GGPP and GG transferase inhibitors on Flv-indued ATP reduction and contraction. A: Summary of ATP contents of myofibers measured by luciferin–lusiferase assay. Myofibers were cultured under 0.1% DMSO (Control), 10 μM Flv, 10 μM Flv + 3 μM GGPP, or 10 μM Flv + 3 μM FPP for 72 h. ATP was normalized by the control in each set of experiments. $^{**}P < 0.01$ vs. Flv. B: Summary of caffeine-induced contraction. Myofibers were cultured with conditions similar to those in panel A. C: Summary of intracellular ATP contents of myofibers cultured with perillyl alcohol (POH) at the indicated concentrations for 4 h. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Control. D: Summary of intracellular ATP contents of myofibers treated with GGTI-298 (GGTI) at the indicated concentrations for 4 h. $^{**}P < 0.01$ vs. Control.

Involvement of Rab1 in loss of $\Delta\Psi$m

Rab1 is an essential regulator of ER-to-Golgi traffic and its inactivation is involved in vacuolation and death of myofibers by statins (10). Since POH, a Rab GG transferase inhibitor, reduced $\Delta\Psi$m (Fig. 6B), we tested the effect of BFA, an inhibitor of ER-to-Golgi, on $\Delta\Psi$m to confirm the involvement of Rab1. Although BFA for 4 and 24 h did not show a significant effect (data not shown), the treatment of BFA for 96 h reduced $\Delta\Psi$m (Fig. 6C), further suggesting that Rab1 inactivation is involved in Flv-induced loss of $\Delta\Psi$m.

Involvement of Rho in loss of $\Delta\Psi$m

Because GGTI-298 mimicked Flv and reduced $\Delta\Psi$m, we suspected that some small GTPase other than Rab might be involved in the maintenance of $\Delta\Psi$m in skeletal myofibers. Maeda et al. (16) reported that statins depolarize $\Delta\Psi$m of human liver carcinoma (HepG2) cells by inactivating Rho, one of the small GTPases geranylgeranylated by GG transferase-I. Therefore, we investigated the effect of a specific Rho inhibitor, mpC3, on $\Delta\Psi$m. mpC3 at 10 μg/ml for 96 h induced a small but significant decrease in $\Delta\Psi$m in myofibers (Fig. 6D). This indicates that together with Rab1, Rho may be involved in the effect of Flv on $\Delta\Psi$m decrease.

Effect of ubiquinones on Flv-induced reduction of ATP and contraction

Since ubiquinones (CoQ9 and CoQ10) are also metabolites of mevalonate, it has been suggested that statins reduce ATP by depleting ubiquinones (17), although some reports denied this idea (18, 19). In the present study, we examined the effect of ubiquinones on Flv-induced reduction of ATP and contractility. There are two different coenzyme Qs; CoQ9 is a major ubiquinone in rodents and CoQ10 is a major ubiquinone in humans (20). After 72 h with 10 μM Flv, neither CoQ9 nor CoQ10 at 10 μM prevented Flv-induced reduction of ATP (Fig. 7A) and contractility (Fig. 7B). This indicates that ubiquinones are not involved in the statin's adverse effects.

Discussion

In the present study, we reproduced our previous result that Flv suppressed caffeine-induced contraction in cultured myofibers (6). To elucidate the mechanisms of impaired contractility by statins, we examined three factors essential for muscle contraction: Ca^{2+} sensitivity of myofilaments, cytosolic Ca^{2+} concentration, and ATP contents. We found that Ca^{2+} sensitivity of myofilaments was unaffected by Flv because the Ca^{2+}-shortening curves obtained in skinned myofibers were identical between control and Flv-treated ones. This was also supported by electronmicrographs, where the structure of myofilaments appeared intact even with Flv-treatment (see Fig. 5A and 5B, see also ref. 6).

Cytosolic Ca^{2+} concentration was reduced in Flv-treated myofibers because caffeine-induced Ca^{2+} release from SR was significantly less in those myofibers (Fig.

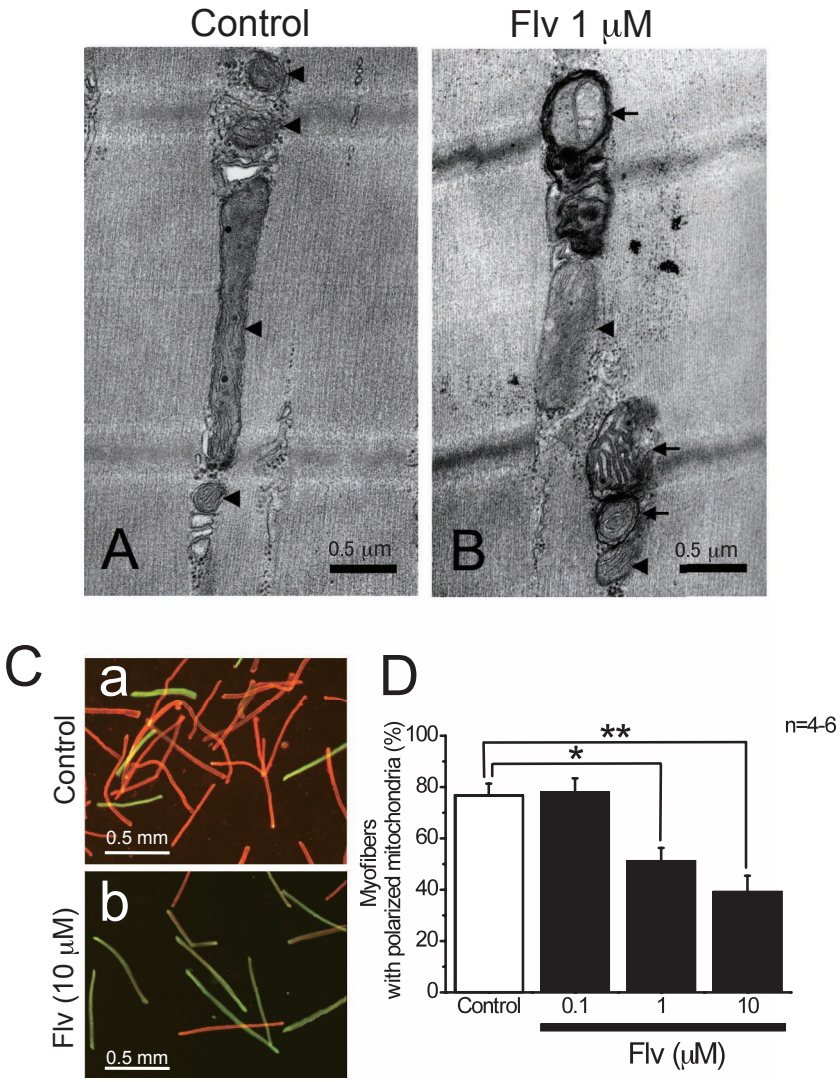

Fig. 5. Morphological and functional changes in mitochondria of Flv-treated myofibers. Transmission electron microscope images of myofibers cultured without (A) or with (B) 1 μM Flv for 96 h. Note that degenerated mitochondria are engulfed by electron-dense autophagosome membranes (arrows). Arrowheads indicate mitochondria with normal morphology. Bars: 0.5 μm. C: Fluorescence images of Control (a) and Flv-treated (b) myofibers stained with JC-1 dye. Myofibers with red fluorescence signifies polarized mitochondria, while those with green fluorescence contain depolarized (uncoupled) mitochondria. D: Summary of JC-1 staining that shows the percentage of myofibers with red fluorescence. Myofibers were treated with Flv at the indicated concentrations for 96 h. $*P < 0.05$, $**P < 0.01$ vs. Control.

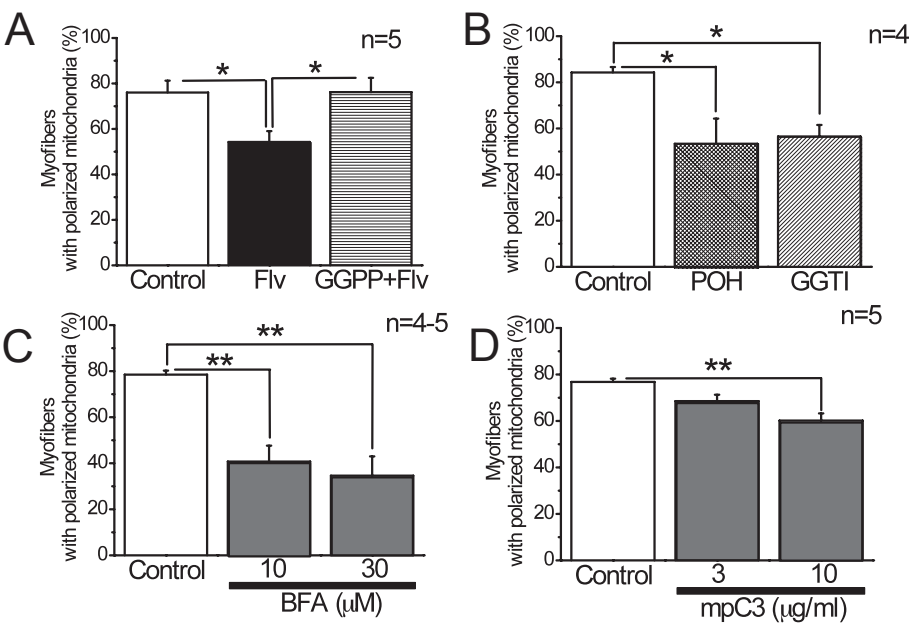

Fig. 6. Effect of Flv, GGPP, and GG transferase inhibitors on mitochondrial membrane potential ($\Delta\Psi$m). A – D: Summary of percentages of myofibers with red fluorescence (depolarized $\Delta\Psi$m) with JC-1 staining. A: Myofibers were treated with 0.1% DMSO (Control), 1 μM Flv, or Flv + 3 μM GGPP for 96 h. $*P < 0.05$ vs. Flv. B: Myofibers were treated with 1 mM perillyl alcohol (POH) or 10 μM GGTI-298 for 4 h. $*P < 0.05$ vs. Control. C: Myofibers were treated with brefeldin A (BFA) for 96 h. $**P < 0.01$ vs. Control. D: Myofibers were treated with membrane permeable exoenzyme C3 transferase (mpC3) at the indicated concentrations for 96 h. $**P < 0.01$ vs. Control.

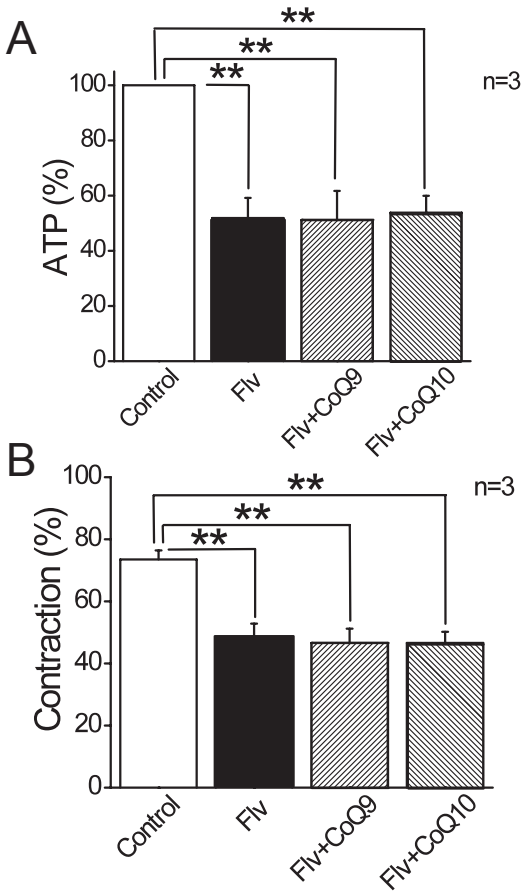

Fig. 7. Ubiquinones do not prevent Flv-induced reduction in ATP and contraction. A: Summary of ATP contents in myofibers measured by the luciferin–lusiferase assay. Myofibers were cultured with 0.1% DMSO (Control), 10 μM Flv, 10 μM Flv + 10 μM CoQ9, or 10 μM Flv + 3 μM CoQ10 for 72 h. $^{**}P < 0.01$ vs. Control. B: Summary of caffeine-induced contraction. Myofibers were treated with conditions similar to those in panel A. $^{**}P < 0.01$ vs. Control.

2). This agrees with the findings of Liantonio et al. (5) who also showed that caffeine-induced Ca^{2+} release was suppressed in skeletal muscles of Flv-administered rats. Since Ca^{2+} mobilization is predominantly from SR in skeletal muscles under physiological conditions, Flv-induced Ca^{2+} depletion in SR should be one of the main reasons why Flv reduced muscle contractility.

Diminished Ca^{2+} mobilization may not appear to underlie Flv-induced contractile dysfunction (Fig. 1) because 10 μM Flv treatment reduced caffeine-induced contraction from about 80% to 60% (Fig. 1A), while the same concentration of Flv almost totally abolished Ca^{2+} release from SR (Fig. 2A). However, in the contraction assay, we measured the length of myofibers at 15 and 60 min after the onset of caffeine application. On the other hand, we measured the Ca^{2+} concentration only for 3 min after caffeine application because myofibers are damaged by a long-time exposure to excitation UV light. As it can be seen in Fig. 2A in Flv treated myofibers, caffeine does not release Ca^{2+} at the beginning, but the Fura-2 ratio gradually increases in the presence of caffeine. Therefore, the apparent difference is not a discrepancy, but it is due to the different time scale of the measurement during caffeine application.

We found less ATP in Flv-treated myofibers. This could be responsible for both contractile and SR dysfunction. Reduced ATP inhibits actin–myosin crossbridge interaction, which could explain our ionomycin result that contraction was suppressed in Flv-treated myofibers even though sufficient Ca^{2+} was supplied to the cytosol by ionomycin. ATP depletion also impairs the activity of sarcoplasmic/ER Ca^{2+}-ATPase (SERCA) and inhibits Ca^{2+} uptake into SR, resulting in reduced Ca^{2+} release. Thus, we suggest that ATP depletion is also one of the main reasons for statin-induced reduction in contractility of myofibers.

As a mechanism for reduced ATP, we found that $\Delta\Psi$m was depolarized in Flv-treated myofibers. The dissipation of $\Delta\Psi$m and subsequent depletion of intracellular ATP levels were both prevented by GGPP. Furthermore, GG transferase inhibitors reproduced the effect of Flv on ATP and $\Delta\Psi$m (Figs. 4C, 4D, and 6B). Therefore, suppression of geranylgeranylation of some small GTPases would be responsible for these adverse effects of statins.

In our previous study, vacuolation by statins was mimicked by the Rab GG transferase inhibitor (POH) but not by the non-Rab GG transferase inhibitor (GGTI-298) (6). This suggests that there is a difference in the types of small GTPases involved in the loss of $\Delta\Psi$m from those in vacuolation.

We have reported previously that Rab1 inactivation was involved in statin-induced vacuolation in myofibers, and this was mimicked by BFA, a suppressor of ER-to-Golgi vesicle trafficking (10). Since BFA also decreased $\Delta\Psi$m (Fig. 6C), interference of intracellular traffic may also be responsible for the loss of $\Delta\Psi$m. What is the relation between the inhibition of ER-to-Golgi traffic and $\Delta\Psi$m loss? It has been reported in mouse embryonic fibroblasts that BFA induces activation of caspase-9 and -12 followed by a release of cytochrome c from mitochondria, which indicates an increased permeability of inner mitochondrial membrane (21). However, it is unclear whether this signaling pathway is also present in skeletal muscles. We observed that myofibers treated with 10 – 30 μM BFA for 5 – 6 days were dead (indicated by trypan blue staining) in a relaxed shape in a similar manner to Flv-treated myofibers (10). This suggests that BFA also depletes ATP and inhibits muscle contraction. Further study is required to clarify the relation between

Rab inactivation and mitochondrial dysfunction.

GGPP supplementation recovered ATP partially (approximately 70% of the control) (Fig. 4A) and yet prevented Flv reduced contraction completely (Fig. 4B). Skeletal muscle contraction is reported to reduce, when intracellular ATP concentration is less than 0.5 mM (22). Therefore, in the experiment shown in Fig. 4, the partially recovered ATP may have been sufficient to induce normal contraction in GGPP-supplemented Flv-treated myofibers.

Our results suggest a possible involvement of Rho inactivation in mitochondrial dysfunction by Flv. Previously, Maeda et al. from our laboratory reported in H9c2 rat cardiac myoblasts that Flv reduced the expression of Na^+/Ca^{2+} exchanger type 1 by inactivating RhoB, an isoform of Rho (23, 24). This suggests a possibility that Rho inactivation also alters intracellular Ca^{2+} signaling by modulating the expression of Ca^{2+}-transporting proteins. In the present study, mpC3, a specific Rho inhibitor, moderately but significantly reduced $\Delta\Psi$m in skeletal myofibers (Fig. 6D). However, this effect of mpC3 was small even at a high concentration, and it took longer than that of Flv and GGTI-298 (Fig. 6: B, D). In HepG2 cells, mpC3 (1 – 4 μg/ml) dramatically reduced $\Delta\Psi$m in 24 h (19). What is the difference in the effects of mpC3 between myofibers and proliferative HepG2 cells? One possibility is smaller membrane permeability of mpC3 in skeletal myofibers that have thick and solid sarcolemma reinforced by extracellular matrix and scaffold proteins (25). However, mpC3 treatment for 24 h revealed floating myofibers (data not shown), which is a typical index of Rho inhibition (26). In fact, Flv also reduced adhesion of myofibers to the bottom of the culture dish in a similar manner to mpC3 (11). This suggests that mpC3 did permeate skeletal sarcolemma. Therefore, the contribution of Rho inactivation may be minor, if at all, in the effect of Flv on the mitochondrial dysfunction in skeletal myofibers.

In human airway mesenchymal cells, Smv reduced binding of Drp1 small GTPase to mitochondria by depleting GGPP (27). Since Drp1 is a critical small GTPase for maintenance of mitochondrial functions (28), its inactivation may also be involved in the loss of $\Delta\Psi$m induced by Flv in myofibers.

We also reported previously that Flv causes swelling of SR (6). The morphological abnormality of the SR membrane could also cause the dysfunction of SERCA and ryanodine-receptor Ca^{2+}-release channels. Our recent finding that Flv induced inactivation of Rab1 small GTPase may also be involved in the morphological changes of SR (10).

Electron micrographs revealed that some mitochondria were included in autophagosomes after Flv treatment (Fig. 5B). It has been reported that a protein called Parkin initiates the formation of autophagosomes around mitochondria by sensing depolarization of $\Delta\Psi$m (29). Parkin was originally discovered in the brain as a factor involved in Parkinson's disease (30), but its function was also recognized in skeletal muscles (31). Thus, Parkin might be involved in Flv-induced mitophagy in our myofibers. Recently, it has been reported that the excessive accumulation of autophagosome in skeletal muscles is associated with myopathic syndrome (Autophagic Vacuolar Myopathy) (32). Therefore, accumulation of autophagosomes may be a new indicator common to some types of myopathy including statin-induced adverse effects.

In early 1990s, it was proposed that statins reduced ATP synthesis by depleting ubiquinone in mitochondria (16). However, recent studies showed that statins reduce ubiquinone only in the serum, but not in tissues including skeletal muscles (reviewed in 33). In our hands, ubiquinones failed to prevent the Flv-induced reductions in ATP and muscle contractility (Fig. 7). In our experiment, extracellularly applied ubiquinones would have reached the intracellular space of myofibers, because by using the same technique as ours, Okamoto et al. reported that extracellular application of ubiquinones prevented contraction-induced loss of ATP in skeletal myofibers (34).

In summary, we found in this study that 1) Flv reduced contractility of cultured skeletal myofibers in a concentration- and time-dependent manner; 2) Flv inhibited contraction even with high cytosolic Ca^{2+} concentration; 3) however, Flv did not affect Ca^{2+} sensitivity of myofibrils; 4) Flv reduced intracellular ATP content; 5) GGPP depletion underlies all the adverse effects of Flv, including ATP depletion; 6) inactivation of Rab and additional small GTPases possibly Rho may be responsible for mitochondrial dysfunction; 7) ubiquinone is not involved in the Flv-induced reduction of contractility and ATP. We suggest that reduction of ATP and impaired Ca^{2+} release from SR underlie the mechanisms responsible for statin-induced reduction in muscular contractility. Further study is required for the molecular entities responsible for the statin-induced dysfunctions of mitochondria and SR.

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