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Involvement of monocarboxylate transporter 4 expression in statin-induced cytotoxicity

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MCT4: monocarboxylate transporter 4, HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A, LDL: low-density lipoprotein, CK: creatine kinase, AMPK: AMP-activated protein kinase, PKC: protein kinase C, CHC: α -cyano-4-hydroxycinnamate, MTT: 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide, OATP: organic anion transporting polypeptide, MRP: multidrug resistance-associated protein

Abstract

Statins, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, are the most widely used cholesterol-lowering agents for prevention of obstructive cardiovascular events. However, statins can cause a variety of skeletal muscle problems, and exercise leads to an increase in statin-induced muscle injury. Exercise induces the protein content of monocarboxylate transporter 4 (MCT4), which is expressed strongly in skeletal muscle and is thought to play a major role in the transport of metabolically important monocarboxylates such as L-lactate. We previously reported that α -cyano-4-hydroxycinnamate (CHC), an MCT4 inhibitor, increased the inhibition of growth of an RD cell line, as a model of *in vitro* skeletal muscle, induced by a statin. However, it is unclear whether statin-induced RD cell cytotoxicity is associated with MCT4 expression. We therefore examined the relationship between statin-induced cytotoxicity and MCT4 expression in RD cells. Atorvastatin reduced the number of viable cells and up-regulated MCT4, but not MCT1, mRNA level in a concentration-dependent manner. MCT4 knockdown suppressed atorvastatin, simvastatin and fluvastatin-induced reduction of cell viability and apoptosis compared with negative control-treated cells. In this study, we demonstrated that MCT4 expression is associated with statin-induced cytotoxicity.

1. Introduction

Statins, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, are the most effective medications for reducing elevated concentrations of low-density lipoprotein (LDL) cholesterol, but statins can cause a variety of skeletal muscle problems including myalgia, creatine kinase (CK) elevation and rhabdomyolysis. Statin-induced rhabdomyolysis is rare, occurring in only ~0.1% of patients taking statin medication. However, the occurrence of myopathy has been estimated to range from 1 to 10%.¹ Although these reactions represent a relatively low risk of complications, many patients use statin medication, which may increase the proportion of myopathic complications.² Risk/benefit analysis of statin therapy is further complicated by the finding that exercise increases the risk of statin-induced myopathy. The prevalence of muscular symptoms is as high as 25% among statin users who exercise²⁻⁴ and may exceed 75% in statin-treated athletes³. However, little is known about the mechanisms by which statins induce skeletal muscle injury.⁵ Monocarboxylate transporter 4 (MCT4) is a pH-dependent lactate transporter⁶ and is expressed in skeletal muscle, especially in fast-twitch glycolytic fibers.⁷ Westwood *et al.* reported that type I and IIB fibers represent metabolic extremes of a continuum of metabolic properties through the fiber types with type I fibers being most oxidative in metabolism and type IIB fibers being

most glycolytic and that simvastatin, one of the statins, impaired type IIB fibers more than type I fibers.⁸ AMP-activated protein kinase (AMPK) is a serine/threonine protein kinase, and skeletal muscle AMPK is activated by exercise.⁹ Protein kinase C (PKC) plays important roles in intracellular signaling involved in many cellular responses.¹⁰ Some PKC isoforms have a role in exercise-mediated glucose transport.¹¹ We previously reported that AMPK activation induced expression of MCT4¹² and that MCT4 expression was mediated through PKC δ in an RD cell line as a model of *in vitro* skeletal muscle.¹³ Although we previously reported that α -cyano-4-hydroxycinnamate (CHC), an MCT4 inhibitor, increased the inhibition of growth of RD cells induced by cerivastatin, one of the statins,¹⁴ it is unclear whether statin-induced RD cell cytotoxicity is associated with MCT4 expression. The aim of this study was to determine the relationship between statin-induced cytotoxicity and MCT4 expression in RD cells.

2. Materials and methods

2.1. Materials

Statins were obtained from Tokyo Chemical Industry (Tokyo, Japan). All other reagents were of the highest grade available and used without further purification.

2.2. Cell culture

RD cells, a prototypic embryonal rhabdomyosarcoma cell line, are tumor cells of skeletal muscle origin that affect children and young adults and express a number of muscle-specific proteins,¹⁵ and these cells have been used as a model for studying the myotoxic effects of statins.^{16,17} RD cells were maintained in plastic culture flasks (Corning Incorporated Corning) as described previously.¹⁸

2.3. MTT assay

The 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) assay was performed as described previously.¹⁹ MTT is normally reduced by dehydrogenases of viable cells and transformed to formazan. Living, but not dead, cells are detected by this assay, and the signal generated is dependent on the degree of activation of cells. For the MTT assay, RD cells were transfected with Negative control or MCT4 siRNA and seeded on 96-well plastic plates. Following cell attachment (24 h), various concentrations of statins were added for the times indicated. Before the end of treatment, 10 μ L of PBS-containing MTT solution (0.5%) was added, and the cells were incubated for a further 1 h. The MTT medium was then replaced with dimethyl sulfoxide, and

absorbance was read at 590 nm. Absorbance measured in MTT assays was expressed as percent of the control (defined as 100%).

2.4. Quantitative real-time PCR

Quantitative real-time PCR was performed using an Mx3000™ Real-time PCR System (STRATAGENE, Tokyo, Japan) with a KAPA SYBR Fast qPCR kit (KAPA Biosystems, Boston, MA) as per the manufacturer's protocol. PCR was performed using human MCT4-specific primers through 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min or using human 18S-specific primers. The primers specific to human MCT4 and 18S were designed on the basis of sequences in the GenBank™ database (accession no.: NM_004207, NM_001530 and NR_003286, respectively). The sequences of the specific primers were as follows: the sense sequence was 5' -ATT GGC CTG GTG CTG CTG ATG-3' and the antisense sequence was 5' -CGA GTC TGC AGG AGG CTT GTG-3' for human MCT4, and the sense sequence was 5' -CGG CTA CCA CAT CCA AGG AA-3' and the antisense sequence was 5' -GCT GGA ATT ACC GCG GCT-3' for human 18S. The PCR products were normalized to amplified 18S, which was the internal reference

2.5. Knockdown of MCT4 using small interfering RNA

A Silencer[®] Validated siRNA targeted to the MCT4 gene (siRNA 1 (siRNA ID#: 117233) or siRNA 2 (siRNA ID#: s17416)) and nontargeting siRNA as a Silencer[®] Negative control siRNA were purchased from Ambion (Austin, TX). The siRNA transfection was optimized using Lipofectamine[™] RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Nontargeting siRNA or MCT4 siRNA (final concentration of 10 nM) and OPTI-MEM[®] I Reduced Serum Medium (GIBCO, Grand Island, NY) were mixed in plastic plates and incubated at room temperature for 15 min after addition of Lipofectamine[™] RNAiMAX. Then suspended RD cells (1.5×10^5 cells/mL) in a growth medium without antibiotics were added. After siRNA transfection (24 h), the medium was replaced with DMEM containing 10% FBS with or without statins for an additional 72 h and then the cells were analyzed using the following assays.

2.6. Western blot analysis

Western blot analysis was performed as described previously.¹² Total protein extracts were prepared from RD cells. The cells were scraped and centrifuged at $1,500 \times g$ for 1 min at 4 °C. The pellet was suspended in 1 mL of PBS and centrifuged at $1,500 \times g$ for

1 min at 4 °C. The resulting pellet was suspended in a lysis buffer containing 1.0% Triton X-100, 0.1% SDS and 4.5 M urea. The suspension was allowed to stand for 5 min and was sonicated for 15 min at 4 °C. The suspension was then centrifuged at 12,000 × g for 15 min at 4 °C, and the protein concentration in the clear supernatant was determined by the method of Lowry *et al.*²⁰ The samples were denatured at 100 °C for 3 min in a loading buffer containing 50 mM Tris–HCl, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.002% BPB and 3.6 M urea and separated on 4.5% stacking and 10% SDS polyacrylamide gels. Proteins were transferred electrophoretically onto polyvinylidene difluoride membranes at 15 V for 90 min. The membranes were blocked with PBS containing 0.05% Tween 20 (PBS/T) and 1% non-fat dry milk for 1 h at room temperature. After being washed with PBS/T, the membranes were incubated with rabbit anti-MCT4 antibody (sc-50329, Santa Cruz Biotechnology, Santa Cruz, CA) (diluted 1:100) or mouse anti-actin monoclonal antibody (Millipore, Bedford, MA, USA) (diluted 1:500) for 24 h at room temperature and washed three times with PBS/T for 10 min each time. The membranes were subsequently incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a

dilution of 1:4000 and washed three times with PBS/T for 10 min each time. The bands were visualized by enhanced chemiluminescence according to the instructions of the manufacturer (Amersham, Buckinghamshire, UK).

2.7. Caspase assay

The caspase assay was performed as described previously.²¹ Cells were lysed with a cell culture lysis reagent (Promega, Madison, WI). Protein concentration of the cell lysate was adjusted to 10 µg/mL, and the cell lysate was subjected to caspase-3/7 colorimetric protease assays for measuring Ac-DEVD-pNa and Ac-LEHD-pNa cleavage as described in the manufacturer's protocol (Promega, Madison, WI).

2.8. Data analysis

Student's t-test was used to determine the significance of differences between two group means. Statistical significance among means of more than two groups was evaluated using Dunnett's test or Tukey's test. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Effects of atorvastatin on cell viability and expression of MCTs in RD cells

We previously reported that alteration of monocarboxylate transporter (MCT) 4 function was associated with MCT4 expression levels in RD cells¹³ and that lipophilic statins, atorvastatin, simvastatin and fluvastatin, significantly reduced RD cell viability in a concentration-dependent manner.¹⁷ We therefore used RD cells and atorvastatin, simvastatin and fluvastatin in this study. First, we examined the effect of atorvastatin on RD cell viability and alteration in MCT4 mRNA level. Atorvastatin reduced the number of viable cells in a concentration-dependent manner and up-regulated MCT4 mRNA level (Fig. 1). Moreover, mRNA level of MCT4 in RD cells was also up-regulated by simvastatin and fluvastatin (Supplemental Fig. 1). On the other hand, MCT1 was reported to be responsible for L-lactate uptake, and L-lactate efflux was shown to be mediated by MCT4 in RD cells¹⁸. We therefore also investigated the effect of atorvastatin on MCT1 mRNA level. Atorvastatin did not affect mRNA level of MCT1 (Supplemental Fig. 2). The results suggest that atorvastatin-induced cytotoxicity is associated with MCT4 mRNA level but not with MCT1 mRNA level.

3.2. Establishment of MCT4 knockdown conditions in RD cells

To clarify whether alteration in MCT4 expression is associated with statin-induced cytotoxicity, we performed MCT4 knockdown in RD cells. In this study, different siRNA constructs targeted to the MCT4 gene, MCT4 siRNA 1 and MCT4 siRNA 2, were used. Quantitative real-time RT-PCR and Western blot analysis were performed to quantify MCT4 mRNA and protein levels at 72 h after transfection of siRNA to RD cells. MCT4 siRNA 1 and 2 (10 nM) significantly decreased MCT4 mRNA and protein levels ($p < 0.01$; Fig. 2A-B) in RD cells. The knockdown effect of MCT4 siRNA 2 was stronger than that of MCT4 siRNA 1. MCT4 seems suited to play a role in the extrusion of lactate from glycolytic fibers. To clarify whether MCT4 siRNA transfection affects extracellular lactate content, we examined lactate content in the medium at 72 h after transfection of siRNA to RD cells. MCT4 siRNA 2 significantly decreased extracellular lactate content compared with the negative control (Supplemental Fig. 3A). Moreover, LDH activity alteration may affect intracellular and extracellular lactate contents. LDH activity was not significantly different in RD cells transfected with the negative control and two siRNAs (Supplemental Fig. 3B). We established MCT4 knockdown conditions using MCT4 siRNA in RD cells and selected the conditions in the following study.

3.3. Suppression of statin-induced cytotoxicity by MCT4 knockdown

We examined the effects of MCT4 knockdown on reduction of RD cell viability by statins. As shown in Fig. 3A, atorvastatin significantly reduced negative control cell viability in a concentration-dependent manner. On the other hand, MCT4 siRNAs 1 and 2 had a relieving effect on atorvastatin-induced reduction of cell viability. We previously reported that statin caused caspase-9, -3/7 activation and mainly induced apoptosis *via* a mitochondrial stress-induced cascade in RD cells.²¹ Next, we compared the caspase-3/7 activation potencies of atorvastatin in RD cells transfected with the negative control or MCT4 siRNAs. As shown in Fig. 3B, atorvastatin significantly enhanced the activity of caspase-3/7 in negative control-treated cells in a concentration-dependent manner. On the other hand, MCT4 siRNA 2 abolished atorvastatin-induced caspase-3/7 activation completely. These results suggest that atorvastatin-induced reduction of cell viability and apoptosis are associated with MCT4 expression. To confirm the effect of MCT4 knockdown on other statins, we examined the effects of simvastatin and fluvastatin on the viability of RD cells transfected with MCT4 siRNA 2. As shown in Fig. 4, simvastatin and fluvastatin significantly reduced cell viability and enhanced the activity of caspase-3/7 in negative control-treated cells. On the other hand, MCT4 siRNA 2 prevented these statin-induced reductions of cell viability and caspase-3/7 activation as in the case of atorvastatin. These observations

indicate that suppression of MCT4 expression leads to prevention of statin-induced reduction of cell viability and apoptosis.

4. Discussion

In a large study in the USA, the incidences of statin-induced rhabdomyolysis leading to hospitalization were zero for pravastatin, approximately 0.5 per 10,000 person-years for simvastatin and atorvastatin, and 5.3 per 10,000 person-years for cerivastatin.²² Moreover, we previously reported that atorvastatin, simvastatin and fluvastatin significantly reduced cell viability more than did pravastatin.¹⁷ Therefore, we used atorvastatin, simvastatin and fluvastatin in this study. Our study showed that 1) statins reduced the number of viable cells in a concentration-dependent manner and up-regulated MCT4 level, but not MCT1 mRNA level, 2) MCT4 siRNA (10 nM) significantly decreased MCT4 mRNA and protein levels in RD cells, and 3) MCT4 knockdown suppressed statin-induced reduction of cell viability and apoptosis compared with negative control-treated cells. Statins induce various forms of muscular toxicity, but the exact mechanism of statin-induced myopathy is unclear. Sirvent *et al.* reported that simvastatin-induced impairment of mitochondria and alteration of Ca²⁺ homeostasis occurred in intact rat skeletal muscle fibers from the flexor digitorum

brevis muscle but not in intact ventricular rat cardiomyocytes and that the simvastatin-induced alteration of Ca^{2+} homeostasis was abolished when MCT4 was inhibited, indicating that the mechanism operates specifically in skeletal muscles.²³

Moßhammer *et al.* reported that it is not clear whether MCT4-mediated transport of statins across membranes of myocytes plays a role in myopathy.²⁴ We found that statin-induced cytotoxicity was associated with intracellular accumulation of statins.¹⁷

We hypothesized that suppression of statin-induced cytotoxicity by MCT4 knockdown is associated with intracellular accumulation of statins. Therefore, we examined the effect of MCT4 knockdown on accumulation of atorvastatin in RD cells. The intracellular accumulation of atorvastatin was not significantly different in negative control-treated cells and MCT4 siRNA-treated cells (Supplemental Fig. 4). These observations indicate that the suppressive effect of MCT4 knockdown on statin-induced cytotoxicity is not associated with intracellular accumulation of statins. The uptake transporter organic anion-transporting polypeptide (OATP) 2B1 (SLCO2B1) and the efflux transporters multidrug resistance-associated proteins 1 (MRP1, ABCC1), 4 (MRP4 ABCC4) and 5 (MRP5, ABCC5) have been shown to be expressed on the sarcolemmal membrane of human skeletal muscle fibers, suggesting a role of OATP2B1 in sensitizing skeletal muscle cells to statin toxicity and roles of the statin efflux

transporters MRP1, MRP4 and MRP5 in protection of muscle from toxicity.²⁵ These transporters may be involved in statin accumulation in skeletal muscle. Recently, Bonifacio *et al.* reported the importance of the AKT/mTOR signaling pathway in statin-induced myotoxicity.²⁶ Statins elicit effects on the AKT signaling pathway by the well-established mitochondrial toxicity of these compounds.²⁷⁻²⁹ By impairing the function of the mitochondrial respiratory chain, statins decrease the skeletal muscle ATP content and increase the ADP content,³⁰ leading to the activation of AMPK. AMPK activation was shown to induce the expression of MCT4.¹² Moreover, atorvastatin induced the expression of phospho-acetyl-CoA carboxylase (p-ACC), resulting in AMPK activation in RD cells (data not shown). We previously reported that L-lactate efflux was mediated by MCT4 in RD cells¹⁸ and that lipophilic statins significantly inhibited [¹⁴C] L-lactate transport in a concentration-dependent manner in MCT4-expressing cells.³¹ Accordingly, inhibitory effects of statins on L-lactate transport mediated by MCT4 suggest the risk of leading to over-accumulation of the acid and disturbance of RD cell pH homeostasis and thereby the possibility of inducing intracellular acidification and apoptosis in RD cells. We also have found that cerivastatin-induced apoptosis was associated with intracellular acidification and that bicarbonate prevented cerivastatin-induced intracellular acidification.²¹ Collectively, the

results suggest that both increased expression of MCT4 and disturbance of RD cell pH homeostasis may play an important role in statin-induced cytotoxicity. In conclusion, our results showed that MCT4 knockdown suppressed statin-induced reduction of cell viability and apoptosis in an RD cell line as a model of *in vitro* skeletal muscle. We hope that the results of this study will contribute to an understanding of the mechanism of statin-induced adverse effects.

Acknowledgments

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Figure legends

Fig. 1. Effects of atorvastatin on cell viability (A) and MCT4 mRNA level (B) in RD cells

(A) RD cells were exposed to atorvastatin (1, 10 μ M) for 72 hours. Each column represents the mean with S.D. of 10 determinations. *; significantly different from the control at $p < 0.01$.

(B) RD cells were exposed to atorvastatin (1, 10 μ M) for 72 hours. Each column represents the mean with S.D. of 7 determinations. *; significantly different from the control at $p < 0.01$.

Fig. 2. Effects of MCT4 siRNA on MCT4 mRNA level (A) and protein level (B) in RD cells

RD cells were transfected with MCT4 siRNA 1 (10 nM) or MCT4 siRNA 2 (10 nM) for 72 hours.

(A) Each column represents the mean with S.D. of 3-12 determinations. *; significantly different from negative control at $p < 0.01$.

(B) Each column represents the mean with S.D. of 3-5 determinations. *; significantly different from negative control at $p < 0.01$.

Fig. 3. Effects of MCT4 siRNA on cell viability (A) and caspase-3/7 activity ratio**(B) in atorvastatin-treated RD cells**

RD cells were transfected with MCT4 siRNA 1 (10 nM) or MCT4 siRNA 2 (10 nM) for 72 hours.

(A) RD cells were exposed to atorvastatin (1, 5 μ M) for 72 hours. Each point represents the mean \pm S.D. of 5-12 determinations. *, significantly different from the control at $p < 0.01$.

(B) RD cells were exposed to atorvastatin (1, 5 μ M) for 72 hours. The cell lysate (100 ng protein) was used to determine caspase-3/7 activity ratio. Each column represents the mean with S.D. of 3-15 determinations. *, **; significantly different from the control at $p < 0.05$, $p < 0.01$.

Fig. 4. Effects of MCT4 siRNA on cell viability (A) and caspase-3/7 activity ratio**(B) in RD cells treated with other statins**

The conditions were identical to those described in the legend to Fig. 3.

(A) RD cells were exposed to simvastatin and fluvastatin (1, 5 μ M) for 72 hours. Each point represents the mean \pm S.D. of 6 determinations. *, significantly different from the control at $p < 0.01$.

(B) RD cells were exposed to 1 μ M simvastatin and fluvastatin for 72 hours. The cell lysate (100 ng protein) was used to determine caspase-3/7 activity ratio. Each column represents the mean with S.D. of 6-13 determinations. *, significantly different from the control at $p < 0.01$.

Supplemental figure legends

Supplemental Fig. 1. Effects of simvastatin (A) and fluvastatin (B) on MCT4 mRNA level in RD cells.

(A) RD cells were exposed to 1 μ M simvastatin for 72 hours. Each column represents the mean with S.D. of 3 determinations. *, significantly different from the control at $p < 0.01$.

(B) RD cells were exposed to 1 μ M fluvastatin for 72 hours. Each column represents the mean with S.D. of 6 determinations. *, significantly different from the control at $p < 0.01$. ”

Supplemental Fig. 2. Effect of MCT4 siRNA on MCT1 mRNA level in RD cells.

RD cells were exposed atorvastatin (1, 10 μ M) for 72 hours. Each column represents the mean with S.D. of 7-8 determinations.

Supplemental Fig. 3. Effects of MCT4 siRNA on extracellular lactate content (A) and LDH activity (B) in RD cells

RD cells were transfected with MCT4 siRNA 1 (10 nM) or MCT4 siRNA 2 (10 nM) for 72 hours.

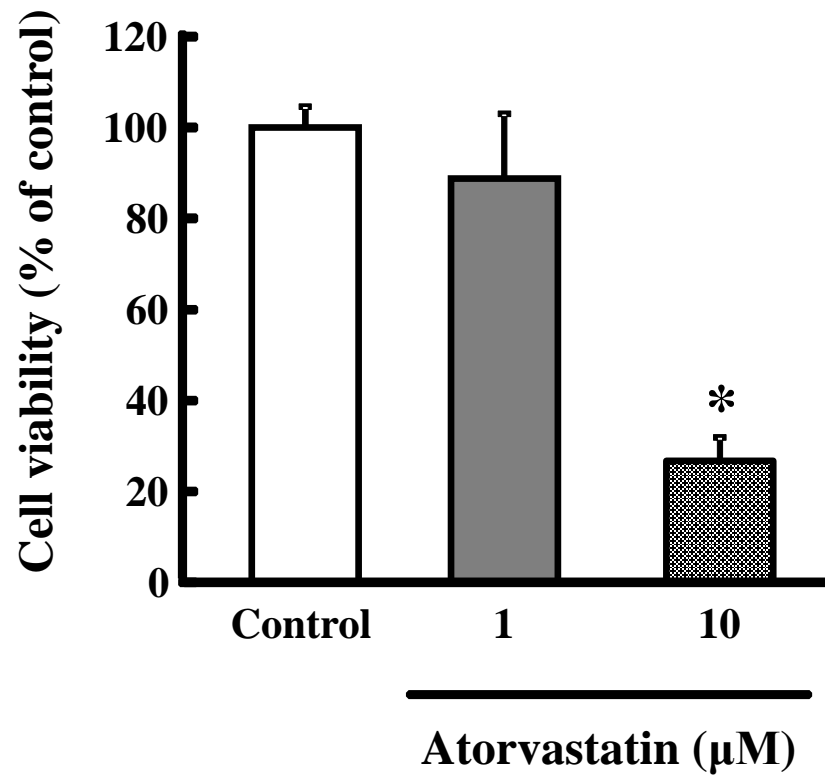
(A) Each column represents the mean with S.D. of 4-10 determinations. *; significantly different from negative control at $p < 0.01$.

(B) Each column represents the mean with S.D. of 3-9 determinations.

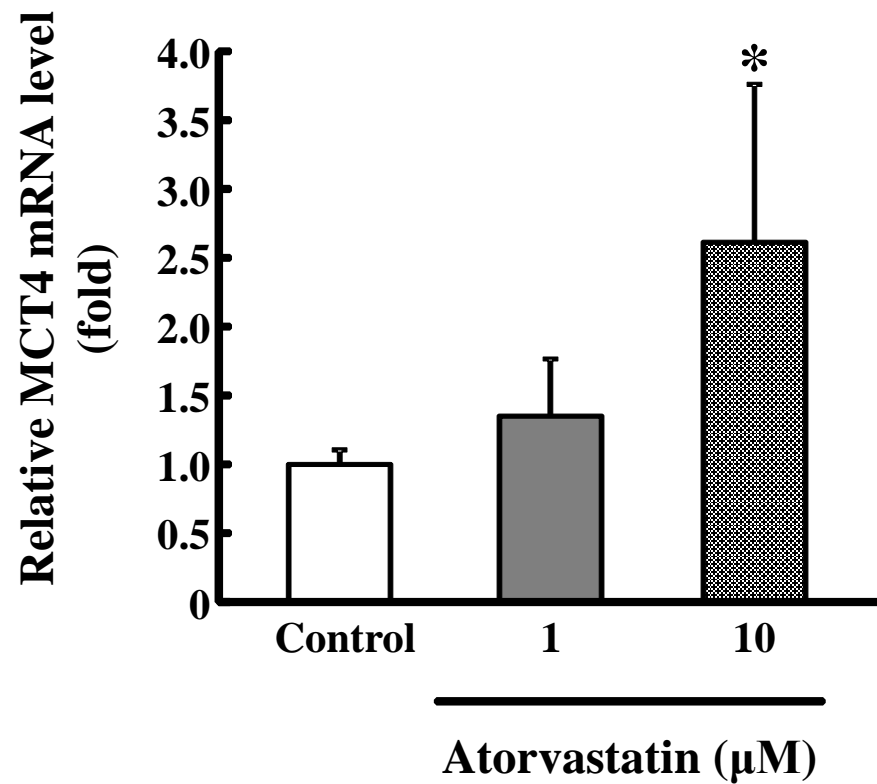
Supplemental Fig. 4. Effect of MCT4 siRNA on atorvastatin accumulation in RD cells.

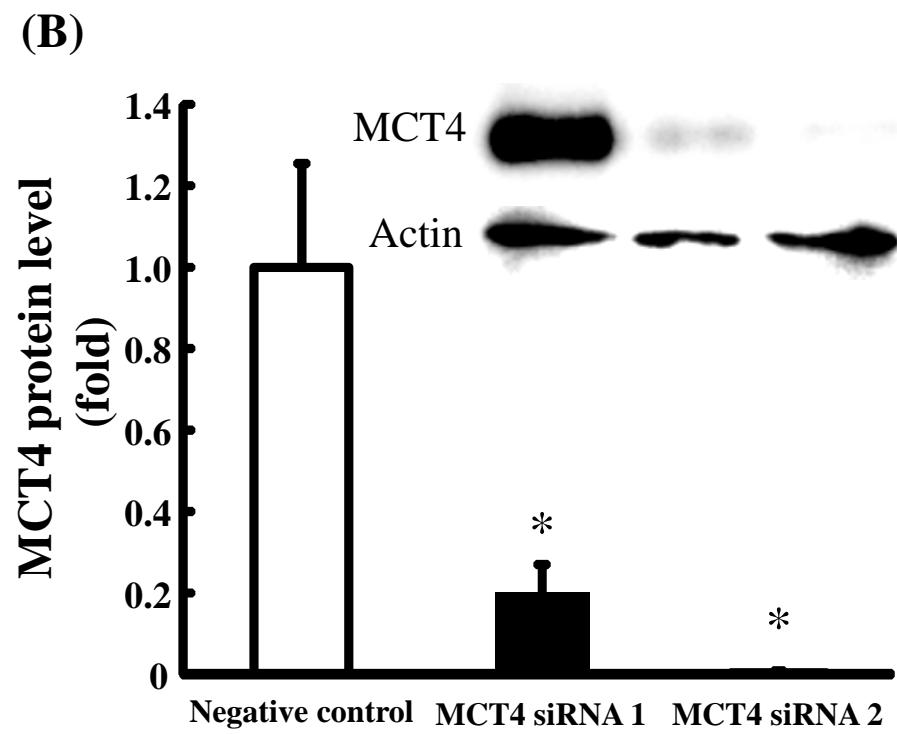
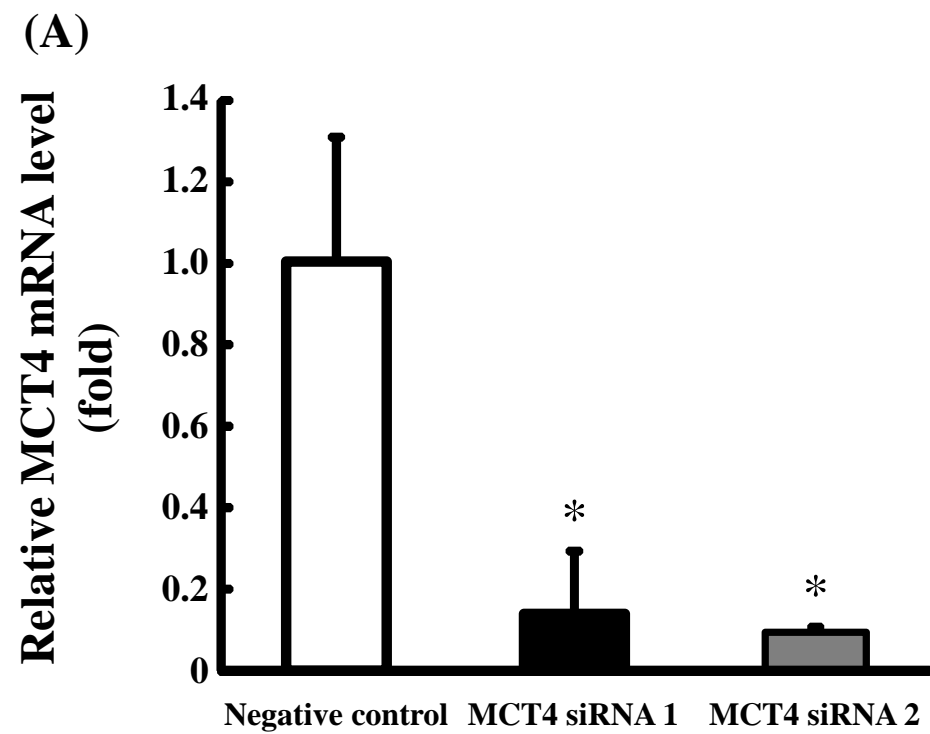
The bars (n=3-4) are given as means with S.E. of three independent experiments.

(A)



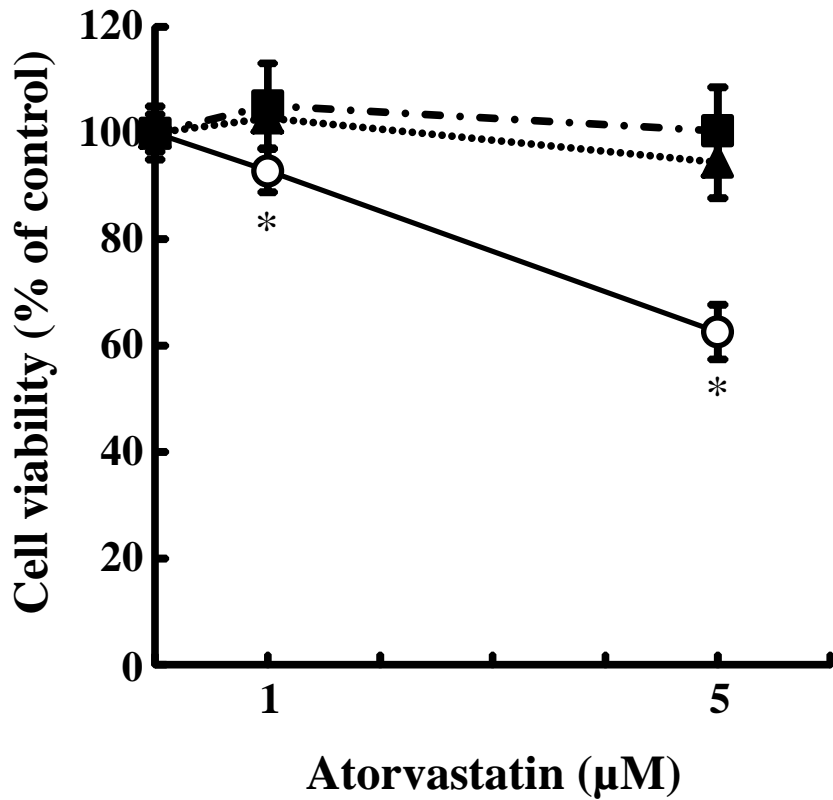
(B)





(A)

—○— Negative control ···▲··· MCT4 siRNA 1
 -■- MCT4 siRNA 2



(B)

□ Negative control ■ MCT4 siRNA 1
 ■ MCT4 siRNA 2

