Statin-Induced Ca\textsuperscript{2+} Release was Increased in B Lymphocytes in Patients who Showed Elevated Serum Creatine Kinase During Statin Treatment

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\textbf{Aim}: Statins are effective in lowering cholesterol levels, but cause fatal rhabdomyolysis in susceptible individuals. Because it has been hypothesized that muscle damage could result from alterations in Ca\textsuperscript{2+} homeostasis in muscle cells, we tested whether measuring statin-induced changes in intracellular calcium ([Ca\textsuperscript{2+}]\textsubscript{i}) is useful for predicting susceptibility to statin-muscle damage, using human CD19\textsuperscript{+} primary B lymphocytes.

\textbf{Methods}: Statin-induced alterations in [Ca\textsuperscript{2+}]\textsubscript{i} were studied using the human THP-1 cell line and CD19\textsuperscript{+} primary B lymphocytes. Changes in [Ca\textsuperscript{2+}]\textsubscript{i} were measured directly in fluo-3-loaded cells using either single or dual-color flow cytometry.

\textbf{Results}: The Ca\textsuperscript{2+} release study suggested that statin-induced changes in [Ca\textsuperscript{2+}]\textsubscript{i} were due to Ca\textsuperscript{2+} release from ryanodine-sensitive Ca\textsuperscript{2+} stores and mitochondrial compartments. Further, statin users who experienced elevated creatine kinase (n=8) exhibited significantly greater statin-induced Ca\textsuperscript{2+} release in B cells than healthy volunteers (n=45) and statin users without elevated creatine kinase (n=16), while no difference was seen between the latter two groups.

\textbf{Conclusion}: Statin-induced Ca\textsuperscript{2+} release from ryanodine-sensitive stores and mitochondria may contribute to myotoxicity. The laboratory test for Ca\textsuperscript{2+} release using CD19\textsuperscript{+} primary B lymphocytes may be useful to predict susceptibility to statin-induced muscle toxicity prior to statin use.

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\textbf{Key words}: Statin drugs, Calcium release, Ryanodine receptor, B lymphocyte
nisms of simvastatin-induced myotoxicity have been reported to be associated with simvastatin-induced Ca\(^{2+}\) release from both ryanodine receptor (RyR) and mitochondria in human skeletal muscle fibers\(^5\).

Type 1 RyR (RyR1) is distributed mainly in skeletal muscle, but has been reported to be also manifested in the THP-1 cell line and human B-lymphocytes\(^16, 17\). Thus, we theorized that statin-induced Ca\(^{2+}\) release could also be observed in human CD19+ cells.

In this study, to test whether the alteration in Ca\(^{2+}\) homeostasis is related to statin-induced muscle damage, we examined rosuvastatin-induced Ca\(^{2+}\) release using flow cytometry in human CD19+ primary B cells from healthy volunteers, patients with elevation of creatine kinase (CK), and patients without elevated CK during statin treatment.

**Materials and Methods**

**Reagents**

4-Chloro-m-cresol (4Cmc) was obtained from Calbiochem (San Diego, CA), and thapsigargin and ionomycin were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Fluo-3 acetoxymethyl ester was obtained from Molecular Probes (Eugene, OR), xestospongin C from BIOMOL International LP (Plymouth Meeting, PA), and cerivastatin from Toronto Research Chemicals (Ontario, Canada). Simvastatin, fluvastatin, rosuvastatin, and pravastatin were a gift from Daiichi-Sankyo Co. Ltd (Tokyo, Japan).

**Subjects**

Blood samples were obtained from 8 rosuvastatin-using patients who had developed elevated CK levels (range: 282–2,193 IU/L), 16 rosuvastatin users with no elevation of CK levels (less than 200 IU/L), and 45 healthy adult volunteers. Mean age at the time of enrollment in the study was 57.4 years (range, 20–80), 53.1 years (range, 31–77), and 37.6 years (range, 24–75), respectively. None of the patients were taking fibrates with statin, and myoglobinuria was not observed. Gifu University Hospital Ethics Committee approved the study protocol, and all patients gave informed consent. Serum CK level was measured using a Quick Auto Neo CK Kit (Shino-Test Co., Tokyo, Japan).

**Human Peripheral Blood Mononuclear Cells (PBMCs) and THP-1 Cell Line**

PBMCs were isolated using Ficoll-Hypaque density gradient centrifugation. The THP-1 cell line was obtained from the Health Science Research Resources Bank (Osaka, Japan), and routinely cultivated in RPMI 1640 (Sigma-Aldrich Chemical Co., St. Louis, MO) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (ICN Biomedical Inc., Aurora, OH) and 100 U/mL penicillin-streptomycin (Gibco BRL, Life Technologies Inc., Rockville, MD), and maintained at 37°C in a humidified atmosphere of 95% air and 5% CO\(_2\), as described previously\(^16, 18\).

**Measurement of Intracellular Calcium**

Relative changes in intracellular calcium ([Ca\(^{2+}\)]) in B cells were derived from changes in the fluorescence intensity of fluo-3-loaded cells\(^17, 19\). PBMCs (2×10\(^6\)/mL) were loaded with 1 μM acetoxy-methyl ester of fluo-3 by incubation in subdued light (30 min, 25°C). The resultant fluo-3-loaded cells were then stained with phycoerythrin (PE)-conjugated anti-CD19 mAb (Beckman Coulter Co., Tokyo, Japan) to selectively label B cells. Cells were then washed three times with Mg\(^{2+}\)-, Ca\(^{2+}\)-free phosphate-buffered solution (PBS), resuspended in 1 mL PBS and analyzed with FACScan (Becton-Dickinson, San Jose, CA). Forward and right angle scatter signals were displayed on a linear scale, with the forward scatter adjusted to gate cells from debris. For dual-color analysis of intracellular fluo-3 and surface-labeled PE, fluo-3 fluorescence (excitation at 488 nm with emission at 525 nm) and PE (excitation at 488 nm, with emission at 585 nm) signals were detected after separation with 530 nm (FL-1) and 585 nm (FL-2) bandpass filters, respectively. FL-1 fluorescence and FL-2 fluorescence were recorded, amplified, and displayed on a logarithmic scale. Crossover of FL-1 fluorescence into the FL-2 detection window was compensated for by analog subtraction at the preamplifier stage. For each experiment, fluo-3-loaded cells were analyzed to obtain an unstimulated baseline. Cells were then exposed to 4Cmc or rosuvastatin, and 5000 cells were analyzed every 30 sec. Transit time required for data acquisition was approximately 10–30 sec. The measurement was performed at room temperature. To analyze the fluo-3 fluorescence shift of PE-labeled cells, FL-1 cytograms of FL-2-positive cells were obtained by gating FL-2-positive clusters in an FL-1 versus FL-2 dot plot display. Relative changes in [Ca\(^{2+}\)] in the THP-1 cell line were derived from changes in single FL-1 fluorescence. The percentage of fluo-3-positive cells relative to the unstimulated baseline was then calculated and analyzed using CellQuest software (Becton-Dickinson). Neither 4Cmc nor statins affected fluo-3 fluorescence properties in this system\(^20\). For dose-response experiments, changes in [Ca\(^{2+}\)] were examined after addition of each statin at the indicated concentrations.
Rosuvastatin, simvastatin, fluvastatin, cerivastatin, and pravastatin were dissolved in 98% N,N-Dimethyl formamide, 99.5% ethanol, 99.7% methanol, Mg$^{2+}$/Ca$^{2+}$-free PBS, and Mg$^{2+}$/Ca$^{2+}$-free PBS, respectively, according to the attached industrial information for each drug. The respective solvents were used as controls and showed no changes in [Ca$^{2+}$] (data not shown). All measurements were performed in triplicate.

Statistical Analysis

Data are expressed as the mean ± SE. The Mann-Whitney U-test was used to compare data between groups, and statistical significance was reached when $p < 0.05$.

Results

Effect of Statins on Ca$^{2+}$ Release in THP-1 Cell Line and CD19+ Primary B Cells

The dose-dependent effects of rosuvastatin, simvastatin, fluvastatin, cerivastatin, and pravastatin on [Ca$^{2+}$]: in the THP-1 cell line and CD19+ primary B cells are shown in Fig. 1. All statins, except pravastatin, induced increases in [Ca$^{2+}$]; dose-dependently in both THP-1 cell line and CD19+ primary B cells. Rosuvastatin was the most potent for increasing [Ca$^{2+}$], followed by simvastatin, cerivastatin, fluvastatin, and pravastatin.

Statin-Induced Ca$^{2+}$ Release from RyR-Sensitive Pool and Mitochondrial Compartment in THP-1 Cell Line

The THP-1 cell line was used to characterize the

Fig. 1. Dose-dependent effects of statins on [Ca$^{2+}$].
Changes in% fluo3+ cells, which reflect changes in [Ca$^{2+}$], were measured 120 sec after the addition of statins to the THP-1 cell line (open circles) and human CD19+ primary B cells (closed circles). (A) Rosuvastatin. (B) Simvastatin. (C) Fluvastatin. (D) Cerivastatin. (E) Pravastatin. Data are represented as the mean ± S.E. ($n$ = 5).
mechanism of statin-induced increases in $[\text{Ca}^{2+}]$, because sufficient CD19+ cells were not easily prepared for the study. For this characterization, we used simvastatin because it does not contain calcium in the formulation while rosuvastatin does contain calcium by the formation of a water-insoluble complex with EGTA solution.

To determine whether simvastatin-induced Ca$^{2+}$ release occurred through a RyR1 channel, we used xestospongin C, a selective inhibitor of IP$_3$ receptor, and 4Cmc, a selective RyR-stimulating agent. In the presence of 1 mM EGTA and 1 μM xestospongin C, 100 μM simvastatin induced robust Ca$^{2+}$ release. No further release was observed by 1 mM 4Cmc treatment and by subsequent application of 1 μM ionomycin (Fig. 2A).

This result suggested that simvastatin depleted not only RyR-sensitive endoplasmic reticulum (ER) stores but also mitochondrial stores. Consistent with this finding, simvastatin was capable of increasing $[\text{Ca}^{2+}]$ after Ca$^{2+}$ release was induced by 4Cmc. To further clarify the effect of simvastatin on mitochondrial store, we tested its ability to increase $[\text{Ca}^{2+}]$ after application of 100 nM thapsigargin. In the presence of 1 mM EGTA, after depletion of Ca$^{2+}$ from ER stores by 100 nM thapsigargin, 100 μM simvastatin but not 4Cmc could increase $[\text{Ca}^{2+}]$. Simvastatin-induced depletion of Ca$^{2+}$ in mitochondrial stores was confirmed by the absence of ionomycin-induced Ca$^{2+}$ release (Fig. 2B). These results strongly suggested that simvastatin-induced Ca$^{2+}$ release was derived from both RyR-sensitive ER and mitochondrial stores.

**Rosuvastatin-Induced Ca$^{2+}$ Release in CD19+ Primary B Cells from Rosuvastatin Users and Healthy Subjects**

Rosuvastatin-induced Ca$^{2+}$ release was tested using two different concentrations: 30 μM, a concentration lower than the dose that caused an obvious rise in fluo-3-positive cells; and 80 μM, the concentration producing a modest Ca$^{2+}$ release (19.7%) in CD19+ primary B cells, as seen in Fig. 1A. To determine a reference value for Ca$^{2+}$ release in CD19+ primary B cells, we analyzed Ca$^{2+}$ release in cells from healthy subjects who had received no statin. The concentration of 30 μM rosuvastatin caused less than a 5% increase, and 80 μM caused increases of 15 to 33% of $[\text{Ca}^{2+}]$ in CD19+ primary B cells from healthy subjects ($n=45$). These values were not significantly different from those in rosuvastatin-using patients without CK elevation ($n=16$) $(3.4 \pm 0.24$ vs. $2.6 \pm 0.31$ at 30 μM and $23.9 \pm 0.92$ vs. $23.0 \pm 1.03$ at 80 μM, respectively) (Fig. 3A). Additionally, Ca$^{2+}$ release was compared between pre- and post-treatment for at least three months in rosuvastatin-using patients without CK elevation ($n=3$). There was no significant differ-
ence between pre- and post-treatment (2.8 ± 0.56 vs. 1.8 ± 0.74 at 30 μM and 23.4 ± 1.50 vs. 22.3 ± 2.77 at 80 μM, respectively) (Fig. 3B).

We demonstrated Ca\(^{2+}\) release in CD19\(^+\) primary B cells from rosuvastatin-using patients with elevated CK (n = 8) (Table 1) and compared them with patients who showed no CK elevation (n = 16). As shown in Fig. 4, 30 μM and 80 μM rosuvastatin-induced Ca\(^{2+}\) release in cells from rosuvastatin-using patients with elevated CK was significantly greater than in cells from rosuvastatin-using patients without elevated CK (2.6 ± 0.31 vs. 5.2 ± 0.83 at 30 μM, p = 0.011; and 23.0 ± 1.03 vs. 35.0 ± 2.39 at 80 μM, p = 0.0008, respectively).

**Discussion**

LDL cholesterol has been incriminated as a causative factor in ischemic heart disease and cerebral vascular disturbance by many epidemiological surveys. Statins, which inhibit HMG-CoA reductase and effect improvements in serum lipid levels, are used worldwide; however, serious side effects of muscle toxicity, such as myopathy and rhabdomyolysis, have been reported. The pathogenic mechanisms of rhabdomyolysis have not yet been elucidated although they have been researched from various aspects; however, it has been reported that statin-induced apoptosis is related to molecular mechanisms for muscle damage.

Because elevation of serum CK levels caused by statin use has been reported in malignant hyperthermia (MH)-susceptible patients, it is possible that rhabdomyolysis triggered by statins involves a mechanism similar to that of MH. MH is a pharmacogenetic disorder of skeletal muscle triggered by volatile anesthetics and depolarizing muscle relaxants. The majority of MH is caused by rare mutations of the RyR1 gene. In fact, RyR1-mediated Ca\(^{2+}\) release by 4Cmc was increased in pig lymphocytes in a study of an MH swine model.

Further, more convincing evidence for the involvement of RyR and mitochondria in statin-mediated changes in [Ca\(^{2+}\)] has recently been demonstrated using human skeletal muscle fibers. Since the localization of RyR has recently been found in mitochondrial membranes, statin-induced Ca\(^{2+}\) release from mitochondria may be related to the RyR on mitochondrial membranes.

In this study, we investigated Ca\(^{2+}\) release by statins using the THP-1 cell line and CD19\(^+\) B primary lymphocytes since both express RyR1. Here we demonstrated that statin-induced Ca\(^{2+}\) release was derived from RyR-sensitive and mitochondria stores in the THP-1 cell line. Although rosuvastatin could not be used for the *in vitro* study due to its compound characteristics, our findings suggest that statins, at least simvastatin, induce Ca\(^{2+}\) release from ryanodine-sensitive stores and mitochondria. We found little effect on Ca\(^{2+}\) release by pravastatin, but it is consistent with the results from a report using mouse skeletal...
It is currently unknown whether pravastatin induces Ca$^{2+}$ release in vivo differently from in vitro, or whether it causes Ca$^{2+}$-independent mechanisms, because there have been cases of rhabdomyolysis and CK elevation in pravastatin-using patients. At present, it is also possible that each drug’s affinities to the molecular targets that cause Ca$^{2+}$ release vary in different tissues. Additionally, although rosuvastatin showed the most potent Ca$^{2+}$ release among the statins tested, this may be due to Ca$^{2+}$-induced Ca$^{2+}$ release caused by calcium salt in the formula.

The results for rosuvastatin-induced Ca$^{2+}$ release in CD19$^+$ primary B cells from healthy subjects and rosuvastatin-users provided important insights regarding the pathogenesis of statin-induced myopathy as well as the development of diagnostic methods. We found the flow cytometric measurement of Ca$^{2+}$ release using two concentrations (30 and 80 μM) of rosuvastatin in CD19$^+$ primary B cells to be straightforward and relatively practical. Moreover, repeated measurements of Ca$^{2+}$ release showed no different values between pre- and post- treatment with rosuvastatin, indicating that this measurement is stable and reproducible. Rosuvastatin users with CK elevation showed a significant increase in Ca$^{2+}$ release compared to those without CK elevation, although no correlation was observed between the CK level and the increase in Ca$^{2+}$ release. It is likely that increased Ca$^{2+}$ release

Table 1. Characteristics of patients with elevated serum CK during the treatment with rosuvastatin

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CK: creatine kinase, reference value (40–200 IU/L).
FH: familial hypercholesterolemia, HC: hypercholesterolemia, HL: hyperlipidemia, FL: fatty liver.

**Fig. 4.** Rosuvastatin-induced Ca$^{2+}$ release in CD19$^+$ primary B cells from rosuvastatin-using patients.

The graph indicates the peak % fluo-3$^+$ cells (mean ± S.E.) after 30 and 80 μM rosuvastatin for rosuvastatin users who showed no CK elevation ($n=16$) and rosuvastatin-using patients who indicated CK elevation ($n=8$). There were significant differences in peak % fluo-3$^+$ cells between groups at 30 μM ($p=0.011$) and 80 μM ($p=0.0008$).

The current knowledge on the mechanisms of muscle fibers is limited. It is currently unknown whether pravastatin induces Ca$^{2+}$ release in vivo differently from in vitro, or whether it causes Ca$^{2+}$-independent mechanisms, because there have been cases of rhabdomyolysis and CK elevation in pravastatin-using patients. At present, it is also possible that each drug’s affinities to the molecular targets that cause Ca$^{2+}$ release vary in different tissues. Additionally, although rosuvastatin showed the most potent Ca$^{2+}$ release among the statins tested, this may be due to Ca$^{2+}$-induced Ca$^{2+}$ release caused by calcium salt in the formula.

The results for rosuvastatin-induced Ca$^{2+}$ release in CD19$^+$ primary B cells from healthy subjects and rosuvastatin-users provided important insights regarding the pathogenesis of statin-induced myopathy as well as the development of diagnostic methods. We found the flow cytometric measurement of Ca$^{2+}$ release using two concentrations (30 and 80 μM) of rosuvastatin in CD19$^+$ primary B cells to be straightforward and relatively practical. Moreover, repeated measurements of Ca$^{2+}$ release showed no different values between pre- and post- treatment with rosuvastatin, indicating that this measurement is stable and reproducible. Rosuvastatin users with CK elevation showed a significant increase in Ca$^{2+}$ release compared to those without CK elevation, though no correlation was observed between the CK level and the increase in Ca$^{2+}$ release. It is likely that increased Ca$^{2+}$ release
reflected muscle damage, but the damage degree may also be influenced by statin-susceptible genes. If the sensitivity and specificity of the test can be thoroughly established, this laboratory test for Ca\(^{2+}\) release using CD19+ primary B lymphocytes may be useful to identify individuals who are susceptible to statin-induced muscle damage, at least for rosuvastatin, prior to statin use. 

More recently, a genome-wide study for statin-induced myopathy showed that an increased risk of statin-induced myopathy was significantly associated with common variants of the SLC01B1 gene, which encodes the organic anion-transporting polypeptide OATP1B1. Since the OATP1B1 has been reported to regulate the hepatic uptake of statins\(^{40, 41}\), the SLC01B1 gene is likely to contribute to increased risk of myopathy through its effect on blood and tissue levels of statins. Although it is unknown how our data relate to SLC01B1 gene variants at present, the results from this elegant genome-wide study give important insights into the pathogenesis of this complex pharmacogenetic disease and also the risk genes. If abnormal increases in myoplasmic Ca\(^{2+}\) are responsible for triggering myopathy, dose-dependent effects of statins on Ca\(^{2+}\) levels in our study suggest possible interactions between the SLC01B1 genes and statins’ molecular targets that are responsible for increasing Ca\(^{2+}\) levels. One such molecular target may be the RyR1 gene, but this gene was apparently difficult to associate with the disease in the genome-wide study because of the rarity of its variants. Considering these difficulties in finding rare risk variants, testing statin-induced Ca\(^{2+}\) responses may be useful to complement SLC01B1 genotyping in order to identify susceptible individuals.

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Ca²⁺ Release Test for Statin-Induced Myopathy


