Simvastatin represses protein synthesis in the muscle-derived C2C12 cell line with a concomitant reduction in eukaryotic initiation factor 2B expression


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Tuckow AP, Jefferson SJ, Kimball SR, Jefferson LS. Simvastatin represses protein synthesis in the muscle-derived C2C12 cell line with a concomitant reduction in eukaryotic initiation factor 2B expression. Am J Physiol Endocrinol Metab 300: E564–E570, 2011.—Statins are a widely prescribed class of cholesterol lowering drugs whose use is frequently associated with muscle-related ailments. A number of mechanisms have been implicated in statin-induced myotoxicity including alterations in both protein synthesis and protein degradation. The objective of the present study was to explore the mechanism(s) contributing to the statin-induced reduction in protein synthesis in the muscle-derived C2C12 cell line. Cells were treated with 10 μM simvastatin or vehicle alone for 24 h in 1% serum. Cells exposed to simvastatin exhibited reduced rates of protein synthesis, as evidenced by [35S]methionine and [35S]cysteine incorporation into protein. The reduction in protein synthesis occurred with a concomitant decrease in expression and activity of eukaryotic initiation factor 2B (eIF2B), a regulated and rate-controlling guanine nucleotide exchange factor known to affect global rates of protein synthesis. The reductions in protein synthesis and eIF2B expression were prevented by coincubation with mevalonate. Simvastatin treatment also resulted in a proteasome-sensitive reduction in the protein expression of all the subunits of the eIF2B heteropentameric complex. Finally, increased phosphorylation of the catalytic e-subunit at Ser535 was observed, an event consistent with an observed reduction in eIF2B activity. These results suggest that repression of eIF2B expression and activity may contribute, at least in part, to the statin-induced reduction in protein synthesis.

messenger RNA translation; 3-hydroxy-3-methylglutaryl coenzyme A-reductase; myoblasts; myotubes

STATINS [3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors] are a widely prescribed and effective class of lipid lowering drugs that have demonstrated enormous clinical benefit not only for reducing cardiovascular risk (26) but also for several additional and unexpected pleiotropic benefits (e.g., anti-inflammatory and immunomodulatory effects) (10, 23). One of the less desirable effects of statin use is the incidence of myopathies ranging from complaints of muscle pain and weakness (i.e., myalgia) to the most severe case of rhabdomyolysis, which is rare but can be fatal (2, 34). The mechanisms implicated in the myotoxic effects of statins are unclear. In this context, it is important to note that eIF2B is implicated in β-adrenergic mediated cardiomyocyte hypertrophy (12) and that overexpression of eIF2B increases GEF activity and/or protein synthesis in a number of different cell types (3, 12, 35, 40, 42). In contrast, reductions in eIF2B expression and/or activity occur in rat skeletal muscle when protein synthesis and muscle growth is impaired (13, 38). Thus, the purpose of the current investigation was to further elucidate potential mechanisms of the statin-associated repression of protein synthesis. Specifically, we investigated the effects of simvastatin treatment on protein synthesis and eIF2B expression and in the muscle-derived C2C12 cell line.

MATERIALS AND METHODS

Cell culture. Simvastatin (Sigma-Aldrich, St. Louis, MO) and MG-132 (CalBiochem/EMD Biosciences, La Jolla, CA) were prepared as 10 mM stock solutions in DMSO. Mevalonate was purchased as mevalonolactone (Wako Pure Chemical Industries, Osaka, Japan), prepared as a 0.5 M stock in water, and when utilized was added to the medium at a final concentration of 100 μM for the entire 24-h treatment period. C2C12 myoblasts (ATCC, Manassas, VA) were routinely maintained in growth medium (GM), consisting of Dulbecco’s modified Eagle’s medium (DMEM; GIBCO-Invitrogen, Carlsbad, CA) containing 25 mM glucose, 10% fetal bovine serum (FBS; Atlas Biologics), and 1% penicillin-streptomycin (Invitrogen), at 37°C and 5% CO2. Myoblasts were initially seeded at 3.75 × 104 (control) or 5.0 × 104 (simvastatin-treated) cells per 60-mm culture dish in GM for 16 h prior to treatment. In experiments utilizing myoblasts, cells were seeded at a higher density in the simvastatin-treated group on the basis of pilot experiments determining that cell
confluence was more similar to the controls on the day of harvest. Myoblasts were differentiated into myotubes by seeding $5.0 \times 10^5$ myoblasts per 60-mm dish and incubating in GM for 24 h followed by 3 days of incubation in serum-free DMEM with 1% FBS and either simvastatin (10 µM final concentration) or an equal volume of DMSO vehicle (control) for 24 h prior to harvest for measurement of the protein synthesis rate or eIF2B activity.

Protein synthesis. Cells were seeded and treated as described above. During the last 30 min of the 24-h simvastatin treatment, 5 µl of $[^{35}S]$EasyTag Express protein labeling mix (11.0 mCi/ml; NEN, Boston, MA) was added directly to the cell culture medium. Global rates of $[^{35}S]$methionine and $[^{35}S]$cysteine into TCA-precipitable protein were measured as described previously (9).

eIF2B activity assay. The eIF2B guanine nucleotide exchange activity of control or simvastatin-treated cell lysates was assayed as previously described (9). Linear regression analysis was performed to calculate the slope for each assay, which was corrected for total protein content of the lysates as determined by detergent-compatible protein assay (Bio-Rad, Hercules, CA).

Western blot analyses. For protein abundance and phosphorylation status, 2.0 × 10^5 cells (for differentiation into myotubes and control myoblasts) or 2.5 × 10^5 (for myoblasts treated with simvastin) were seeded into six-well culture dishes and treated as described above. In some experiments, MG-132 (final concentration 25 µM) or an equal volume of DMSO vehicle was added directly to the medium for the final 8 h of simvastatin or DMSO treatment (i.e., hours 16–24). At the time of harvest, cells were washed with ice-cold PBS, harvested directly into 1× Laemml sample buffer (200 µl), and boiled for 5 min at 100°C. An equal volume of sample was loaded into each lane of a 4–15% gel and subjected to SDS-PAGE and subsequent Western blotting as previously described (18). Membranes were incubated overnight at 4°C with one of the following primary antibodies diluted in TBS-T: eIF2B (rabbit polyclonal generously provided by Glen N. Barber, University of Miami); eIF2Bβ, -γ, -δ, and -ε (mouse monoclonal); eIF2α (mouse monoclonal (32)); eIF2Be phospho-Ser$^{355}$ and eIF2α phospho-Ser$^{51}$ (rabbit polyclonal; Biosource-Invitrogen); Akt phospho-Ser$^{473}$ and total (rabbit polyclonal; Cell Signaling Technology, Danvers, MA); GSK-3β total (rabbit polyclonal; Calbiochem-EMD Biosciences, La Jolla, CA); GSK-3β phospho-Ser$^{9}$ (rabbit polyclonal, Cell Signaling); and GAPDH (rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA). All proteins analyzed for total protein abundance were normalized to GAPDH density for the corresponding sample, while phosphorylation states were quantitated as a ratio of phosphospecific band density to the density for the corresponding total protein abundance.

RNA isolation and quantitative real-time PCR analysis. Mouse C2C12 myoblasts were seeded and treated as described in the procedures for Western blot analyses. At the time of harvest, cells were washed with ice-cold PBS and total cellular RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. RNA pellets were resuspended in 20 µl of Ambion RNA Storage Solution, and RNA quantity and purity were assessed via NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). RNA (1 µg) was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The resulting cDNA was diluted 1:16 in nuclease-free water, and 2 µl of the diluted cDNA was used in a 20-µl reaction using the Quantitect SYBR Green (Qiagen, Valencia, CA) master mix according to the manufacturer’s protocol with gene-specific primers. The quantitative real-time PCR (qRT-PCR) analysis was performed in triplicate on a StepOnePlus Real-Time PCR system (Applied Biosystems) with a relative standard curve for each primer set. The mRNA abundance for each of the eIF2B subunits was normalized to GAPDH mRNA abundance for the corresponding sample (mouse GAPDH forward primer 5’-GTTGCTTCCCTGCGACTTCA-3’, reverse primer 5’-TGCTGATGCCATATTGCT-3’). Samples were analyzed from three independent experiments, each performed in triplicate, and are expressed as a ratio of the control values (mean ± SE). The primers directed against the mouse eIF2B subunits were as follows: eIF2Bβ, forward 5’-TAATGAGCTGCTGGAACTGGA-3’, reverse 5’-ACGGCACTTGCAAAATCAATG-3’; eIF2β, forward 5’-TGAGGACTCTACATGGAAGCCACAAC-3’, reverse 5’-AGGATGACGCAATGAAGATTGC-3’; eIF2Be, forward 5’-AGTTCTAAGGCGGATACCT-3’, reverse 5’-AGCAGCAAAAGAATAATTGG-3’.

Statistical analyses. Data were analyzed with Prism version 5 (GraphPad Software). Data between two groups (i.e., control and simvastatin-treated samples) were compared via unpaired t-tests. For mevalonate and proteasome inhibition experiments comparing multiple treatment groups, a one-way analysis of variance (ANOVA) was employed followed by Newman-Keuls post hoc multiple comparisons (when the ANOVA detected significant differences among treatments). A value of $P < 0.05$ was considered statistically significant.

RESULTS

Treatment of cells with simvastatin for 24 h resulted in a significant reduction in global rates of protein synthesis as measured by incorporation of $[^{35}S]$methionine and $[^{35}S]$cysteine into protein, with similar effects observed for myoblasts ($-26.43 \pm 5.33\%$ vs. control, $P < 0.001$) and myotubes ($-22.40 \pm 4.91\%$ vs. control, $P < 0.001$). The reduction in protein synthetic rate due to simvastatin treatment was prevented by coinubcation with 100 µM mevalonate (i.e., the product of HMG-CoA reductase; Fig. 1).

As described in the introduction, altered expression of the guanine nucleotide exchange factor eIF2B, particularly of its catalytic ε-subunit, is one mechanism implicated in the dysregulation of protein synthesis in skeletal muscle. In the presence of simvastatin, the rate of incorporation of $[^{35}S]$Met/Cys was significantly reduced in myoblasts and myotubes when compared to the control group ($P < 0.001$). The results of these experiments are summarized in Table 1. The data are expressed as a percentage of the control group, with the values presented as the mean ± SE. The reduction in protein synthesis was dose-dependent, with a significant reduction observed at simvastatin concentrations of 1 µM (Fig. 1A) and 10 µM (Fig. 1B). The results were consistent across multiple experiments performed in triplicate, and the data were analyzed using a one-way ANOVA followed by Newman-Keuls post hoc multiple comparisons. The data are presented as a bar graph with error bars indicating the standard error of the mean. The results are consistent with previous studies, which have demonstrated that simvastatin inhibits protein synthesis in skeletal muscle by reducing the rate of eIF2B guanine nucleotide exchange activity.
ent investigation, simvastatin treatment of myoblasts and myotubes significantly decreased the relative protein abundance of eIF2Bε (Fig. 2). The reduction in eIF2Bε expression was a direct result of the inhibition of HMG-CoA reductase, as coincubation of simvastatin-treated cells with 100 μM mevalonate prevented the decrease in eIF2Bε expression (Fig. 2). To examine the possibility that decreased eIF2Bε protein expression was a consequence of reduced mRNA abundance, qRT-PCR analysis was performed on control and simvastatin-treated myoblasts. The relative abundance of eIF2Bε mRNA was unaltered (1.00 ± 0.03 control vs. 1.02 ± 0.04 simvastatin treated). Another potential mechanism for reduced expression of eIF2Bε relative to other proteins in the simvastatin-treated cells is via degradation by the 26S proteasome. Several groups have implicated induction of components of the ubiquitin-proteasome system in skeletal muscle with statin use (11, 21, 36), which can lead to selective degradation of ubiquitinated target proteins. To examine this possibility, cells were treated with the proteasome inhibitor MG-132 during the final 8 h of simvastatin treatment. Inhibition of the proteasome during simvastatin treatment restored eIF2Bε protein expression to control values (Fig. 3, A and F). Based on the decrease in total eIF2Bε protein expression in response to simvastatin treatment, the effect of the drug on mRNA and protein expression of the other subunits of the holoenzyme was also assessed. Simvastatin treatment resulted in significantly reduced protein expression of the other four subunits of eIF2B following 24 h of treatment (Fig. 3, A–E). As with eIF2Bε, the presence of proteasome inhibitor for the final 8 h prevented the simvastatin-induced decrease of the four remaining subunits (α, β, γ, δ). The relative mRNA abundance for eIF2Bβ was unaffected by simvastatin treatment (1.00 ± 0.02 control vs. 0.97 ± 0.02 simvastatin treated); in contrast, the eIF2Bγ mRNA exhibited a small but significant reduction (1.00 ± 0.05 control vs. 0.81 ± 0.06 simvastatin treated; P < 0.05).

Since eIF2B activity is a rate-controlling step in translation initiation and has been implicated in the control of protein synthesis in skeletal muscle (7, 19) and myoblasts (16), we measured the GEF activity of eIF2B by use of an in vitro assay. The activity of eIF2B in the lysates of cells treated with simvastatin was repressed by ~20% compared with control cells (Fig. 4A). To examine additional potential mechanisms that might account for repression of eIF2B activity, Western blots were performed to assess the phosphorylation of Akt (Ser473), GSK-3β (Ser9), eIF2α (Ser51) and eIF2Bε (Ser535). Simvastatin treatment resulted in decreased Akt phosphorylation at Ser473 (Fig. 4B), with concomitant reductions in GSK-3β phosphorylation at Ser9 (Fig. 4C). Reduced phosphorylation of GSK-3β at Ser9 leads to increased activity of the kinase toward its substrates, including eIF2Bε. In accord with the changes in Akt and GSK-3β phosphorylation, the relative phosphorylation of eIF2Bε at Ser535 was significantly increased in response to statin treatment (Fig. 4D). Phosphorylation of eIF2Bε at Ser535 is considered to be repressive with regard to the enzyme’s activity (14, 41), which is consistent with the observed results. It is important to note that the absolute amount of eIF2Bε present in the phosphorylated form was unchanged in response to simvastatin treatment. However, because total eIF2Bε abundance was reduced, the relative phosphorylation was increased. Thus, the change in relative phosphorylation may not necessarily reflect a change in GSK-3 activity toward eIF2Bε. Another well-known mechanism resulting in inhibition of eIF2B activity is phosphorylation of the α-subunit of its substrate (eIF2α) at Ser51 (30). In the experiments performed herein, simvastatin treatment did not alter eIF2α phosphorylation at Ser51 (Fig. 4E), suggesting that phosphorylation of eIF2α was not a factor contributing toward the observed reduction in eIF2B activity.

![Fig. 2. Simvastatin treatment reduces eukaryotic initiation factor (eIF2Bε) protein expression. C2C12 myoblasts (A) or myotubes (B) were treated with vehicle (control) or 10 μM simvastatin for 24 h in the absence or presence of 100 μM mevalonate as indicated. Equal volumes of cell lysates were subjected to Western blot analysis using antibodies against eIF2Bε and normalized to GAPDH abundance for the corresponding sample. Results in each panel represent means ± SE of 3 experiments, each performed in triplicate, and are expressed as %mean control values. Differences among treatment groups (one-way ANOVA, P < 0.05) were subsequently analyzed via Newman-Keuls multiple comparison tests. Different letters denote statistical differences among treatment groups (P < 0.05); treatment groups with similar letters are not statistically different.](http://ajpendo.physiology.org/Downloadedfrom9/20,2017http://ajpendo.physiology.org/Downloadedfrom9/20,2017)
DISCUSSION

The mechanisms behind statin-associated myopathies remain perplexing, and a number of possibilities have been proposed and/or implicated, including mitochondrial toxicity and induction of apoptosis (37), autophagy (1), enhanced proteolytic degradation via multiple pathways (21), and repressed protein synthesis (8, 22, 28). In the present investigation, we show that simvastatin treatment of the muscle-derived C2C12 cell line results in a repression of global rates of protein synthesis, in part via alteration in eIF2B expression and guanine nucleotide exchange activity. The reduction in eIF2B activity was associated with increased relative phosphorylation of eIF2Bα as well as a proteasome-sensitive reduction in eIF2B subunit protein expression. The effect of MG-132 in statin-treated cells implies that eIF2B may be targeted for degradation by the proteasome during simvastatin treatment. Little is known regarding the stability of the eIF2B complex in mammalian cells; however, a single mutation in any one of several of the eIF2B subunits can lead to decreased protein stability of the subunit as well as the stability of other subunits (29). In the current investigation, it is possible that one subunit may have been ubiquitinated and targeted to the proteasome, whereupon the remaining subunits of the complex become more labile, resulting in reduced expression of the additional subunits (which can be prevented by proteasome inhibition). An alternative explanation is an indirect effect whereby the proteasome targets interacting or upstream regulatory protein(s) that affects eIF2B subunit expression.

The direct mechanism of action of the statin family of drugs is the inhibition of HMG-CoA reductase resulting in reduced cholesterol biosynthesis; however, an additional effect is reduced production of the isoprenoid intermediates of the cholesterol biosynthetic pathway. By reducing substrate availability, proteins that are normally isoprenylated (i.e., modified with farnesyl or geranyl-geranyl moieties) are affected by the statins, and their function and/or membrane localization may be impaired (19). Particularly susceptible to such effects are the small GTPases (e.g., Ras, Rac, Rho). With regard to protein synthesis, impaired farnesylation of the Rheb GTPase (an upstream activator of the mTORC1 complex), has been dem-
onstrated in response to atorvastatin (7) and lovastatin treatment (39) in different cell types. Our laboratory has reported that mTORC1 activation is necessary for stimulation of eIF2B/H9280 subunit protein synthesis (18); in light of reduced Akt phosphorylation as well as impaired farnesylation of Rheb with statin treatment, the synthesis of eIF2B/H9280 may be repressed. However, repressed translation of the eIF2B/H9280 mRNA is unlikely to explain the results of the present investigation on the basis of the ability of MG-132 to prevent the decrease in eIF2B/H9280 protein expression as well as several other subunits of eIF2B during simvastatin treatment. While none of the eIF2B subunits possesses the canonical carboxyl-terminal CaaX isoprenylation motif, at least one subunit (eIF2B/H9251) has been reported to colocalize with adrenergic receptors along the plasma membrane (17). Consequently, one possibility is that an interacting protein of one or more of the eIF2B subunits is normally isoprenylated and that simvastatin, by impairing this modification, alters the normal stability (i.e., synthesis and/or degradation) of eIF2B subunits and/or the activity of the eIF2B holoenzyme, perhaps by altering cellular localization. It is also important to consider that some of the effects of statins occur in an HMG-CoA reductase inhibition-independent manner. For example, IGF-I signaling is impaired in differentiating C2C12 myoblasts treated with simvastatin, but the effects are not rescued by addition of mevalonate (the product of HMG-CoA reductase) or its isoprenoid derivatives (27). However, in the present investigation, the simvastatin-induced repression of protein synthesis and eIF2B protein expression were both completely prevented by the addition of mevalonate, implicating the product of HMG-CoA reductase as a mediator of these effects.

Although treatment with MG-132 was able to rescue eIF2B subunit expression, we did not measure eIF2B activity or protein synthesis in such conditions, because treatment with MG-132 has been shown to increase eIF2α (Ser51) phosphorylation and reduce protein synthesis rates in several cell types including C2C12 myoblasts (6, 15, 44). Indeed, we observed an approximately threefold increase in eIF2α phosphorylation at Ser51 after incubation with MG-132 (data not shown). Thus, preventing the loss of eIF2B subunit expression via proteasome inhibition would not be expected to rescue the reduction in eIF2B activity or protein synthesis rates due to the concomitant increase in eIF2α phosphorylation that would directly inhibit eIF2B activity.

Fig. 4. Repression of eIF2B guanine nucleotide exchange activity in response to simvastatin treatment is associated with altered phosphorylation of Akt, GSK-3β, and eIF2Be. C2C12 myoblasts were treated with vehicle (control) or 10 μM simvastatin for 24 h. Aliquots of control and simvastatin-treated myoblasts were subjected to eIF2B guanine nucleotide exchange activity assay. Results represent means ± SE of 3 experiments, each performed at least in triplicate, and are expressed as %mean control values. * P < 0.05 vs. control. B–F: equal volumes of cell lysates were subjected to Western blot analysis using antibodies specific for proteins phosphorylated at a specific site or for the total protein. Phosphorylation of Akt on Ser473 (B), GSK-3β on Ser9 (C), eIF2B on Ser535 (D), and eIF2α on Ser51 (E) were normalized to the respective total protein and expressed as %mean control values. Representative blots are shown in each panel with control and simvastatin-treated samples in triplicates. Results represent quantitation (means ± SE) of 3 independent experiments with 3 replicates per experiment. * P < 0.05 vs. control.
One important regulator in common to both the protein synthesis and degradation pathways is the AGC serine-threonine kinase Akt. Decreased phosphorylation of Akt has been reported in response to statin treatment in the mouse C2C12 myoblast cell line (11, 27) as well as in rat skeletal muscle in vivo (21). One downstream consequence of reduced Akt activity in response to statins is increased expression of the E3 ligases MAFbx and MuRF1 that are associated with muscle protein degradation (11, 21). However, another consequence to a reduction in Akt activity is enhanced activity of the Akt substrate GSK-3β. Reduced phosphorylation and thus increased activity of GSK-3β can lead to increased phosphorylation of eIF2B at an inhibitory site of its catalytic ε-subunit (14, 41). In the present investigation, we confirmed the reduction in Akt Ser\textsuperscript{473} phosphorylation and observed a significant reduction in GSK-3β phosphorylation at Ser\textsuperscript{9}. Although we cannot directly implicate GSK-3β activity in the phosphorylation of eIF2Bε in the conditions used in the present investigation, the reduction in total eIF2Bε expression resulted in a relative increase in eIF2Bε phosphorylation at Ser\textsuperscript{535}. Finally, reduced Akt phosphorylation at Ser\textsuperscript{473} would also be expected to reduce the activity of the mTORC1 complex, another critical effector of the control of protein synthesis. The effect of statins on reducing mTORC1 signaling has been reported by others in a number of different cell types (7, 31, 39). Accordingly, the results of the present study regarding eIF2B expression represent only one potential effector of reduced protein synthesis rates.

Another potential limitation of the present investigation is that the concentration of simvastatin used was 10 μM; this dose was chosen on the basis of preliminary experiments as well as an abundance of published investigations in which similar or higher concentrations of statins were used in cell culture experiments, including C2C12 cells. The reported concentration of statins in the plasma of human patients with normal therapeutic doses is usually submicromolar. However, in some patients who experience statin-related myopathies (e.g., due to drug interactions, polymorphisms in organic anion-transporting polypeptides, etc.), statin concentrations may indeed reach the low micromolar range (25, 33). Importantly, we have observed the reduction in eIF2Bε protein expression with 10 μM but not 0.1 μM simvastatin treatment for 24 h, whereas 1 μM resulted in an intermediate effect (Supplementary Fig. S1). Nevertheless, the implication of the dose-related effect of simvastatin on eIF2B that we observed in cell culture in the context of skeletal muscle of human patients remains unclear.

In summary, the results of the present study implicate reduced eIF2B holoenzyme expression in the reduction of protein synthesis during statin treatment in C2C12 myoblasts. Repression of eIF2B guanine nucleotide exchange activity is a well-known mechanism for the regulation of global rates of protein synthesis. The reduction in eIF2B activity appears to be primarily the result of decreased eIF2B subunit abundance, with a resulting relative increase in eIF2Bε phosphorylation at the inhibitory Ser\textsuperscript{535} residue of eIF2Bε. The precise mechanism for the reduction in eIF2B subunit expression is not known but is dependent on the ubiquitin-proteasome system. Future studies directed at further examining the mechanism(s) of reduced protein synthesis and its effects on statin-associated myopathies will help in understanding the complexity of this class of beneficial pharmacological agents.

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DISCLOSURES

No conflicts of interest are reported by the authors.

REFERENCES


