Human Skeletal Muscle Drug Transporters Determine Local Exposure and Toxicity of Statins

Michael J. Knauer, Bradley L. Urquhart, Henriette E. Meyer zu Schwabedissen, Ute I. Schwarz, Christopher J. Lemke, Brenda F. Leake, Richard B. Kim, Rommel G. Tirona

Rationale: The 3-hydroxy-3-methylglutarate coenzyme A reductase inhibitors, or statins, are important drugs used in the treatment and prevention of cardiovascular disease. Although statins are well tolerated, many patients develop myopathy manifesting as muscle aches and pain. Rhabdomyolysis is a rare but severe toxicity of statins. Interindividual differences in the activities of hepatic membrane drug transporters and metabolic enzymes are known to influence statin plasma pharmacokinetics and risk for myopathy. Interestingly, little is known regarding the molecular determinants of statin distribution into skeletal muscle and its relevance to toxicity.

Objective: We sought to identify statin transporters in human skeletal muscle and determine their impact on statin toxicity in vitro.

Methods and Results: We demonstrate that the uptake transporter OATP2B1 (human organic anion transporting polypeptide 2B1) and the efflux transporters, multidrug resistance–associated protein (MRP)1, MRP4, and MRP5 are expressed on the sarcolemmal membrane of human skeletal muscle fibers and that atorvastatin and rosuvastatin are substrates of these transporters when assessed using a heterologous expression system. In an in vitro model of differentiated, primary human skeletal muscle myoblast cells, we demonstrate basal membrane expression and drug efflux activity of MRP1, which contributes to reducing intracellular statin accumulation. Furthermore, we show that expression of human OATP2B1 in human skeletal muscle myoblast cells by adenoviral vectors increases intracellular accumulation and toxicity of statins and such effects were abrogated when cells overexpressed MRP1.

Conclusions: These results identify key membrane transporters as modulators of skeletal muscle statin exposure and toxicity. (Circ Res. 2010;106:297-306.)

Key Words: statins • drug transporters • myopathy

HMG-CoA (3-hydroxy-3-methylglutarate coenzyme A) reductase inhibitors, statins, are highly effective drugs for the treatment of hypercholesterolemia, a major risk factor of cardiovascular disease. Statins inhibit the synthesis of mevalonate, the rate-limiting step in cholesterol biosynthesis.1,2 Although statins are generally well tolerated,3 skeletal muscle side effects are commonly reported among those treated. One such side effect, myalgia, which is defined as muscle aches or weakness in the absence of blood creatine kinase elevation, occurs in 5% to 15% of statin-treated patients.2,4–8 In rare cases, potentially life-threatening statin-induced rhabdomyolysis may occur, a condition characterized by acute muscle damage, resulting in pronounced elevation in creatine kinase levels and possible renal failure.9

The pathophysiology of statin-induced myopathy is not completely understood. The leading mechanism suggests a role for cellular depletion of secondary metabolic intermediates of mevalonate in the development of statin-induced myotoxicity.10 In addition to decreased cholesterol synthesis, HMG-CoA reductase inhibition by statins causes a commensurate reduction in the levels of downstream metabolic products including isoprenoids, dolichol, and ubiquinone (coenzyme Q10).10–13 Among these are the isoprenoid secondary metabolic intermediates geranylgeranylpyrophosphate and farnesylpyrophosphate that are involved in protein isoprenylation and activation of small GTPases such as Rho and Rab. The important role for diminished isoprenylation in the mechanism of statin myotoxicity is related to induction of the muscle atrophy-linked protein atrogin-1.12 This is highlighted by the findings that supplementation of geranylgeranylpyrophosphate to cultured skeletal myotubes or isolated myofibers treated with statins leads to attenuation of toxicity,11,13–15 whereas inactivation of a Rab and RhoA induces toxicity.11,13 Decreased geranylgeranylation of small GT-
Non-standard Abbreviations and Acronyms

<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>BCRP</td>
<td>breast cancer resistance protein</td>
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<tr>
<td>HMG-CoA</td>
<td>3-hydroxy-3-methylglutaryl coenzyme A</td>
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<tr>
<td>HSMM</td>
<td>human skeletal muscle myoblast</td>
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<tr>
<td>MRP</td>
<td>multidrug resistance–associated protein</td>
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<tr>
<td>OATP</td>
<td>organic anion transporting polypeptide</td>
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<td>OAT</td>
<td>organic anion transporter</td>
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Pases by statins appears to stimulate the mitochondrial apoptotic cell death pathway in skeletal myotubes. In addition to isoprenoids, coenzyme Q10 levels in plasma and skeletal muscle are decreased with statin treatment. Although depletion of coenzyme Q10 is thought to affect oxidative phosphorylation and protection from statin-induced oxidative stress, compelling clinical evidence is lacking regarding the efficacy of coenzyme Q10 treatment of statin myopathy. There is recent evidence to indicate that fatty acid oxidation is perturbed in cultured myotubes of statin intolerant (myalgic) patients, a finding that differs from patients with rhabdomyolysis. Such data suggest that the mechanisms of statin toxicity are different between those affected with myalgia and rhabdomyolysis.

It is well documented that myotoxicity is statin dose-dependent, and myopathy risk increases when statins are coadministered with drugs that either interact to increase plasma statin levels or themselves have propensity for muscle damage. Indeed, macrodilic antibiotics and azole antifungals are well-known drug inhibitors of hepatic statin metabolism via cytochrome P450 enzymes, dramatically increasing plasma statin levels. Moreover, inhibition of statin liver uptake (transport) mediated by multiple members of the organic anion transporting polypeptide (OATP) family by drugs such as gemfibrozil, cyclosporine A and rifampin can elevate drug levels. Furthermore, we have previously reported that a common genetic polymorphism resulting in a single nucleotide difference in SLCO1B1 gene encoding hepatic OATP1B1 is associated with increased plasma levels of a number of statins. In a genome-wide association study, the rs4149056 polymorphism in SLCO1B1 was found to be the most robust predictor of the risk for simvastatin-induced myopathy. In addition, efflux transporters in liver that mediate secretion of statins into bile could play a role in risk for statin myopathy. For example, genetic variation in the statin biliary efflux transporters multidrug resistance–associated protein (MRP) and breast cancer resistance protein (BCRP) is associated with variability in pravastatin and rosuvastatin plasma levels, respectively.

Despite that the currently marketed statins have varying physicochemical characteristics, membrane transporters that act to facilitate drug uptake or efflux in tissues appear to have significant influence on the pharmacokinetics of most statins. This is evidenced by marked changes in plasma drug levels following transporter inhibition or through their attendant genetic polymorphisms. Considerable attention has been given to drug transporters in the small intestine, kidney, and liver, which affect systemic exposure to statins. However, there is a paucity of studies that have examined statin transporters within skeletal muscle and their influence on myotoxic side effects of statins, despite that this has been considered conceptually. Given that plasma drug levels do not entirely predict risk for statin myopathy, we hypothesize that factors which control local skeletal muscle statin concentrations, such as muscle transporters, may be more relevant. Specifically, we propose that the interplay between statin uptake versus efflux transporters modulates the response to skeletal muscle statin exposure.

In this study, we identify drug transporters in human skeletal muscle that affect the distribution of 2 prototypic lipophilic and hydrophilic statins, atorvastatin and rosuvastatin. We demonstrate that the uptake transporter, OATP2B1, and the efflux transporters, MRP1, MRP4, and MRP5 are expressed in skeletal muscle and are capable of transporting atorvastatin and rosuvastatin. Importantly, we show that by affecting drug transporter activity in a model of human skeletal muscle, statin toxicity can be modulated.

Methods
An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Gene Expression Analysis
Tissue and cell culture expression of transporters was determined by quantitative polymerase chain reaction, and Western blotting and immunofluorescence microscopy were performed as described in the Online Data Supplement.

Identification of Statin Transporters
Heterologous expression of transporters in human cervical cancer cells (HeLa) by recombinant vaccinia virus method and statin transport activity assays were performed as described previously with modifications detailed in the Online Data Supplement.

Statin Accumulation and Toxicity in Skeletal Muscle Cells
Overexpression of transporters in cultured primary differentiated human skeletal muscle myoblast (HSMM) (Lonza, Walkersville, Md) cells by adenoviral gene delivery, statin cellular accumulation assays, as well as toxicity assays (ATP content, mitochondrial methylthiazolyldiphenyl-tetrazolium bromide reduction, caspase 3/7 activation) are described in detail in the Online Data Supplement.

Results
Identification of Statin Transporters in Skeletal Muscle
Little is known about the expression of drug transporters in human skeletal muscle. Therefore, we screened a cDNA library of human skeletal muscle for expression of a wide variety of drug transporters including OATPs, organic anion transporters (OATs), organic cation transporters, MRP1, MRP4, and BCRP, and P-glycoprotein (P-gp). The known statin uptake transporters such as OATP1B1, OATP1B3, OAT1, and OAT3, as well as efflux transporters such as MRP2, P-gp, and BCRP, were not detected in skeletal muscle (not shown). However, we detected mRNA expression of the known statin uptake transporter OATP2B1 (Figure...
The mRNA level of OATP2B1 was highly detectable in skeletal muscle, although it is significantly lower than tissues with very high expression (liver, kidney and small intestine). Given that MRP2 transports statins, the expression of other members of this efflux transporter family was examined revealing high expression of MRP1, MRP4, and MRP5 in skeletal muscle. Quantitative mRNA analysis and Western blot confirmed the expression of these drug transporters in human skeletal muscle (Figure 1A and 1B). Immunolocalization of OATP2B1, MRP1, MRP4, and MRP5 in normal human skeletal muscle was determined by immunofluorescence confocal microscopy. Transporters are shown in green, whereas nuclei are shown in blue. Scale bar=50 μm.

We then determined whether the transporters identified in skeletal muscle were capable of statin transport using a recombinant (vTF-7) vaccinia virus overexpression system in a human cervical cancer cell line (HeLa). As we previously described, OATP2B1 is capable of rosuvastatin transport and here we confirm that atorvastatin is also a transport substrate (Figure 2). Indeed, in this model, OATP2B1 increases the cellular accumulation of rosuvastatin and atorvastatin by 2-fold. Although OATP2B1 is the relevant transporter for uptake of statins into skeletal muscle, we present new and confirming data that other OATPs (1B1, 1B3 and 1A2) transport both atorvastatin and rosuvastatin (Figure 2). It should be noted that the differences in statin transport between OATP2B1 and other transporters as shown in this model (Figure 2) likely do not reflect the relative statin uptake efficiencies in vivo, because transporter expression was not normalized and the absolute expression of these transporters in different tissues is undetermined.

Drug interactions involving inhibition of the major liver OATPs (1B1 and 1B3) are associated with elevated plasma statin levels. Similarly, OATP2B1 is susceptible to inhibition by coadministered medications. Here, we show that stimulated intracellular accumulation of atorvastatin and rosuvastatin by OATP2B1 is attenuated after coincubation with cerivastatin, gemfibrozil, gemfibrozil-glucuronide, fenofibrate, rifampin, and glyburide (Figure 3). Interestingly, incubation with cyclosporine A caused a significant reduction in rosuvastatin accumulation but increased atorvastatin levels (Figure 3).

The transport efficiency of human OATPs compared to rodent OATPs is lower in this experimental system.
instance, the rat ortholog of the human transporters OATP1B1 and OATP1B3, rOatp1b2, appeared capable of mediating a significantly greater accumulation of rosvastatin and atorvastatin into cells using this technique, in relation to human OATP2B1 (Figure 2). For this reason, we used rOatp1b2 as the model transporter to maintain statin uptake into cells thereby allowing for the identification of pertinent transporters capable of statin efflux (see below).

Although highly expressed in skeletal muscle, it was not known whether MRP1, MRP4, or MRP5 transported statins. Hence, HeLa cells were double transfected with the uptake transporter rOatp1b2 and various efflux transporters. In this system, modulation of cellular retention of atorvastatin and rosvastatin into cells using this technique, in relation to human OATP2B1 (Figure 2). For this reason, we used rOatp1b2 as the model transporter to maintain statin uptake into cells thereby allowing for the identification of pertinent transporters capable of statin efflux (see below).

Although highly expressed in skeletal muscle, it was not known whether MRP1, MRP4, or MRP5 transported statins. Hence, HeLa cells were double transfected with the uptake transporter rOatp1b2 and various efflux transporters. In this system, modulation of cellular retention of atorvastatin or rosvastatin served as an indicator for efflux transport activity. Indeed, we confirm that rosvastatin and atorvastatin are transported by MRP2, P-gp, and BCRP using this double transporter (uptake/efflux) transfection system (Figure 3). Rosuvastatin retention in cells expressing rOatp1b2 together with MRP1, MRP4, or MRP5 was lower (77%, 80%, and 27% lower, respectively) than cells expressing rOatp1b2 alone (Figure 4). This was also true for atorvastatin when MRP1 and MRP4 (63% and 47% lower, respectively), but not MRP5, were double transfected with rOatp1b2. These findings demonstrate that MRP1, MRP4, and MRP5 are novel statin transporters.

Statin Disposition in an In Vitro Model of Human Skeletal Muscle
To evaluate the role of statin transport in toxicity, we used differentiated, primary human skeletal muscle myoblast (HSMM) cells as an in vitro model. First, we assessed whether HSMM cells expressed statin efflux transporters and found that MRP1, MRP4, and MRP5 are constitutively expressed (Figure 1A and 1B), whereas MRP2, BCRP, and P-gp are absent. Cellular localization studies revealed that some MRP1 is expressed on the cell surface of HSMM cells, although significant levels of the transporter are found in intracellular spaces. By contrast, MRP4 and MRP5 are not found on the plasma membrane, but localize within the Golgi in HSMM cells (Figure 5A). To test whether functional MRP activity is present in HSMM cells, statin accumulation was examined after chemical inhibition of efflux transport. When
we overexpressed OATP2B1 and MRP1 in HSMM cells using adenoviral vectors (Ad-OATP2B1 and Ad-MRP1, respectively). Examination by confocal microscopy demonstrated robust overexpression of these transporters in HSMM cells (Figure 6). There was significant plasma membrane expression of MRP1, whereas for OATP2B1 there was some transporter on the cell membrane, but the majority was confined intracellularly.

Adenoviral overexpression of OATP2B1 in HSMM cells caused a significant increase in the cellular retention of both atorvastatin and rosuvastatin over 60 minutes (Figure 6B). After transduction with Ad-OATP2B1 and Ad-MRP1, the cellular retention of both rosuvastatin and atorvastatin were significantly attenuated compared to Ad-OATP2B1 alone. Transduction with Ad-MRP1 alone did not significantly reduce the levels of rosuvastatin in HSMM cells; however, there was a trend toward reduced atorvastatin accumulation. Consequently, the effect of statin efflux transporters on intracellular statin levels is not pronounced in the absence of influx transporters. These results indicate that overexpression of OATP2B1 and MRP1 leads to changes in intracellular statin levels in an HSMM cell model.

Preliminary studies were performed to define the time- and concentration dependency for atorvastatin and rosuvastatin cytotoxicity in HSMM cells. Cell viability, as evaluated by intracellular ATP levels, declined after 5 days of statin treatment. At comparable concentrations (eg, 100 μmol/L), atorvastatin was more toxic than rosuvastatin (Figure 7A). The cytotoxicity of atorvastatin and rosuvastatin in HSMM cells after adenoviral-mediated transporter gene delivery was assessed by 2 measures of mitochondrial function, ATP content and MTT reduction to formazan (Figure 7B through 7D), as well as activation of Caspases 3/7, a marker of apoptosis induction (Figure 7D). Transduction of HSMM cells with Ad-OATP2B1 sensitized HSMM cells to atorvastatin toxicity as demonstrated by signals from all 3 toxicity end-points (Figure 7B through 7D). There was a similar trend for rosuvastatin toxicity but only the increased activity of caspases 3/7 reached statistical significance (Figure 7D). This result is likely because overexpressed OATP2B1 was not well localized on the plasma membrane, leading to modest elevation of intracellular statin concentrations. Similarly, when HSMM cells were transduced with Ad-MRP1 alone, there was a lack of effects on statin cytotoxicity, consistent with the absence of significant changes in intracellular statin accumulation. However, when cells were transduced with both Ad-OATP2B1 and Ad-MRP1, there was protection against toxicity by both statins when compared to HSMM cells transduced with Ad-OATP2B1 alone to viabilities similar to those cells transduced with Ad-MRP1 alone (Figure 7B through 7D). Taken together, these results indicate that OATP2B1 expression promotes statin toxicity, whereas MRP1 is cytoprotective in human skeletal muscle cells.

**Discussion**

Drug transporters have recently been implicated in statin-induced myopathy. However, those that have been previously considered have been the transporters located in the liver and small intestine, which are largely responsible for controlling

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**Role of Transporters in Statin Toxicity in an In Vitro Model of Human Skeletal Muscle**

We next examined the effect of transporters on skeletal muscle exposure and toxicity of statins. In this experiment, HSM cells were coincubated with known MRP inhibitors MK-571, dipyridamole, quercetin, or verapamil, there was a significant increase in cellular retention for both atorvastatin and rosuvastatin when compared to cells treated with vehicle (Figure 5B). Together with membrane localization studies, the results indicate that HSM cells natively express functional MRP1 transporter whose activity determines the intracellular accumulation of statins. Coincubation with cerivastatin also significantly increased the cellular retention of atorvastatin and rosuvastatin in HSM cells (Figure 5B), suggesting that this statin interacts with and is a possible substrate of MRPs. With respect to statin uptake, none of the known statin uptake transporters was expressed in HSM cells, and, importantly, OATP2B1 is not expressed despite that it is present in vivo (Figure 1).

**Figure 4.** Intracellular accumulation of statins in HeLa cells transiently transfected with rOatp1b2 and various efflux drug transporters. Intracellular accumulation of [3H]rosuvastatin and [3H]atorvastatin in HeLa cells expressing rOatp1b2 and/or MRP1, MRP2, MRP4, MRP5, P-gp, or BCRP. Results are presented as percentages of rOatp1b2-mediated uptake±SEM (n = 3 to 8). ***P < 0.001 compared to vector control and efflux transporter alone; †††P < 0.001 compared with rOatp1b2 mediated uptake. The presence (+) or absence (–) of transporter expression in various tissues is shown below.

**Figure 5.** Intracellular accumulation of statins in HSMM cells expressing rOatp1b2 and/or various efflux drug transporters. Intracellular accumulation of [3H]atorvastatin and [3H]rosuvastatin in HSMM cells, and, importantly, OATP2B1 is not expressed
plasma statin concentrations. Indeed, genetic polymorphisms in the hepatic statin uptake transporter, OATP1B1, leading to reduced transport function are associated with a dramatic increase in risk for simvastatin-related myopathy.32 Moreover, inhibition of both OATP1B1 and liver glucuronidation activity by concomitant treatment with the antilipidemic drug gemfibrozil causes elevation of cerivastatin plasma concentrations conferring greater predisposition to rhabdomyolysis.29,56 Although high plasma statin level is thought to be a risk factor, it does not entirely predict myopathy. In fact, there are individuals who exhibit high statin plasma levels but do not develop myopathy, suggesting that other factors including skeletal muscle fiber statin concentration may have an impact on side effect risk.38 Despite the recognition that drug transporters control intracellular statin concentrations, the relevant transporters in human skeletal muscle have long been overlooked.

In this report, we identified drug transporters in human skeletal muscle capable of transporting statins. Previous reports have shown that OATP2B1 is a high-affinity uptake transporter for both atorvastatin and rosuvastatin.40,49 OATP2B1 is expressed on the apical and basolateral membranes of enterocytes and hepatocytes, respectively, and contributes to the oral absorption and hepatic distribution of statins. In addition, OATP2B1 is localized on the plasma membrane of cardiac endothelial cells,49 as well as in platelets,50 where it is thought to be involved in the pleiotropic cardiovascular effects of statins. Here, we show for the first time, OATP2B1 is similarly expressed on human skeletal muscle sarcolemmal membrane. These findings are consistent with a report that suggested the presence of Oatp1a4 and Oatp2b1 in rat skeletal myofibers at the mRNA level.57 However, demonstration of rat Oatp1a4 and Oatp2b1 protein expression in muscle was not confirmed, nor were data presented to show that these transporters mediate statin uptake.57 Despite that direct measurement of statin accumulation was not monitored, cotreatment of rat skeletal myofibers with the OATP inhibitor estrone sulfate afforded protection against the toxicity of the hydrophilic and lipophilic statins pravastatin and fluvastatin, respectively.57 The known statin efflux transporters, namely P-gp, MRP2 and BCRP are not expressed in human skeletal muscle (Figure 1A). However, isoforms of the MRP transporter family such as MRP1, MRP4, and MRP5 are highly expressed in skeletal muscle, although previous to this report, their capacity for statin efflux was unknown. Here, we demonstrate that the 3 human skeletal muscle MRPs (MRP1, MRP4, and MRP5) transport rosuvastatin and/or atorvastatin. These transporters are expressed on the sarcolemmal membrane of muscle fibers, indicating a protective role against intracellular statin accumulation. There is wide substrate overlap among MRPs,58 and this is certainly also the case for statins that are transported by the skeletal muscle MRPs, albeit at differing efficiencies. Recently, a role for rat Mrp1 in statin-induced myopathy has been suggested in studies that demonstrate precipitation of rosuvastatin-mediated skeletal

Figure 5. A, Immunofluorescence localization of MRP1, MRP4, and MRP5 (green), Golgi (red), and nuclei (blue) in differentiated HSMM cells using confocal microscopy. Scale bar=20 μm. B, Intracellular accumulation of [3H]rosuvastatin and [3H]atorvastatin after 30 minutes in HSMM cells cotreated with or without 100 μmol/L cerivastatin or the nonspecific MRP inhibitors dipyridamole, MK-571, quercetin, and verapamil at 100 μmol/L. Results are presented as percentages of DMSO control±SEM (n=3 to 4). **P<0.01, ***P<0.001 compared with DMSO control.
muscle toxicity in rats cotreated with the MRP inhibitor, probenecid. Interpretation of these findings remains difficult for a number of reasons, including a lack of demonstration that rat Mrp1 transports rosuvastatin, absence of Mrp1 expression data in tissues such as skeletal muscle, and a deficiency of information regarding differences in plasma and tissue concentrations of rosuvastatin after probenecid cotreatment.

The dynamic interplay between uptake and efflux transporter activities likely controls muscle fiber statin concentrations, which determines susceptibility to toxicity. We have shown that the toxicity of rosuvastatin and atorvastatin in primary human skeletal muscle cells is dependent on the achieved intracellular drug concentrations. This is highlighted by the findings that reduction of cellular statin accumulation by MRP1 overexpression in cultured skeletal muscle cells heterologously expressing OATP2B1 (Figure 6B) afforded cytoprotection against statin exposure (Figure 7C and 7D). In the guinea pig, skeletal muscle concentrations of rosuvastatin and atorvastatin are less than 10% of that found in plasma, suggesting that the balance is tipped toward higher efflux than uptake activity. In our evaluation of the literature, the plasma-to-skeletal muscle concentration ratio of statins in humans is not known, but this value will undoubtedly be dependent on the relative expression and intrinsic activities of the attendant uptake (OATP2B1) and efflux (MRP1, MRP4, MRP5) transporters. The present results would also suggest that drug–statin interactions occurring not only at the level of the hepatocyte cell membrane but also in skeletal muscle fibers could contribute to myopathy. Certainly, a number of clinically used drugs are substrates/inhibitors of the here identified skeletal muscle statin transporters (Figures 3 and 5B). Our data suggest that skeletal muscle statin uptake by OATP2B1 can be inhibited by concomitantly administered drugs such as gemfibrozil, fenofibrate, and glyburide (Figure 3). This finding could be considered contradictory to the increased risk of statin myopathy in patients cotreated with gemfibrozil. However, it should be mentioned that the gemfibrozil inhibits hepatic statin clearance to increase systemic statin exposure and there remains the possibility that inhibition of skeletal muscle efflux transport could offset the protection provided by OATP2B1 inhibition. Indeed, we demonstrate that statin efflux can be blocked by concurrent treatment of HSMM cells with known inhibitors of MRPs (Figure 5B). Moreover, one must consider not only pharmacokinetic but also pharmacodynamic interactions in extrapolating the current transport inhibition findings to myopathy risk.

There are limitations with the differentiated HSMM cell model for the study of statin toxicity. Firstly, although MRP1, MRP4, and MRP5 are constitutively expressed in HSMM cells, only MRP1 localized to the plasma membrane (Figure 5A). This is in contrast to immunofluorescence data that show these MRPs are expressed on the sarcolemmal membrane of intact skeletal muscle fibers (Figure 1C). It is for this reason that we are only able to assess the effect of MRP1 but not MRP4 or MRP5 on cytoprotection against statins in this model of skeletal muscle. Furthermore, HSMM cells do not natively express OATP2B1, as is found in vivo; hence, we required viral gene delivery to assess the role of uptake
transport on statin toxicity. That OATP2B1 is not expressed in HSMM cells compares well with other skeletal muscle genes that we have found at very low levels in relation to intact skeletal muscle, including creatine kinase M isoform (Online Figure I). Therefore, in interpreting the present toxicity findings, it should be considered that this in vitro model of skeletal muscle differs phenotypically to muscle fibers in vivo.

In conclusion, statin transporters are present in human skeletal muscle that control intracellular drug exposure. We propose a role for OATP2B1 in sensitizing skeletal muscle cells to statin toxicity and that the novel statin efflux transporters MRP1, MRP4, and MRP5 protect muscle from toxicity. The dynamic functional interplay between these uptake and efflux transporters in vivo likely determines risk for statin-induced myopathy.

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Disclosures
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SUPPLEMENT MATERIAL

Detailed Methods

Reagents. [3H] atorvastatin (5 Ci/mmol, 99% radiochemical purity) and [3H] rosuvastatin (5 mCi/mmol, 99% radiochemical purity) was purchased from American Radiolabeled Chemicals (St. Louis, MO). [3H] rosuvastatin (79 Ci/mmol, 97.1% radiochemical purity) and unlabeled rosuvastatin were also kindly provided by Dr. Yi Wang (AstraZeneca, Wilmington, DE). Unlabeled atorvastatin, cerivastatin and gemfibrozil-glucuronide were obtained from Toronto Research Chemicals (North York, ON). All other chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

Plasmids. Expression plasmids for OATP1A2, OATP1B1, OATP1B3, OATP2B1, rOatp1b2, BCRP and P-gp are described elsewhere. MRP2 and MRP4 cDNA was provided by Dr. R.H. Ho (Vanderbilt University) and Dr. J.D. Schuetz (St. Jude Children’s Research Hospital), respectively. MRP1 cDNA was obtained by PCR, using Expand Long Template PCR System (Roche Applied Science, Indianapolis, IN), from a cDNA library of human skeletal muscle cDNA using oligonucleotide primers 5'-ACGCCATGCGCTCCGGGCGCTCAGC-3' and 5'-GTCCTGCTCGTGCTGGGCTCAACCA-3'. Similarly, OAT3 was amplified from human kidney using the primers: 5'-AGTGCCATGCGCTTCTGGAAGATCCTGGG-3' and 5'-GTTGTCCCTGCAAGCAGCCCAGGCCCTGG-3'. MRP5 cDNA was amplified in two parts from skeletal muscle with the following primers: 5'-CTCCACCTGAGAGATGAAGGATATCAGC-3' and 5'-CCACATAGCGAAGGTGTCCTGCAACCA-3'; 5'-AACCCTCTCTATTTTCCACCGCTTCTGG-3' and 5'-GGGTGTAGATCTACAAGGGGAGGTACGCCC-3'. Full-length MRP5 was assembled from the two parts by ligation at the XbaI site. The PCR products for MRP1, MRP5 and OAT3 were cloned into pcDNA3.1/V5-His-TOPO vector (Invitrogen).

Adenoviral vectors. Adenoviral vectors containing LacZ, OATP2B1, or MRP1 were generated in pAD/CMV/V5-DEST using the ViraPower Adenoviral Expression System (Invitrogen, Carlsbad, CA). The titer of the virus preparations were measured by infection of HEK293 cells on 24-well plates with limiting dilutions of the viral stock using Adeno-X Rapid Titer Procedure (Clontech, Mountain View, CA).

Gene Expression Analysis. The mRNA expression of OATP2B1, MRP1, MRP2, MRP4, MRP5, and breast cancer resistance protein (BCRP) were measured by SYBR green quantitative real-time PCR with an ABI Prism 7700 sequence detection system (Applied Biosystems). Human skeletal muscle total RNA was sourced from BioChain (Hayward, CA). Total RNA was extracted from cultured cells using Trizol reagent (Invitrogen) and RNA quality and quantity was determined using Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA). cDNA was synthesized using Multiscribe reverse transcriptase (Applied Biosystems) according to manufacturer’s instruction and 38 ng of cDNA was used in each PCR reaction. The sequences of primers used for quantitative PCR are listed in Online Table I. The amount of the transporter was normalized to 18S-rRNA and relative expression was determined using the ΔΔCt method.

Immunoblot Analysis. HSMM cells were harvested in 5 mmol/L Tris-HCl (pH 7.4) flash frozen in liquid nitrogen and homogenized to obtain total cellular protein. Normal human skeletal protein lysate was purchased from BioChain (Hayward,CA). Protein samples were separated by SDS-PAGE and transferred to NuPAGE nitrocellulose membrane Western blotting system (Invitrogen, Carlsbad, CA). HeLa cells overexpressing OATP2B1, MRP1, MRP4 or MRP5 using a vtf7 vaccinia virus method, described below, was used as positive control. Membranes were probed with custom-made rabbit polyclonal OATP2B1 antibody based on a C-terminus epitope (CSPAVEQQLVSGPGKPEDSRV), MRP1 (Alexis Biochemicals), MRP4 M5I-80 (Kamiya), or MRP5 H-100 (Santa Cruz). Anti-rat and rabbit horseradish
peroxidase-labeled antibodies (Bio-Rad, Hercules, CA) were used as the secondary. The immobilized secondary antibody was detected using the ECL Plus Western Blotting Detection System (GE Healthcare) and KODAK ImageStation 4000 MM (Mandel).

**Immunohistochemistry.** Paraffin-embedded sections from normal human skeletal muscle tissue (5 μm) were obtained from BioChain. The tissue sections were deparaffinized in xylol and rehydrated with graded solutions of ethanol/water. For heat-induced epitope retrieval, the tissue sections were boiled in citrate buffer (10 mmol/L, pH 6.0). After washing twice in ice-cold PBS, the slides were blocked with 2% fetal bovine serum (FBS) – phosphate buffered saline (PBS). Thereafter, the slides were incubated with diluted anti–OATP2B1, MRPr1, MRP4 M4I-80 or MRP5 H-100 in a humidified atmosphere for 2h at room temperature. After several washing steps with PBS, the sections were incubated with the fluorescent-labeled secondary antibody (Invitrogen). After washing the slides with PBS, the tissue was mounted in antifading mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Images were obtained by confocal fluorescence microscopy. As negative control, the primary antibody was omitted.

**Cell Culture.** Primary human skeletal muscle myoblast (HSMM) cells were obtained from Lonza (Walkersville, MD). HSMM cells were cultured in SkBM-2 medium (Lonza) according to the manufacturer’s instructions at 37°C in a humidified atmosphere of 5% CO₂/95% air. The cells were plated onto 12-well plates for transport studies and 96-well plates for toxicity assays at a density of ~1 x 10⁶ cells/mL. When the HSMM cells achieved ~70% confluence in SkBM-2 growth medium, cells were cultured in differentiation medium, DMEM-F12 (Lonza) supplemented with 2% horse serum (Invitrogen) for one week. The resulting differentiated cells were then cultured in SkBM-2 growth medium for two weeks before experiments.

**Identification of Statin Transporters.** HeLa cells (human cervical cancer cell line) were grown in 12-well plates (approximately 0.8 x 10⁶ cells/well) and infected with vaccinia (vtf-7) at a multiplicity of infection of 10 plaque-forming units/cell in serum-free Opti-MEM I medium (Invitrogen) and allowed to absorb for 30 minutes. Cells were transfected with 1 µg of transporter cDNA or parental plasmid lacking insert as control using Lipofectin (Invitrogen) and incubated at 37°C for 16h. Double transfection experiments of both uptake and efflux transporters used 1 µg total transporter cDNA in a 1:4 ratio of uptake to efflux. The parental plasmid lacking insert was used in place of uptake or efflux transporter cDNA in the single transfections. Transport was evaluated using labeled substrate as previously reported⁴. Drug accumulation was determined at selected time intervals by washing cells three times with ice-cold PBS followed by lysis with 1% sodium dodecylsulfate. Retained cellular radioactivity was quantified by liquid scintillation spectrometry.

**Statin Accumulation and Toxicity in Skeletal Muscle Cells.** HSMM cells were transduced with the adenoviruses containing the transporter coding region in SkBM-2 medium containing a total of 1 x 10⁷ infectious units per mL (IFU/mL). For single transporter overexpression, cells were transduced with 5 x 10⁶ IFU/mL of Ad-MRP1 or Ad-OATP2B1 as well as 5 x 10⁶ IFU/mL of Ad-LacZ, whereas for overexpression of two transporters, 5 x 10⁶ IFU/mL of each adenovirus was used. After 24h, cells were treated with various concentrations of atorvastatin or rosuvastatin for 48-72h. Cellular viability was evaluated by methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay (see below), measurement of ATP content using CellTiter-Glo Luminescent Cell Viability Assay (Promega Biosciences, San Luis Obispo, CA) and assessment of caspase 3 and 7 activation using Caspase-Glo 3/7 Assay (Promega).

**MTT Assay.** The MTT assay was performed on HSMM cells in 96-well plates with approximately 1 x 10⁵ cells per well. The cells were treated with rosuvastatin and atorvastatin 24h after transduction with adenovirus. After the cells were incubated for 72h, 25 μL of 5 mg/mL MTT in PBS was added to each well. The plates were incubated for 4 h at 37°C and the formazan formed was dissolved in 50
µL DMSO. The background absorbance at 670 nm was subtracted from the absorbance at 560 nm to obtain the raw absorbance data.

**Statistical Analysis.** Statistical differences between group parameters was determined by 1-way ANOVA or 2-way ANOVA, using Bonferroni’s multiple comparison test, as appropriate (GraphPad Software Inc, San Diego, CA). A $P$ value of $<0.05$ was considered statistically significant.
Online Figure I. Characterization of primary cultured human skeletal myoblasts (HSMM). Morphology of HSMM cells pre-differentiation (A) and post-differentiation (B). HSMM cells were stained with phalloidin Alexa 488 (Invitrogen) to visualize actin and DAPI for nuclei. Expression of the skeletal muscle-selective genes MyoD (C) and creatine kinase isoform M (CK-M) (D) in pre-differentiated and post-differentiated cultured HSMM cells, as well as, intact human skeletal muscle was determined by quantitative PCR.
Online Table I. Real-time PCR primers used for gene expression quantitation.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tbody>
<tr>
<td>18S</td>
<td>5'-GTAACCCCGTTGAACCCATT-3'</td>
<td>5'-CCATCCAATCGGTAGTAGCG-3'</td>
</tr>
<tr>
<td>OATP2B1</td>
<td>5'-CTTCATCTCGGAGCCATACC-3'</td>
<td>5'-AGATGCTGGTTTCTGTGTA-3'</td>
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<td>MRP1</td>
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<td>5'-ACCTCCTCATTCGCATCCACCTTG-3'</td>
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<td>MyoD</td>
<td>5'-AGTAAATGAGCTTTTGACACT-3'</td>
<td>5'-TCGATATAGCAGGATGCGTT-3'</td>
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Supplemental References.


