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Biphasic Regulation of Intracellular Calcium by Gemfibrozil Contributes to Inhibiting L6 Myoblast Differentiation: Implications for Clinical Myotoxicity

Aiming Liu^{†,‡,§}, Julin Yang^{§,¶}, Frank J. Gonzalez^{||}, Gary Q. Cheng[⊥], and Renke Dai^{*,†}

[†]South China University of Technology, Guangzhou 510641, China

[‡]Medical School of Ningbo University, Ningbo 315211, China

[§]Ningbo College of Health Sciences, Ningbo 315100, China

^{||} National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, USA

[⊥]South China Center for Innovative Pharmaceuticals, Guangzhou 510006, China

Abstract

Gemfibrozil is the most myotoxic fibrate drug commonly used for dyslipidemia, but the mechanism is poorly understood. The current study revealed that gemfibrozil inhibits myoblast differentiation through the regulation of intracellular calcium ($[Ca^{2+}]_i$) as revealed in L6 myoblasts by use of laser scan confocal microscopy and flow cytometry using Fluo-4 AM as a probe. Gemfibrozil at 20–400 μM , could regulate $[Ca^{2+}]_i$ in L6 cells in a biphasic manner, and sustained reduction was observed when the concentration reached 200 μM . Inhibition of L6 differentiation by gemfibrozil was concentration-dependent with maximal effect noted between 200 and 400 μM , as indicated by creatine kinase activities and the differentiation index, respectively. In differentiating L6 myoblasts, gemfibrozil at concentrations below 400 μM led to no significant signs of apoptosis or cytotoxicity, whereas differentiation, inhibited by 200 μM gemfibrozil, was only partially recovered. A good correlation was noted between gemfibrozil concentrations that regulate $[Ca^{2+}]_i$ and inhibit L6 myoblasts differentiation, and both are within the range of total serum concentrations found in the clinic. These data suggest a potential pharmacodynamic effect of gemfibrozil on myogenesis as a warning sign, in addition to the complex pharmacokinetic interactions. It is also noteworthy that mobilization of $[Ca^{2+}]_i$ by gemfibrozil may trigger complex biological responses besides myocyte differentiation. Information revealed in this study explores the mechanism of gemfibrozil-induced myotoxicity through the regulation of intracellular calcium.

Graphical Abstract

*Corresponding Author: School of Bioscience and Bioengineering, South China University of Technology, Guangzhou 510641, China. Phone: +86-13527857328. Fax: +86-20-39380601. renke_dai@yahoo.com.

#These authors contributed equally to this work.

INTRODUCTION

Fibrates and statins are commonly used lipid lowering agents for the treatment of dyslipidemia.¹ The former drug reduces serum triglycerides by activating peroxisome proliferator-activated receptor α (PPAR α), while the latter regulates cholesterol metabolism through inhibiting 3-hydroxy 3-methylglutaryl coenzyme A reductase (HMG-CoAR). Clinically, these drugs are prescribed as monotherapy or in combination on the basis of their complementary pharmacological actions. However, both of them were known to induce myotoxicity.² Combinatory therapy with fibrates and statins can significantly increase the risk of myotoxicity, and coadministration of gemfibrozil and cerivastatin has been reported to cause fatalities.³ Because of the risk of rhabdomyolysis mortality, cerivastatin was withdrawn in 2002, shortly after its approval.

Studies of myotoxicity induced by statins have been carried out for decades. Myotoxic mechanisms of simvastatin, pravastatin, lovastatin, and cerivastatin were studied in multiple species,⁴ including a number of studies in myoblast cell lines.⁵ CoQ10 deficiency, disturbance of intracellular calcium, and inhibition of protein geranylgeranylation have been implicated in their myotoxic actions.⁶ Lovastatin and mevastatin were found to be potent inhibitors of L6 myoblast fusion in 1990s.⁷ Recently, the rise in HMG-CoAR level was revealed to be crucial for the differentiation of L6 myoblasts, thus making the consensus that statins inhibiting myoblast differentiation is mechanism-based.⁸ Nevertheless, it has also been reported that statins cause myotoxicity through pathways independent of m PPAR α agonists by the use of L6 myoblasts.⁹

While fibrates have been clinically applied for about 40 years, the mechanism for their myotoxicity is not fully understood. The withdrawal of cerivastatin in 2002 has triggered a new wave of fibrate mytotoxicity investigations, especially on gemfibrozil. On the basis of pathological analysis in rats, findings of differences between clofibrate and cerivastatin in muscle susceptibility and fiber selectivity have been reported.^{10,11} In homogenates of rat skeletal muscle, fibrates induce mitochondrial dysfunction via different molecular mechanisms including the inhibition of the respiratory chain.¹² In L6 myoblasts, a significantly increased TUNEL staining was induced upon exposure to 500 μ M gemfibrozil for 48 h, but it is not correlated to caspase 3/7 activation, thus indicating that apoptosis was not involved in the myotoxicity.⁹ Complicated drug–drug interactions (DDI) between gemfibrozil and statins have been studied,^{13–16} but the increase of statin levels caused by gemfibrozil in clinics only revealed the pharmacokinetic basis for the increased myotoxicity risk associated with their combination.¹⁷ Pharmacodynamic interactions between fibrates and statins may still play a role in their synergism toward myotoxicity. Interestingly, a high incidence of myotoxicity in cynomolgus monkeys induced by gemfibrozil was reported,

where sustained exposure to higher concentrations of gemfibrozil was suggested to be important for the occurrence of myotoxicity.¹⁸

The regeneration and repair of skeletal muscle tissue are physiologically important for maintaining normal function and are enhanced by pathological and toxic stimuli. Formation of new myofibers and the reconstitution of the functional contractile apparatus includes the activation of quiescent myogenic cells, proliferation, differentiation, and cell fusion.¹⁹ The mouse C2C12 myoblast model was found to resemble much of embryonic myogenesis where a temporal and functional hierarchy is formed.¹⁹ Disruption of cell cycle withdrawal, proliferation, differentiation, and fusion may impair the correct muscle regeneration process and result in toxicity.²⁰

Regulation of intracellular calcium concentration ($[Ca^{2+}]_i$) may modify a multitude of biological reactions which may be involved in the pathogenesis of biochemical disorders and toxicity.²¹ In muscles, calcium regulates the function of phosphatases and kinases (e.g., calcineurin, CaMK) that can alter the localization and function of transcription activators including MEF2, Myf5, MyoD, MRF4, and myogenin.²² In C2C12 myoblasts, differentiation into mature myotubes is inhibited by calcium channel blockers, which reduce $[Ca^{2+}]_i$ through different mechanisms.²³ In L6 rat skeletal myoblast cells, clofibrate was found to elevate $[Ca^{2+}]_i$ leading to the appearance of apoptotic DNA fragments.²⁴ Intracellular calcium homeostasis and its biological regulation are thus important for myoblast differentiation.

In this study, the potential of gemfibrozil to regulate $[Ca^{2+}]_i$ in L6 myoblasts and inhibit its differentiation was revealed. A correlation between systemic total concentration of gemfibrozil in the clinic and concentrations regulating $[Ca^{2+}]_i$ and L6 myoblast differentiation was uncovered. The action of gemfibrozil on $[Ca^{2+}]_i$ motivation provides a molecular basis for the inhibition of L6 differentiation and the mechanism of myotoxicity.

MATERIALS AND METHODS

Materials.

Gemfibrozil was purchased from Hunan Qianjin Xiangjiang Pharmaceutical Co., Ltd. (Zhuzhou, Hunan, China). Fluo-4 AM was purchased from Molecular Probes, Inc. (Eugene, Oregon, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and insulin were obtained from Hyclone (Logan, UT, USA). Biochemical analysis kit of CK was purchased from Shanghai Kehua Bioengineering Co. Ltd. (Shanghai, China). Cell counting kit-8 (CCK-8) and Hoechst 33342 kit were both purchased from Dojindo Laboratories (Shanghai, China). All other chemicals were of analytical or HPLC grade from commercial sources.

L6 Culture and Its Differentiation Model.

Rat L6 skeletal myoblasts (ATCC no. CRL-1458) were grown on plastic substrates in growth medium (DMEM supplemented with 10% FBS, 100 units/L penicillin G sodium, and 100 mg/L streptomycin sulfate). Cells were cultured at 37 °C in a humidified incubator (Thermo Scientific) containing 5% CO₂, and the growth medium was refreshed every other

day. Confluency was maintained at less than 40% to ensure that the cells would not differentiate spontaneously. Passage was performed at a 1:6 dilution, and cells at passages 4 to 8 were used for experiments.

L6 cells were allowed to reach 65–70% confluency for the induction of differentiation, which was performed using differentiation medium (DMEM supplemented with 2% FBS, 100 units/mL penicillin G sodium, 100 mg/L streptomycin sulfate, and insulin 5 mg/L). Once differentiation was initiated and/or when gemfibrozil was added, the differentiation medium was refreshed once daily. In the differentiation medium, L6 cells showed no obvious morphological changes between 0 and 48 h, and myotubes formed between 48 and 72 h. Because DMSO was used as the solvent for Fluo-4 AM and gemfibrozil, 0.2, 0.4, 0.6, 0.8, and 1% DMSO (v/v) was added following the induction of differentiation in a preliminary experiment. The final DMSO concentration used in this study was 0.1% unless otherwise specified.

Cell fusion was monitored every day using phase-contrast microscopy, and quantification of the differentiation index was performed according to processes in earlier literature with minor modifications.^{7,23,25} Specifically, cells were fixed in 10% phosphate-buffered formalin and hematoxylin, and eosin (HE) staining was performed. The number of nuclei inside and outside the fused myotubes was quantified using ImageJ (NIH, Bethesda, MD) software. The differentiation index was calculated as the ratio of the number of nuclei in myotubes and the total number of nuclei in each image. In every experiment, two images were taken in randomly selected fields for each well, and 6 results from 3 wells were averaged per condition.

Regulation of L6 $[Ca^{2+}]_i$ by Gemfibrozil.

Profiles of $[Ca^{2+}]_i$ fluctuation were recorded by the use of a Leica SP2 AOBS spectral laser scanning confocal microscope (LSCM) system (Leica Microsystems, Mannheim, Germany) using the calcium-specific dye, Fluo-4 AM. Both the dye and gemfibrozil were dissolved in DMSO as stock solution, which led to a final concentration of 1% DMSO in this experiment. Cells were grown to about 60% confluence at least 2 days after passage and incubated with 1 μ M Fluo-4 AM in 0.1 mL of physiological salt solution (pH 7.2–7.4; 1% bovine serum albumin; with the following composition in mM, NaCl 145.0, KCl 3, $CaCl_2$ 2, $MgCl_2$ 1, glucose 10, Hepes 10) for 30 min at 37 °C in a humidified incubator. The excess dye was washed away by 1 mL of physiological salt solution three times before measurement. The baseline level of $[Ca^{2+}]_i$ in 2 mL of physiological salt solution was measured before the addition of gemfibrozil. To reveal the relationship between $[Ca^{2+}]_i$ fluctuation and drug exposure, 20, 100, and 200 μ M gemfibrozil was added discretely or cumulatively. Fluorescence was detected at an excitation wavelength of 496 nm and emission wavelength in the range 505–551 nm using an argon laser. All data were acquired once per second in Live Date Mode, and images were collected at a 40 $\times$ magnification.

To verify whether the influx of extracellular calcium was involved in the reduction of $[Ca^{2+}]_i$ by gemfibrozil, calcium-free physiological salt solution (calcium in physiological salt solution substituted with 1 mM EGTA) was used during detection. The cells were first loaded with Fluo-4 AM in normal physiological salt solution but subsequently washed and

scanned in calcium-free physiological salt solution. The detection was performed as described above.

In order to obtain data from a larger cell population for a longer duration, flow cytometry (FCM) (FCM Caliber, BD Biosciences, CA, USA) was used to study the influence of gemfibrozil on $[Ca^{2+}]_i$. Differentiating L6 cells cultured in 24-well plate format were used for this purpose, and 200 μM gemfibrozil was used because previous results showed that this concentration of drug reduces $[Ca^{2+}]_i$. L6 differentiation was initiated by the replacement of differentiation medium with 200 μM gemfibrozil added 48, 24, 12, 3, and 0.5 h before measurement was performed. Before staining, L6 cells were washed with DMEM medium three times and incubated with 1 μM Fluo-4 AM for 30 min at 37 °C. After washing cells with calcium-free physiological salt solution three times, one drop of 0.25% trypsin was added to each well. Digestion was stopped by the addition of 0.5 mL of ice-cooled phosphate buffered saline (PBS). The cells were then suspended and immediately introduced into FCM for determination.

Inhibition of L6 Differentiation.

After demonstrating that gemfibrozil regulates L6 myoblast $[Ca^{2+}]_i$ levels in a biphasic manner, the potential of gemfibrozil to inhibit myocyte differentiation and its correlation with reduction of $[Ca^{2+}]_i$ was examined. The concentration-dependent inhibition of L6 differentiation was first assayed in terms of both morphology and biochemistry. In the established model, L6 differentiation was initiated by replacement with differentiation medium containing 0, 20, 100, 200, and 400 μM gemfibrozil added and stopped 72 h after induction following which the differentiation index quantified. To monitor creatine kinase (CK) expression, differentiation was stopped at 48 and 72 h. The cell cultures were washed by PBS (3 min · 3) and lysed by sonication (8 min · 3) in 0.3 mL of ice-cooled PBS supplemented with protease inhibitor cocktails (sc-29130, Santa Cruz Biotechnology, Inc.) added. The lysate was immediately subject to a standard CK biochemical assay.

Since 200 μM gemfibrozil was found to reduce $[Ca^{2+}]_i$ and then potently inhibit L6 differentiation, the temporal effect of gemfibrozil on L6 differentiation was analyzed. Experiment groups were established where gemfibrozil was either added or removed from the differentiation medium at 0, 12, 24, 36, and 48 h during a time course of 72 h in total. A vehicle group was used as the negative control, and cells incubated with gemfibrozil throughout the time-course experiment were used as the positive control. Differentiation indices of all samples were measured at the end of the whole time course of 72 h.

Toxic Effect of Gemfibrozil on Differentiating L6Myoblasts.

Induction of apoptosis in L6 myoblasts by 0–400 μM gemfibrozil was evaluated using Hoechst staining. L6 myoblasts were seeded on 24-well plates, and differentiation was allowed when 65–70% confluency was reached. Here a serial concentration of 0 to 400 μM gemfibrozil was used and refreshed every 24 h. Cells at 24 and 48 h were fixed in 10% phosphate-buffered formalin and stained using Hoechst 33342 working solution. After washing with PBS, the stained cells were photographed under a fluorescence microscope.

The normal nuclei and those showing apoptotic features were counted using ImageJ and the apoptotic rate calculated.

The cytotoxicity of gemfibrozil 0–400 μM on differentiated L6 myoblasts was analyzed using CCK-8. Similar to the previous procedure, differentiating cells seeded in 96-well plates were incubated in differentiation medium with a serial concentration of 0 to 400 μM gemfibrozil, which was refreshed every 24 h. After incubation at 37 °C for 2 days, 10 μL of CCK-8 was added to each well and incubated for 2 h. Absorbance at 450 nm was measured using a microplate reader to evaluate the cytotoxic effect.

To determine the potential of myotoxicity by differentiation inhibition, the reversibility of L6 differentiation after inhibition by gemfibrozil was studied. Two hundred micromolar gemfibrozil was removed after incubation with L6 cells for 48 h in differentiation medium, and the cell culture was allowed to differentiate for another 72 h. The positive and negative controls were identical to those in the above procedures. Three groups of cells were fixed to produce differentiation indices for statistics.

Data Analysis.

Except where noted, each value was derived from six replicate wells. Two separate experiments were performed for each condition and typical results shown. Data were presented as mean values $\pm$ SD. Statistical analysis was performed with SPSS11.5 for Windows. One-way ANOVA followed by Student-Newman-Keuls' multiple comparison test was applied. Statistically significant differences between groups were indicated by *p* values.

RESULTS

Biphasic Regulation of $[\text{Ca}^{2+}]_i$ by Gemfibrozil.

Two phases of responses in $[\text{Ca}^{2+}]_i$ were found when L6 myoblasts were exposed to gemfibrozil in physiological salt solution (final DMSO was 1%). The control $[\text{Ca}^{2+}]_i$ indicated by the green fluorescence with fluo-4 AM is displayed in Figure 1A. Exposure of L6 myoblasts to gemfibrozil elicited an immediate increase of fluorescence emission intensity, indicating a transient elevation of $[\text{Ca}^{2+}]_i$ in L6 cells (Figure 1B and C). The emission intensity correlated with gemfibrozil concentrations where higher concentrations triggered higher fluorescence emission. Moreover, when L6 myoblasts were treated with 20 and 100 μM gemfibrozil, the fluorescence intensity was reduced to a baseline level after the initial transient elevation (Figure 1C). However, when L6 myoblasts were treated with 200 μM or higher gemfibrozil concentrations, the fluorescence intensity dropped below the baseline level (Figure 1B and C), and no recovery was observed during the scan period. The pictures of the $[\text{Ca}^{2+}]_i$ profile displayed in Figure 1B are specifically shown in Figure 1D, E, and F.

The calcium free physiological salt solution was also used as bathing medium to investigate the role of Ca^{2+} influx in mobilization of $[\text{Ca}^{2+}]_i$ by gemfibrozil. A similar regulation of $[\text{Ca}^{2+}]_i$ was noted following exposure to varying concentrations of gemfibrozil (data not shown), suggesting that the influx of extracellular calcium is not involved in the regulation of L6 $[\text{Ca}^{2+}]_i$ by gemfibrozil.

Gemfibrozil Reduces $[Ca^{2+}]_i$ in Differentiating L6Myoblasts.

The L6 myoblast differentiation model has been widely used for mechanistic investigations. In the current study, the L6 myoblast model in differentiation medium would experience two major stages: a stage within the 48 h period for biochemical changes and a stage in the post time period (48–72 h) for cell fusion. In the first stage, the cells became increasingly stereognostic. During the second stage, large and densely aligned myotubes formed. The morphology of the L6 myoblasts cultured in growth medium and differentiation medium for 72 h is shown in Figure 2A and B. By HE staining, approximately 50% of total nuclei presented in matured myotubes as shown in Figure 2C and D.

The differentiation of L6 affected by the vehicle solvent was first evaluated. When 0.2–1.0% DMSO was added to L6 myoblast cultures, differentiation was inhibited in a concentration-dependent manner. Typically, when applying 0.4% or higher DMSO, the differentiation index manifested an inhibition of 72% or more. For 0.2% DMSO, no significant inhibitory effect on L6 differentiation was observed (data not shown).

When the concentration of 200 μM gemfibrozil was found to be critical to reduce $[Ca^{2+}]_i$ in L6 cells (Figure 1), FCM was used to study the reduction of $[Ca^{2+}]_i$ by gemfibrozil during L6 differentiation. The average $[Ca^{2+}]_i$ fluorescence intensity was 1380 ± 154 for the vehicle control, and it was reduced to be 1100 ± 150 after 3 h of incubation in the presence of 200 μM gemfibrozil. This lowered $[Ca^{2+}]_i$ lasted for at least 12–24 h. $[Ca^{2+}]_i$ recovery was observed 24 h after treatment with gemfibrozil. The fluorescence intensity of 1340 ± 161 was recorded at 48 h, indicating full recovery (Figure 3A). FCM pictures showing the reduction of $[Ca^{2+}]_i$ during differentiation are shown in Figure 3B and C.

Inhibition of L6 Differentiation by Gemfibrozil.

The concentration range where gemfibrozil regulates $[Ca^{2+}]_i$ in L6 myoblast cells was found to potently inhibit L6 differentiation as well (typical pictures shown as Figure 2E and F). Gemfibrozil inhibited L6 differentiation in a concentration-dependent manner between 100 and 400 μM ($p < 0.01$) as shown in Figure 4A, where the differentiation of 85% and 96% of L6 cells that were inhibited was observed by treatment with 200 and 400 μM gemfibrozil, respectively.

CK, a biochemical marker for muscle differentiation, has also been used for tracking differentiation. CK expression in this study was significantly inhibited by 20–200 μM gemfibrozil, with the maximum inhibitory effect at 200 μM (Figure 4B). This biochemical marker seems to be more sensitive than the morphological differentiation index to describe L6 differentiation. Our time course study manifested that cell fusion or myotube formation took place during 48–72 h after the initiation of differentiation, in accordance with low CK expression levels in the initial 48 h and an increase during 48–72 h. Thus, these results demonstrated a good correlation of the temporal effect of gemfibrozil on L6 differentiation on the basis of both the morphological differentiation index and the biochemical CK assay.

Temporal Action of Gemfibrozil on L6 Differentiation.

Distinct stages are involved in establishing the skeletal muscle phenotype, and the differentiation of myoblasts is directed by sequential expression and activation of specific transcription factors. Since 200 μM gemfibrozil reduced $[\text{Ca}^{2+}]_i$ and inhibited L6 differentiation, the functional time period during which gemfibrozil inhibits L6 differentiation was examined.

The individual groups, to which gemfibrozil was added at different times, were allowed to differentiate for 72 h, and differentiation indices was quantified (Figure 5A). The 12 h group and the 24 h group exhibited a similar effect with the positive control ($p > 0.05$), indicating that inhibition took place after 24 h. Similarly, the 48 h group exhibited no difference with the negative control, thus establishing that no inhibitory effect on differentiation was exerted from 48 to 72 h during differentiation ($p > 0.05$). A difference was, however, observed among the 24 h, 36 h, and 48 h groups ($p < 0.01$). These data suggested that the effective time period for gemfibrozil to inhibit L6 differentiation was between 24 and 48 h during the differentiation process.

To verify the above results, L6 myoblasts were first treated with gemfibrozil at the onset of differentiation, and gemfibrozil was removed at 12, 24, 36, and 48 h after differentiation was initiated. The differentiation index was measured for each group at the end of 72 h in this experiment. The removal of gemfibrozil within 24 h showed no difference with the negative control ($p > 0.05$ for the 12 and 24 h groups versus the negative control group), indicating that incubation with gemfibrozil within 0–24 h did not significantly inhibit L6 differentiation (Figure 5B). In contrast, the differentiation indices from the groups where gemfibrozil was removed at 36 and 48 h were statistically different from those of the vehicle control ($p < 0.01$). Therefore, we concluded that L6 differentiation was inhibited only when gemfibrozil was added between 24 and 48 h after differentiation induction (Figure 5A and B).

Toxic Effect of Gemfibrozil on L6Myoblasts.

Results of Hoechst staining and CCK-8 analysis are shown in Figure 6A and B. ANOVA analysis indicated that treatment with 0–400 μM gemfibrozil for 24 and 48 h caused neither significant cytotoxicity nor apoptotic effect on differentiating L6 myoblasts. Thus, the gemfibrozil concentration (200–400 μM) that reduces $[\text{Ca}^{2+}]_i$ does not cause a direct cytotoxic effect on L6 myoblasts.

In the recovery study of inhibited differentiation, the negative group differentiated normally, while the positive control cells were totally inhibited as expected. However, as shown in Figure 6C, only 56% differentiation was recovered after gemfibrozil treatment ($p < 0.01$), indicating the relevance for studying gemfibrozil myotoxicity.

DISCUSSION

Clinically, the reported trough concentration of gemfibrozil is 4.8–6 μM , and the average C_{max} range is 88–196 μM .^{26,27} Epidemiology studies reported that rhabdomyolysis usually happens in elderly patients (41–84 years old).² This patient population has decreased metabolism and excretion capability that may lead to higher exposure levels than the average

$C_{\max}$ described above. In the current study, biphasic regulation of $[Ca^{2+}]_i$ in L6 myoblasts was found by treatment with 20–200 μM gemfibrozil, where a concentration of 200 μM sustainably reduced $[Ca^{2+}]_i$. Moreover, 20–200 μM gemfibrozil was also revealed to inhibit L6 differentiation indicated by both morphological and biochemical markers. As summarized in Figure 7, the total serum concentration of gemfibrozil in clinics and the concentrations vital for *in vitro* activation of PPAR α and the resultant clinical response are correlated and within the range that regulates $[Ca^{2+}]_i$ and inhibits L6 differentiation as demonstrated in this study. The fact that gemfibrozil regulates $[Ca^{2+}]_i$ and inhibits myogenesis is highly relevant for its clinical myotoxicity, especially for those patients whose exposure levels might be increased above 200 μM due to the lower capability of elimination or DDI (Figure 7).

Calcium is involved in chromatin remodeling, initiation, phenotypic differentiation, and myoblast fusion during myogenesis.²⁸ In IGF-1 transfected C2C12 myoblasts, $[Ca^{2+}]_i$ in myotubes is higher than control myotubes.²⁹ A 70% increase in the subsarcolemma calcium concentration following insulin stimulation was observed in isolated mouse skeletal muscle.³⁰ C2C12 myoblasts failed to form myotubes in the presence of calcium channel blockers such as nifedipine, verapamil, dantrolene, and thapsigargin which lowers $[Ca^{2+}]_i$ via different mechanisms.²³ These studies demonstrate that regulation of $[Ca^{2+}]_i$ is required for the proper differentiation of skeletal myoblasts. Considering the concentration-dependent effect of gemfibrozil revealed in this study, the reduced $[Ca^{2+}]_i$ caused by gemfibrozil treatment might have directly attributed to the observed inhibition of L6 differentiation.

Disruption of intracellular calcium homeostasis may mediate pathological, pharmacological, and toxicological processes.²⁹ Calcium was proposed to be involved in myotoxicity induced by fibrates, but little mechanism evidence has been reported.³¹ In isolated mouse skeletal muscle fibers, clofibric acid induces calcium release from the sarcoplasmic reticulum and/or endoplasmic reticulum.³² Another study reported that clofibrate elevated $[Ca^{2+}]_i$ in L6 skeletal myoblasts and caused apoptotic DNA fragmentation through activation of caspase 12.²⁴ In differentiated myotubes of L6 myoblasts, treatment with gemfibrozil at concentrations below 250 μM only caused a mild concentration-dependent apoptosis. However, a sharp increase of TUNEL staining was observed when treated with 500 μM gemfibrozil.⁹ In concert with the above report, exposure of L6 cultures to gemfibrozil 200–400 μM for 48 h did not result in an increase of apoptosis as indicated by Hoechst staining (Figure 6A). As revealed by CCK-8, no cytotoxic effect of gemfibrozil on L6 myoblast was observed under the concentration range studied. These results suggested that mobilization of $[Ca^{2+}]_i$ by gemfibrozil below 400 μM does not cause the apoptosis and cytotoxicity of L6 myoblasts.

Differentiation is vital in maintaining the balance and stability of muscle tissues. Growth and repair of muscle tissues is linked to a group of myogenic precursor cells called satellite cells.^{8,33} Upon activation, satellite cells proliferate and differentiate into myoblasts which differentiate and fuse with existing myofibers to repair damaged muscle.²⁰ Alteration of the dynamic balance of muscle tissues may cause or aggravate pathological and toxicological reactions. The partial recovery of inhibited L6 differentiation after gemfibrozil treatment

suggested that the drug may lead to the obstruction of normal or induced myogenesis in muscle tissues, thus unveiling a potential mechanism of clinical myotoxicity.

Synergism of statins and fibrates in combination to aggravate myotoxicity as well as their myotoxicity mechanism in monotherapy has attracted attention. Most investigations have focused on the pharmacokinetic interactions, *in vitro* or *in vivo*. Intake of statins by hepatocyte transporter organic anion transporting polypeptide 2 is inhibited by both gemfibrozil and its metabolite,^{15,16} indicating the potential of transporter-mediated drug–drug interaction (DDI). For phase I metabolism, gemfibrozil potently inhibits the cytochrome P450 CYP2C9, and its major metabolite, gemfibrozil 1-*O*- β -glucuronide, is a mechanism-dependent inhibitor of P450 2C8, which is crucial for the elimination of cerivastatin and contributes to the elimination of simvastatin.¹³ For UDP-glucuronide transferases (UGT), gemfibrozil inhibits UGT 1A1 and UGT 1A3, both of which are largely responsible for the elimination of statins.¹⁴ Clinical studies indicated gemfibrozil greatly increased the exposure levels of simvastatin, pravastatin, and cerivastatin. These pharmacokinetic DDI may partly explain the high incidence of myopathy observed with the combination of fibrates and statins, but the possibility of pharmacodynamic modification cannot be ruled out.^{17,27} The mechanism-based potential of statins to inhibit L6 differentiation⁸ and the data in this study revealed that the inhibition of myocyte differentiation may be common for both statins and gemfibrozil. Thus, pharmacodynamic evidence could account for increased myotoxicity risk following combined therapy. Pharmacodynamic together with pharmacokinetic DDIs are both contributory elements to the clinical myotoxicity caused in the combined therapy of gemfibrozil and statins.

In the C2C12 model, skeletal myogenesis is a highly ordered process, and obvious temporal events govern the transitions from myoblasts to myotubes.²⁵ Pathways involving calcineurin, CaMK, ERK5, and p38 contribute to the regulation of myogenesis signals including MRFs and MEFs, in which $[Ca^{2+}]_i$ plays an important role.^{34,35} Cell fusion is also strictly Ca^{2+} -dependent where a net Ca^{2+} influx is a prerequisite for myotube formation.³⁶ In this study, 24–48 h after differentiation initiation is the effective temporal phase in which gemfibrozil inhibits L6 differentiation. Therefore, the calcineurin pathway, typically activated by Ca^{2+} and involved in myocyte differentiation, does not seem to mediate the inhibition of L6 differentiation by gemfibrozil because it is required at the onset of differentiation.³⁷ Phenotypic differentiation and then cell fusion are both temporally late events, where the former includes the expression of acetylcholine receptors, CK, MHC, and other marker proteins.³⁸ Thus, protein expression for phenotypic differentiation (such as CK expression as indicated) and/or the Ca^{2+} signaling pathway mediating cell fusion are possible targets of gemfibrozil. Unveiling the underlying molecular mechanism requires further investigation.

In conclusion, the potential of gemfibrozil to regulate $[Ca^{2+}]_i$ and inhibit L6 myoblast differentiation was revealed in the present study. The effective concentrations of gemfibrozil showing the above activities correlate well with its total serum concentration in clinics, indicating both a warning sign and relevance for mechanistic investigations of clinical myotoxicity. Biological responses besides differentiation following the regulation of $[Ca^{2+}]_i$ by gemfibrozil, as well as their molecular pathways, are still under investigation.

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ABBREVIATIONS

PPARα	peroxisome proliferator-activated receptor α
HMG-CoAR	3-hydroxy 3-methylglutaryl coenzyme A reductase
DDI	drug–drug interaction
[Ca²⁺]_i	intracellular calcium concentration
DMEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum
HE	hematoxylin and eosin
LSCM	laser scanning confocal microscope
FCM	flow cytometry
PBS	phosphate buffered saline
CK	creatine kinase
CCK-8	cell counting Kit-8
UGT	UDP-glucuronide transferases

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Figure 1.

Gemfibrozil regulates $[Ca^{2+}]_i$ in L6 myoblast cells. (A) $[Ca^{2+}]_i$ profile when DMSO was added. (B) $[Ca^{2+}]_i$ profile when 200 μM gemfibrozil was added. (C) $[Ca^{2+}]_i$ profile when 20, 100, and 200 μM gemfibrozil was added cumulatively. (D) $[Ca^{2+}]_i$ before 200 μM gemfibrozil was added in panel B. (E) Transient elevation of $[Ca^{2+}]_i$ following the addition of 200 μM gemfibrozil in panel B. (F) Reduced $[Ca^{2+}]_i$ after the addition of 200 μM gemfibrozil in panel B. Pictures were taken under LSCM, and arrows indicated the time when the vehicle or gemfibrozil was added.

Figure 2.

L6 myoblast differentiation and inhibition by gemfibrozil. (A and B) L6 myoblasts cultured in growth medium and differentiation medium, respectively, for 72 h. (C and D) HE staining of L6 myoblasts cultured in growth medium and differentiation medium, respectively, for 72 h. (E and F) L6 differentiation in the absence (E) and presence (F) of 200 μ M gemfibrozil, respectively. (Gem: gemfibrozil).

Figure 3.

Regulation of $[Ca^{2+}]_i$ by 200 μM gemfibrozil during differentiation. (A) Reduced $[Ca^{2+}]_i$ (16%, 20%, and 19% reduction versus the control for 0.5, 3, and 12 h, respectively) following the addition of 200 μM gemfibrozil and the recovery 24 h later (10% and 3% reduction versus the control for 24 and 48 h, respectively). Data were produced from 4 wells of a 24-well plate and expressed as the mean $\pm$ SD ($n = 4$), and one-way ANOVA analysis was performed. (B) Typical scan of control where DMSO was added. (C) Typical scan 12 h after 200 μM gemfibrozil was added. (* indicates $p < 0.05$, and O indicates $p > 0.05$ compared with the control.)

Figure 4.

Concentration-dependent inhibition of L6 myoblast differentiation by gemfibrozil. (A) Differentiation index of L6 was inhibited by gemfibrozil at serial concentrations of gemfibrozil. Data were produced from 3 wells of a 24-well plate and expressed as the mean $\pm$ SD ($n = 6$, two visual fields for each well). One-way ANOVA was performed. (B) CK expression of L6 myoblasts was inhibited by serial concentrations of gemfibrozil during 48–72 h following the addition of 200 μM gemfibrozil. Data were produced from 3 wells of a 24-well plate and expressed as the mean $\pm$ SD ($n = 3$). Two-way ANOVA was performed.

(** indicates $p < 0.01$, and * indicates $p < 0.05$ compared with the control group. Gem, gemfibrozil; CK, creatine kinase.)

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Figure 5.

Effective temporal phase when gemfibrozil inhibited L6 differentiation. (A) 200 μ M gemfibrozil was added at 12, 24, 36, and 48 h during differentiation. Treatment during 24–48 h showed an inhibitory effect. (B) 200 μ M gemfibrozil was removed at 12, 24, 36, and 48 h during differentiation. Removal of gemfibrozil from the medium during 24–48 h led to decreased differentiation recovery. Data were produced from 3 wells of a 24-well plate and expressed as the mean $\pm$ SD ($n = 6$, two visual fields for each well). One-way ANOVA indicated significance (** indicates $p < 0.01$, and O indicates $p > 0.05$ between compared groups). These data indicate that 24–48 h of differentiation was the effective time period when 200 μ M gemfibrozil exerted inhibition. (Pos., positive control; Neg., negative control.)

Figure 6.

Toxic effect of 0–400 μM gemfibrozil on differentiating L6 myoblasts. (A) Differentiating L6 myoblasts in 24-well plates were incubated with 0–400 μM gemfibrozil for 24 and 48 h. Apoptosis was analyzed using Hoechst 33342 staining. Two-way ANOVA indicated no significance ($n = 5$). (B) Differentiating L6 myoblasts in 96-well plates were incubated with 0–400 μM gemfibrozil for 48 h. Direct cytotoxicity was analyzed using CCK-8. One-way ANOVA indicated no significance ($n = 6$). (C) Recoverability of L6 differentiation inhibited by 200 μM gemfibrozil. The negative (Neg.) group was cultured in differentiation medium for

72 h and the positive (Pos.) group in the presence of 200 μM gemfibrozil in the differentiation medium. In the recovery (Rec.) group, L6 myoblast cells were treated with 200 μM gemfibrozil for 48 h and allowed to differentiate for another 72 h after the removal of gemfibrozil. Independent sample *t* tests indicated significance (** indicates $p < 0.01$ between compared groups, $n = 3$. Gem, gemfibrozil; Pos., positive control; Rec., recovery group; Neg., negative control).

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Figure 7.

Summary about the correlationship between *in vitro* and *in vivo* biological responses and exposure concentrations of gemfibrozil (μM). (A) Reported human systemic concentration in the clinic.²⁷ (B) TUNEL staining significantly increased when L6 was exposed to 500 μM gemfibrozil or higher.⁹ (C) Trough concentrations in cynomolgus monkeys where a high incidence of myotoxicity was observed.¹⁸ (D) EC_{50} for PPAR α activation by Gemfibrozil.³⁹ (E) Gemfibrozil inhibits L6 differentiation morphologically between 100 and 400 μM with maximal effect at a concentration of 400 μM . (F) Gemfibrozil inhibits L6 CK expression between 20 and 200 μM with maximal effect at a concentration of 200 μM . (G) Biphasic regulation of L6 $[\text{Ca}^{2+}]_i$ by gemfibrozil between 20 and 200 μM with a reductive effect at 200 μM . Arrow symbols indicate that higher gemfibrozil concentrations may be applied to produce toxicity or maximal biological effect. It could be seen that 200 μM gemfibrozil is a concentration with relevance for clinical myotoxicity. Data were summarized from indicated references and the present study. ($C_{\min}$, trough concentration of a drug observed after its administration; $C_{\max}$, peak concentration of a drug observed after its administration; PPAR α , peroxisome proliferator-activated receptor α ; EC_{50} , concentration with half maximal effective; CK, creatine kinase; Gem, gemfibrozil.)