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Running Title: Autophagy Flux and Myotoxicity

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Abstract

Statins are some of the most widely used drugs worldwide, but one of their major side effects is myotoxicity. Using mouse myoblast (C2C12) and human alveolar rhabdomyosarcoma cell lines (RH30) in 2-dimensional (2D) and 3-dimensional (3D) culture, we investigated the mechanisms of simvastatin's myotoxicity. We found that simvastatin significantly reduced cell viability in C2C12 cells compared to RH30 cells. However, simvastatin induced greater apoptosis in RH30 compared to C2C12 cells. Simvastatin-induced cell death is dependent on Geranylgeranyl pyrophosphate (GGPP) in C2C12 cells, while in RH30 cells it is dependent on both Farnesyl pyrophosphate (FPP) and GGPP. Simvastatin inhibited autophagy flux in both C2C12 and RH30 cells and inhibited lysosomal acidification in C2C12 cells, while autophagy inhibition with Bafilomycin-A1 increased simvastatin myotoxicity in both cell lines. Simvastatin induced more cell death in RH30 cells compared to C2C12 in 3D culture model with similar effects on autophagy flux as in 2D culture. Overall our results suggest that simvastatin-induced myotoxicity involves both apoptosis and autophagy, where autophagy serves a pro-survival role in both cell lines. The sensitivity to simvastatin myotoxicity is different in 2D versus 3D culture, demonstrating that the cellular microenvironment is a critical factor in regulating simvastatin-induced cell death in myoblasts.

INTRODUCTION

The statin drugs ('statins') are competitive inhibitors of HMG-CoA (3-hydroxy-3-methylgutarylcoenzyme A) reductase, and thus attenuate cholesterol and isoprenoid biosynthesis in the mevalonate (MA) pathway (Endo et al., 1977). They are used clinically as lipid-lowering drugs that prevent and treat cardiovascular diseases including atherosclerosis, coronary artery disease, and stroke (Grundy and Vega, 1985; Illingworth and Sexton, 1984; Tikkanen and Nikkila, 1987). The MA pathway is an essential contributor to mammalian cell homeostasis, as it is involved in the regulation of a multitude of cellular processes that require cholesterol and the isoprenoid intermediates (Cartocci et al., 2017; Hashemi et al., 2017). Cholesterol is the final sterol product of the MA cascade but several upstream isoprenoid metabolites including Farnesyl pyrophosphate (FPP) and Geranylgeranyl pyrophosphate (GGPP) are necessary for the prenylation of monomeric small GTPase proteins (e.g. Rho, Ras, Rac, Cdc42, Rab, Rap) (Hashemi et al., 2017; Sheikholeslami et al., 2019). These prenylated GTPases are critical cell signaling molecules involved in many basic cellular processes including proliferation, growth, migration, cytoskeletal dynamics, vesicular trafficking, barrier integrity, and smooth muscle contraction, to name a few. Thus, the MA pathway is tightly regulated to maintain these precise cellular functions under varied conditions in many cell types critical to health and disease (Jiao et al., 2017; Yeganeh et al., 2014).

Statins are generally well-tolerated medications, however, there are side effects associated with these compounds which are dose-dependent. One of the most important and clinically relevant side effects is skeletal muscle myopathy which occurs in 1–5% of patients who take statins. Rarely, this can lead to lethal rhabdomyolysis if it is not diagnosed promptly (Ballantyne et al., 2003; Graham et al., 2004; Staffa et al., 2002; Thompson et al., 2003). According to recent

investigations, statin-related muscle disorders are potentially dependent on the inhibition of FPP and GGPP (Bhardwaj et al., 2013; Matzno et al., 1997). Treatment of C2C12 cells with GGPP can reverse the inhibitory effects of statins on myotube formation (Baba et al., 2008). In support of these findings, there is evidence that statin-induced muscle toxicity is connected to the inhibition of protein geranylgeranylation (Johnson et al., 2004), where prenylation of small GTPases is essential to their signaling function, including RAP GTPase. This reaction exclusively requires geranylgeranylation of RAP1A small Rho-GTPase protein, which is catalyzed by the prenyltransferases (Crick et al., 1997). Although these investigations have been illuminating regarding statin-induced muscle toxicity, the exact mechanisms underlying this phenomenon remain incompletely understood.

Macroautophagy (hereafter listed as autophagy) is a multi step ‘‘self-eating’’ physiological process that regulates cellular response to stress (Amiri et al., 2019). Autophagy can be involved in both survival and death mechanisms based on the type of the cells and stimuli (Hombach-Klonisch et al., 2018; Mokarram et al., 2017). Once it has been induced, tightly regulated sequential steps direct the formation of a bilayer vesicle called the autophagosome to consume cytoplasmic cargo (Klionsky et al., 2016; Mehrbod et al., 2019). This cargo is then ubiquitinated and recognized by autophagy receptors like p62. The cargo receptor later binds to the cargo and LC3-II, a component of the autophagosome membrane, which facilitates the isolation of the cargo and its delivery to lysosomes.

Autophagy can be involved in regulation of programmed cell death I (apoptosis) under different scenarios: i) as a positive controller (autophagy increases apoptosis), ii) as a negative controller (autophagy decreases apoptosis), or iii) parallel to apoptosis (autophagy does not change cellular apoptosis) (Ghavami et al., 2010a; Ghavami et al., 2010b; Ghavami et al., 2012a;

Ghavami et al., 2011; Ghavami et al., 2014; Ghavami et al., 2012b; Liu et al., 2017; Song et al., 2017). Investigators have used autophagy and apoptosis cross regulation to develop new therapeutic approaches for cancer. For example, different autophagy inducers and inhibitors have been used with chemotherapy agents, and radiotherapy to increase the efficiency of cancer therapy in some patients (Hombach-Klonisch et al., 2018; Mokarram et al., 2017).

We have previously studied cell death mechanisms of statins in airway smooth muscle and recently established a research program in developing new therapeutic approaches for Rhabdomyosarcoma (RMS). Previous investigations have used C2C12 mouse myoblasts (Jaskiewicz et al., 2019; Schirris et al., 2015a) as a model for investigation of statins myopathy. In the current study, we aim to understand the myotoxic effects of statins, using the rhabdomyosarcoma cell line (RH30) (Moghadam et al., 2018) as well as C2C12 cell lines to address this clinically relevant question.

MATERIALS and METHODS

Chemicals and Antibodies

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (M2128), simvastatin (S6196), FPP (F6892), GGPP (G6025), cholesterol (47129), mevalonate (68519), LC3β antibody (L7543), and beta actin antibody (A2228) were purchased from Sigma/Aldrich (Canada, Ontario) Dimethyl sulfoxide (DMSO) (4948-02) were purchased from VWR (Canada, Ontario). SQSTM1/p62 antibody (5114) were purchased from cell singling (Canada Ontario). Cleaved PARP (Asp214) (D64E10) XP® Rabbit mAb, LC3B (D11) XP® Rabbit mAb, and SQSTM1/p62 (D5L7G) Mouse mAb were purchased from Cell Signaling Technology. Alexa Fluor® 488 AffiniPure Donkey Anti-Rabbit IgG, and Alexa Fluor® 647 AffiniPure Donkey Anti-Mouse IgG secondary antibodies and IgG-free bovine serum albumin (BSA) were purchased from Jackson ImmunoResearch Inc. DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) was purchased from Thermo Fisher Scientific. Bovine type 1 collagen (10 mg/mL) was purchased from Advanced BioMatrix Inc. Live/dead viability staining kit was purchased from Millipore Sigma.

Cell Lines and Cell Culture

The human rhabdomyosarcoma cell line (RH30) [RC13, RMS 13, SJRH30] (ATCC® CRL¬ 2061™) (Human muscle cancer cells) and mouse muscle cell line (C2C12) (ATCC® CRL¬1772™) were used in this project. Cells were cultured in Roswell Park Memorial Institute (RPMI- 1640) with L-glutamine and 25mM HEPES (BioWhittaker; Cat #: 12-115Q) and Dulbecco's Modified Eagle's Medium (DMEM) (CORNING; Cat #: 50-003-PB) with 10% fetal bovine serum (FBS) (Gibco™; Cat #: 16000044). RH30 cell lines were cultured in RPMI-1640 with L-glutamine and 25 mM HEPES media, and C2C12 cells were cultured in DMEM with high glucose media. Both media were supplemented with FBS (10%), penicillin (1%), and

streptomycin (1%). Cells were grown to 35–40% confluency on a 100 mm cell culture plate, 6-well plates, and 96-well plates. Cells were maintained in a humidified incubator with 95% air and 5% CO2 at 37 °C and were passed once every 2–3 days. Cell culture plastic ware, penicillin, and streptomycin were purchased from VWR (Toronto, ON, Canada).

MTT Assay

The MTT assay was performed based on a protocol established in our group (Alizadeh et al., 2017; Ghavami et al., 2004; Ghavami et al., 2010b; Ghavami et al., 2012a; Ghavami et al., 2011; Ghavami et al., 2014). Briefly C2C12 (20,000 cells/mL) and RH30 (30,000 cells/mL) were seeded in 96-well plates and treated with varying concentrations of simvastatin (Simva, 0-20 μM). At each time-point (24, 48, 72, and 96 hrs), 20 μL of (MTT, 5 mg/ml) was added to each well. The cells were incubated at 37°C for 4 hours, after which the media was gently aspirated. Then, 200 μL of DMSO was added to each well and mixed with the cells by pipetting to dissolve the MTT formazan crystals. Lastly, the plates were incubated for 20 min at room temperature. Absorbance was measured at 570 nm using a Synergy H1 Microplate Reader.

Mevalonate Cascade Rescue Assay

Rescue experiments were done according to previously reported protocols (Alizadeh et al., 2017; Ghavami et al., 2012a; Ghavami et al., 2011; Ghavami et al., 2014). Briefly, cells were seeded and grown in 96-well plates at a density of 2,000 cells per well, up to 50% confluence. Cells were pre-treated with 5 mM mevalonate (MeV), 30 μM FPP, 30 μM GGPP, and 50 μM cholesterol, and were incubated at 37°C for 4 hrs. These cells were then co-treated

with 10 μM Simvastatin and incubated at 37°C for 96 hrs. Cell viability was then measured using the MTT assay after 96 hrs, as described in the previous section.

Immunoblotting

Western blotting analysis of C2C12 and RH30 cell lysates was used to assess markers of autophagy (LC3 (1:2500), p62 (1:1000)) as has been described in our previous studies (Ghavami et al., 2010b; Ghavami et al., 2012a; Ghavami et al., 2011; Ghavami et al., 2014). Cells were grown to 40-50% confluency in 100 mm dishes and either treated with 10 μM Simva or with a drug vehicle control (DMSO). At the appropriate time point, cells were collected, and protein extracts were made using NP-40 Lysis Buffer (0.5% (v/v) Nonidet P-40, 20 mM Tris-HCl (pH 7.5), 0.5% (v/v) PMSF, 100 μM β-glycerol 3-phosphate and 1.5% (v/v) protease inhibitor cocktail). Once the protein concentration was known, samples were prepared for western blotting with a total protein concentration of 1 μg/μL (15 μL of each sample was used). After electrophoresis, the membranes were developed for LC3β and p62 proteins.

Measurement of Apoptosis with Flow Cytometry

We measured apoptosis using the Nicoletti method. C2C12 and RH30 cells were cultured in 6-well plates and treated with either Simva (5 or 10 μM) or with a drug vehicle control (DMSO) for 48 hrs. After drug treatment, cells were detached using EDTA buffer and centrifuged at 1,500×*g* for 5 min at 4°C. Cells were washed with cold PBS before they were permeabilized and stained with a hypotonic Propidium Iodide (PI) buffer (0.1% Triton X-100, 1% sodium citrate, 0.5 mg/ml RNase A, 40 μg/ml propidium iodide). Samples were then incubated for 1 hour in the dark at 4°C to prevent photo-bleaching. Flow cytometry was carried

out at 460 nm for 10,000 cells. Residual debris were gated out accurately to obtain accurate data. The resulting histogram was analyzed to determine the percentage of normal and apoptotic nuclei; the nuclei of apoptotic cells were located on the left side of the G1 peak as they have less DNA compared to the nuclei of healthy G0/G1 cells. For each sample, the sub-G1 peak was measured and statistically compared with other samples to determine significance (Hashemi et al., 2007; Moghadam et al., 2018).

Live Cell Imaging

LC3 is a specific marker for autophagosomes, which are key structures in the process of autophagy. LC3-GFP is a fusion of green fluorescent protein (GFP) and LC3, and it can behave in the same manner as endogenous LC3. LC3-GFP is localized on the autophagosome membrane and emits green light when excited. In a normal cell, LC3 is dispersed evenly through the cytosol. However, when autophagic flux is initiated, LC3 is recruited to autophagosome membranes, resulting in sharp green puncta and LC3-GFP-containing cells. To confirm autophagy findings seen in western blots, C2C12 or RH30 cells were grown in 6-well plates and transfected with a plasmid containing LC3-GFP (Addgene #24920) using Qiagen's Effectene reagent, as per manufacturer's instructions. After transfecting cells for 18 hrs, the cells were treated with either Simva (10 μM), Bafilomycin-A1 (Baf-A1, 100 nM), Simva + Baf-A1, or a vehicle control (DMSO). After 24 hrs, the cells were incubated with LysoTracker red dye (Molecular Probes; LysoTracker Red DND-99; L7528) at a concentration of 50 nM to detect lysosomal activity and counterstained with Hoechst. Cells were stained for 30 min in a 37°C incubator. After the 30 min period, the cells were washed with PBS and fresh media was added. The cells were imaged on an epifluorescence microscope. Images were analyzed to determine the

percentage of cells with distinct LC3-GFP puncta and to see if green and red puncta co-localization had occurred, which was assumed to signify the fusion of autophagosomes with lysosomes (Field et al., 2018; Moghadam et al., 2018).

Transmission Electron Microscopy (TEM)

TEM was used to evaluate autophagy in both C2C12 and RH30 cell lines. The TEM protocol is the same as previously described[1]. Briefly, cells were grown in 10 mm dishes (300,000 cells/dish) and treated with either DMSO (drug vehicle control) or 10 μM Simva for 48 hrs. At the time point, cells were detached with EDTA and centrifuged at 1,500×*g*. The samples were then fixed with 3% glutaraldehyde in PBS (pH 7.4) for 3 hrs at room temperature. The samples were later treated post-fixation with 1% osmium tetroxide in PBS for 2 hrs, followed by an alcohol dehydration series, and then embedded in Epon and stained with uranyl acetate. They were counterstained with lead citrate for 3 min sequentially and finally washed with water for 1 min and dried. The samples were imaged on a Philips CM10 at 80kV on ultra thin sections (100nm on 200 mesh grids) (Alizadeh et al., 2018a; Moghadam et al., 2018).

Culturing C2C12 and RH30 cells in 3D cultures

C2C12 and RH30 cells were grown in 3D culture according to the same protocol explained in previous publications (Moghadam et al., 2018). Briefly, cells were grown in culture medium (DMEM or RPMI with 10% FBS and 0.5% Pen-Strep) until they reached 80% confluency. Cells were detached with Trypsin-EDTA, spun down, and re-suspended in fresh media. Collagen and media were gently added to the cell suspension at 4°C to reach a final collagen concentration of 3 mg/mL and a cell density of 2 million cells/mL. Then, 20 μL of this

solution was added to cylindrical wells with 5 mm diameter and 1 mm depth in PDMS holders which were placed in 12 well-plates and put in a 37°C incubator for 45 min to cure the collagen. Afterwards, 2 mL of media was added to each well and they were incubated overnight. The next day, cells were treated with either DMSO or Simva (Moghadam et al., 2018).

Live-Dead Assay in 3D Culture

Cells were grown and treated as explained above. Live-dead assay solution was prepared as per the supplier's instructions, where 5 μL of calcein and 20 μL of ethidium homodimer-1 were added to 10 mL of DPBS. After treatment, media was removed, and the live-dead solution was added to the wells. After incubating for 2 hrs at room temperature in the dark, the solution was removed and the cells were gently rinsed with DPBS three times, each time with 5 min incubation. Stained cells were then imaged on a confocal microscope and quantified (Moghadam et al., 2018).

Immunofluorescence (IF) in 3D Culture

IF was used to confirm apoptosis and autophagy findings by evaluating cleaved-PARP, LC3, and p62 levels. C2C12 and RH30 cells were treated with DMSO or 10 μM Simva for 96 or 48 hrs, respectively. At the appropriate time point, media was removed, and cells were fixed by incubating them with 4% paraformaldehyde in PBS for 15 min at room temperature. Paraformaldehyde was then removed, and cells were washed 3 times with PBS. Cells were blocked with blocking buffer (5% goat serum, 0.3% Triton-X in RBS) at room temperature. After 60 to 120 min, blocking buffer was removed and the appropriate primary antibody—p62, LC3, or c-PARP –was diluted 1:300 in Antibody Buffer (1% BSA, 0.3% Triton-X in PBS) and

incubated with the samples overnight at 4°C. The next day, the antibody solution was removed, and cells were washed three times with PBS. Cells were then incubated with the appropriate fluoro-conjugated secondary antibody which was diluted 1:400 in Antibody Buffer. Samples were incubated in the dark for 2 hrs at room temperature, and then washed with PBS. Finally, the cells were incubated with DAPI solution for 1 hr in the dark. After washing three times with PBS, cells were immediately taken for imaging (Moghadam et al., 2018).

Statistical Analysis

All results were presented as mean ± SD, and the differences between the groups were tested by one-way ANOVA or two-way ANOVA analysis (non-parametric, Brown–Forsythe test), using GraphPad Prism 7.0. The confidence interval in each analysis was 95%, and $p < 0.05$ was considered statistically significant.

RESULTS

Mevalonate Cascade Inhibition Induces Cell Death in Both RH30 and C2C12 Cells

We previously showed that the MA cascade inhibitor simvastatin induces cell death in a broad range of primary cells (primary airway mesenchymal cells, and primary atrial fibroblasts) (Ghavami et al., 2010b; Ghavami et al., 2011; Ghavami et al., 2014) and tumor cell lines

(Alizadeh et al., 2018b; Alizadeh et al., 2017; Sheikholeslami et al., 2019) including breast (MCF-7, MDA-MB231), brain (U87, U251), and lung (A549, H1965), as well as medulloblastoma brain tumor cell lines (Daoy, D283, and D341 cells). Lovastatin and mevalonate cascade inhibitors (GGTi-298, 6-Fluoromevalonate) also inhibit ovarian cancer tumor growth (Kobayashi et al., 2017; Kobayashi et al., 2015).

We now demonstrate that simvastatin induces dose- (0-20 μM) and time- (0-96 hr) dependent cell death in both RH30 (Fig. 1 A-D) and C2C12 cells (Fig. 1 E-H). In RH30 cells, simvastatin (20 μM) significantly induced cell death ($p < 0.05$) in 24 hrs (Fig. 1 A), simvastatin (10, 20 μM) significantly induced cell death ($p < 0.0001$) in 48 hrs (Fig. 1 B), simvastatin (5,10, 20 μM) significantly induced cell death ($p < 0.05$, $p < 0.0001$) in 72 and 96 hrs (Fig. 1 C&D). Interestingly, simvastatin (0.5-20 μM) induced significant cell death ($p < 0.001$, $p < 0.0001$) in all time points (24-96 hr) in C2C12 cells (Fig.1 E-H). The morphology of RH30 cells treated with simvastatin (10 μM) is shown in Fig. 1J and compared with RH30 time-matched control. Simvastatin at concentrations of ≥2.5 μM induced significant cell death in C2C12 cells as compared to RH30 cells ($p < 0.01$).

Prenylation Precursors Differentially Control Simvastatin-Induced Cell Death in C2C12 and RH30 Cells.

We know that mevaloante (MEV) can reverse statin-induced cell death in many cell models, and GGPP is the major regulator of prenylation events among the isoprenoid intermediates. We now show that MA (5 mM) significantly ($p < 0.0001$) inhibits simvastatin-(10 μM) induced cell death in both C2C12 (Fig. 2 A) and RH30 (Fig. 2E) cells. While GGPP (30

μM) significantly ($p < 0.0001$) inhibited simvastatin-induced cell death in both C2C12 (Fig. 2B) and RH30 (Fig 2F) cells, it was more effective in rescuing RH30 cells than C2C12 cells (Fig. 2J). We found that FPP (30 μM) did not significantly inhibit simvastatin-induced cell death in C2C12 cells (Fig. 2C), but it did significantly ($p < 0.01$) inhibit simvastatin-induced cell death in RH30 cells (Fig. 2G). FPP was also more effective in rescuing RH30 cells against simvastatin-induced cell death (Fig. 2K). Furthermore, cholesterol (50 μM) did not significantly inhibit simvastatin-induced cell death in either C2C12 (Fig. 2D) or RH30 (Fig. 2I).

Mevalonate Cascade Inhibition Induces Apoptosis in Both C2C12 and RH30 Cells

Mevalonate cascade inhibition can induce apoptosis in many cell models (Alizadeh et al., 2018b; Alizadeh et al., 2017; Ghavami et al., 2010b; Ghavami et al., 2012b). In this study, we show that simvastatin induces dose- (5, 10 μM) and time-depended (48, 72 hr) apoptosis in both C2C12 (Fig. 3A-C) and RH30 (Fig. 3D-F) cells ($p < 0.01$, $p < 0.0001$). We also show that simvastatin significantly induces greater apoptosis in RH30 cells as compared to C2C12 cells (Fig. 3G) ($p < 0.01$).

Mevalonate Cascade Inhibition Induces Blockage of Autophagy Flux in Both C2C12 and RH30 Cells While Inhibiting the Acidification of Lysosomes in C2C12 Cells

Statins can induce autophagy in different types of cells (Ghavami et al., 2011; Ghavami et al., 2014; Ghavami et al., 2012b). Our current study shows that statins inhibit autophagy flux in both RH30 and C2C12 cells. We show that simvastatin (10 μM) increases LC3 lipidation and induced p62 accumulation in both RH30 and C2C12 cells (Fig. 4A). To further confirm our results, using GFP-LC3 and lysotracker immunostaining, we show that simvastatin induced

significant increase of LC3 puncta in both C2C12 and RH30 (Fig. 4B-D) while in C2C12 cells prevented acidification of lysosomes (Fig. 4B) (lack of lysotracker red activity in simvastatin-treated cells). We further confirmed our results using the autophagy inhibitor Bafilomycin A1 (Baf-A1, 100 nM for 1 hr) and show that adding Baf-A1 does not significantly increase the number of LC3 puncta in both C2C12 (Fig. 4 E&G) and RH30 cells (Fig 4 F&H). We also confirmed increased numbers of autophagosomes in both C2C12 (Fig. 4I) and RH30 cells (Fig 4J). We then used Baf-A1 (4 nM) in presence and absence of simvastatin (10 μM, 24 hrs) in C2C12 and RH30 cells (Fig 4K). Immunoblotting results confirmed further inhibition of autophagy flux in both C2C12 and RH30 cells (increase of LC3β lipidation and decrease of p62 degradation) (Fig 4K). Further, inhibition of autophagy significantly increased simvastatin-induced myotoxicity in both C2C12 and RH30 cells (Fig 4L&M).

Simvastatin Induces Apoptotic Cell Death and Inhibits Autophagy in Both C2C12 and RH30 Cells in 3D Culture

Cells cultured in 3D configurations using hydrogel biomaterials display a more physiologically-relevant phenotype (Seyfoori et al., 2018). We recently showed that 3D-cultured C2C12 and RH30 cells can be used to screen drugs (Moghadam et al., 2018). In this study, we used this same 3D technique to evaluate the effect of simvastatin on C2C12 and RH30 cells. We performed live/dead assays in 3D culture of C2C12 and RH30 cells and show that simvastatin (5, 10 μM) induces both dose- and time- (48, 96 hrs) dependent cell death (Fig. 5 A-H). Also, simvastatin induced significant ($p < 0.0001$) cell death in both C2C12 (Fig. 5 A, C, E&F) and RH30 (Fig. 5 B, D, G, H) cells in the 3D model. However, when cultured in the 3D hydrogel,

simvastatin induced more cell death in RH30 cells as compared to C2C12 cells. This was opposite to the effect we observed when such cells were culture in standard 2D conditions. In addition, simvastatin (10 μM) induces apoptotic cell death in both C2C12 and RH30 cells (cleavage of PARP) in the 3D culture model (Fig. 5 I, J). We further investigated the effects of simvastatin on autophagy in both C2C12 and RH30 3D culture cells. We showed that simvastatin inhibits autophagy flux in 3D culture model (increase of LC3 puncta and lack of localization with p62) in both C2C12 and RH30 cells (Fig. 5 K&L).

DISCUSSION

Our previous studies have demonstrated that the HMG-CoA reductase inhibitor simvastatin induces endoplasmic reticulum stress/unfolded protein response, autophagy, and apoptosis in human airway smooth muscle (HASM) cells, human airway fibroblasts (HAF), and human atrial fibroblasts through inhibition of GGPP biosynthesis (Ghavami et al., 2012a; Ghavami et al., 2011; Ghavami et al., 2014). Previously, we also showed that simvastatin induces apoptotic cell death in a wide variety of tumor cells (lung, brain, and breast) via inhibition of geranylgeranylation of small Rho GTPases (Alizadeh et al., 2017) .

Statin-induced myotoxicity is a major concern for clinicians and basic scientists alike, and several recent investigations have focused on the possible underlying mechanisms involved in statin myotoxicity. In the current investigation, we used C2C12 as our non-cancerous cell line and RH30 as a cancer skeletal muscle cell line to elucidate the mechanisms underlying simvastatin-induced myotoxicity. Our experiments utilized both monolayer 2D and 3D cell culture models, which are more physiologically relevant accounting in part for the cellular microenvironment.

Previous investigations have demonstrated that statin-induced myotoxicity occurs via vacuolation of skeletal muscle fibers, blebbing of sarcolemma, and cell necrosis (Sakamoto et al., 2007). Inhibition of the mitochondrial complex III is involved in statin-induced myotoxicity in C2C12 cells (Schirris et al., 2015b). Other reports indicated that mitochondria (Bouitbir et al., 2012; Kwak et al., 2012; Schirris et al., 2015b), Ca^{2+} homeostasis (Sirvent et al., 2012), plasma membrane mono-carboxylate transporter (Kobayashi et al., 2006), plasma membrane receptors (Dricu et al., 1997; Siddals et al., 2004), and ubiquitin ligases (Cao et al., 2009) are statins' primary targets for myotoxicity. Here we show that simvastatin induced cell death in both C2C12 and RH30 cells. However, there were significant differences between C2C12 and RH30 in cell viability (MTT assay) after treatment with simvastatin in 2D monolayer cell culture (simvastatin caused significantly greater reduction in C2C12 cell viability as compared to RH30). Since the MTT assay is based on the measurement of mitochondrial reductase activity to produce formazon, our results suggest that simvastatin-induced cell death may be dependent on the decrease of reductase activity in C2C12 cell lines. Of note, statin-induced myotoxicity (Graham et al., 2004) is augmented with the combination of drugs that block metabolic pathways and decrease mitochondrial reductase activity in cells, such as cytochrome P450 and UDP-

393 glucuronyltransferase 1A1 and 1A3 systems (Prueksaritanont et al., 2002). Consistent with
394 previous studies, our results demonstrate that simvastatin has greater myotoxicity in C2C12
395 (non-cancerous muscle) cells than RH30 (skeletal muscle cancer) cells.

396 We also show that simvastatin induced apoptosis in a time- and dose-dependent manner in
397 both C2C12 and RH30 cells. Interestingly, RH30 was more susceptible to apoptosis than C2C12
398 in 2D monolayer culture model (Figure 3). The rate of simvastatin-induced apoptosis in RH30
399 cells was ~2-fold greater than the C2C12 cell line. Therefore, while simvastatin caused a
400 reduction in cell viability in C2C12 cells, these cells were also less susceptible to apoptosis than
401 RH30 cells. This is not unexpected given that in the broader statin-cancer literature, cancer cells
402 are predominantly more sensitive to statin-induced cell death than their normal or non-cancerous
403 controls. These results intriguingly show that simvastatin-induced apoptosis in skeletal muscle
404 cells does not correlate with loss of cell viability as measured by the MTT assay; this suggests an
405 effect mediated via other mitochondrial factors. For example, in our previous investigations we
406 showed that simvastatin-induced apoptosis is dependent on the release of Smac/Diablo and
407 Omi/Htr2 from mitochondria in HASM cells and HAF (Ghavami et al., 2010b) and is
408 independent of the release of cytochrome c from mitochondria.

409 The role of cholesterol biosynthesis in statin-induced cell death has been widely
410 investigated and those results are consistent with ours (Graham et al., 2004; Sakamoto et al.,
411 2007; Schirris et al., 2015b). Previous studies showed that statins reduce GGPP levels and
412 production of ubiquinones which are used as electron carriers in the electron transport chain
413 (Harper and Jacobson, 2007; Thompson et al., 2003). Consequently, the decrease of ubiquinone
414 production in cells leads to dysfunction of the electron transport chain, which reduces muscle
415 cell ATP levels, elevates free radical production, and induces apoptosis (Harper and Jacobson,

2007; Thompson et al., 2003). Further, the impaired geranylgeranylation of proteins may be a root cause in statin-associated myopathy (Cao et al., 2009; Johnson et al., 2004; Mullen et al., 2010), a concept contested by work carried out in rhabdomyosarcoma rather than normal skeletal muscle cells (Gee et al., 2015). Therefore, we decided to investigate the MA pathway in both C2C12 and RMS cells. In our study, co-treatment with MA or GGPP inhibited simvastatin-induced cell death in the C2C12 cell line while co-treatment with cholesterol and FPP did not. Takeda et al. demonstrated that the reduction of smooth muscle cell proliferation by simvastatin was inhibited by GGPP, but not by FPP (Takeda et al., 2006). Their findings are compatible with our results which show simvastatin signaling is dependent on GGPP in C2C12 cells. Our present study also shows that in RH30 cells simvastatin-induced cell death is inhibited by MA and GGPP, but not cholesterol (Fig 2). Unlike in C2C12 cells, we show that in RH30 cells simvastatin-induced cell death was inhibited by FFP (Fig 2). These findings confirm that the effect of simvastatin on cell death in both cells was mediated via inhibition of the MA pathway, in particular, GGPP. In addition, we discovered that FPP may play an important role in simvastatin-induced death mechanisms in RH30 cells.

GGPP and FPP are necessary for the prenylation of small Rho GTPase proteins including Rho, Rac, Cdc42, Rab and Rac (Alizadeh et al., 2018b; Alizadeh et al., 2017; Ghavami et al., 2010b; Ghavami et al., 2012a; Yeganeh et al., 2014). We show that simvastatin-induced cell death is dependent on GGPP in C2C12 cells. This indicates that Rho, Cdc42, and Rac GTPases may be involved in cell death induction mechanisms in C2C12 cells. Conversely, in RH30 cells both FPP and GGPP mediate simvastatin-induced cell death, suggesting that Ras GTPases may also be involved via farnesylation pathways.

Several recent investigations have shown that HMG-CoA reductase inhibitors such as simvastatin either induce or inhibit autophagy in different cell models (Ghavami et al., 2014; Hwang et al., 2015; Vilimanovich et al., 2015; Whitehead, 2016). There are two recent articles that showed hydrophobic statins induced autophagy in A204 RMS cells (Araki and Motojima, 2008; Gee et al., 2015). But, the exact molecular mechanisms of the autophagy flux, autophagosome fusion and degradation steps of autophagy have not been investigated in RMS cells. Many studies demonstrated that the LC3-II/LC3-I ratio is often used to determine the activation of autophagy (Mizushima et al., 2010). The present results show that simvastatin increased the conversion of light chain 3 (LC3)-I to LC3-phosphatidylethanolamine conjugate (LC3-II) in both C2C12 and RH30 cells by increasing the number of LC3 puncta (immunofluorescence) and autophagosome formation (Figure 4). The protein p62 facilitates the degradation of ubiquitinated protein aggregates by autophagy (Guo et al., 2013) and is a selective substrate for autophagy and directly interacts with LC3 to mediate the degradation of ubiquitinated protein aggregates by autophagy (Pankiv et al., 2007). Our results show that simvastatin increases p62 accumulation in both RH30 and C2C12 cell lines, therefore, simvastatin inhibits autophagy flux in both cells lines. Moreover, our results showed that simvastatin induced acidification of lysosomes in RH30 cells, but in C2C12 cells simvastatin inhibited acidification of lysosomes (Fig. 4B). Taken together, our data demonstrates that simvastatin inhibits autophagy flux in a time-dependent manner in both non-cancer C2C12 and RMS RH30 cells. Therefore, we conclude that simvastatin inhibits autophagy flux in both C2C12 and RH30. In RH30 cells, autophagy inhibitory activity occurs via inhibition of lysosomal acidification, however, further investigation is required to prove this hypothesis. Further blockage of autophagy flux increases the myotoxicity of simvastatin in both C2C12 and

RH30 cells. These findings confirm the importance of autophagy flux inhibition in the myotoxicity of statins. These results are inconsistent with our findings in HAF and HASM (Ghavami et al., 2011; Ghavami et al., 2014). Also, Gu et al showed that simvastatin induces autophagy in bronchial smooth muscle cells (BSMCs) and increases autophagy-related protein Atg5, LC3B, and Beclin1 expression and autophagosome formation in lung tissue (Gu et al., 2017).

The effect of chemical compounds on cells have mostly been performed using 2D cell culture models, where cell-cell interaction, extracellular matrix, and cellular morphology significantly differ from their natural structure in tissues (Levinger et al., 2014). These differences highly influence cellular growth and their response to different chemical compounds (Levinger et al., 2014). Three-dimensional (3D) culture models have been introduced for drug assessment to improve the relation between cell cultures and cellular microenvironment (Friedrich et al., 2009). Recently, 3D culture models have been used as clinically relevant models for the study of cell death and autophagy (Gomes et al., 2015; Ma et al., 2011). We examined the effects of simvastatin in RH30 and C2C12 3D culture models (Fig. 5), which showed that simvastatin induces significantly greater cell death in RH30 cells as compared to C2C12 cells. Whereas, the cell death effects of simvastatin were greater in C2C12 cells as compared to RH30 cells in the 2D cell culture model. This shows how the cell microenvironment and 3D structure can affect fundamental cellular response(s) including to chemical compounds or drugs. We also observed that simvastatin inhibits autophagy flux in 3D culture of RH30 and C2C12 cells (absence of localization of p62 and LC3 puncta) which correlates with our 2D observations. Overall, our results indicate that 3D culture models are important tools for screening cytotoxic

effects of chemical compounds as they can account for some of the effects of cellular matrix in response to extracellular stress.

There are several limitations to this study that are important to mention. The dose of simvastatin used in our experiments (10 μM) is significantly higher than pharmacologic concentrations found in human blood which is in the low nanogram/mL (nM) range (Ucar et al., 2000). However, we don't know if statins accumulate in human skeletal muscle, and whether they reach micromolar concentrations. Furthermore, simvastatin's half-life is approximately 2 hours in plasma, and results can vary according to which statin is selected. In addition, normal human skeletal muscle behaves differently than rhabdomyosarcoma cell line, so the effects observed could manifest differently in human normal skeletal muscle.

In conclusion, we found that simvastatin induces cell death in both RH30 and C2C12 cells in both 2D and 3D culture. Our results showed that simvastatin significantly decreases cellular viability in C2C12 cells compared to RH30 cells while it also significantly induces greater apoptosis in RH30 cells compared to C2C12 cells. In addition, simvastatin inhibits autophagy flux in both RH30 and C2C12 cells with differential effects on lysosomal acidification. We also showed that simvastatin-induced cell death is dependent on both FPP and GGPP in RH30 cells while it is only dependent on GGPP in C2C12 cells. Our current investigation provides solid evidence that both autophagy and apoptosis are involved in statin-induced myotoxicity, and further, autophagy flux inhibition varies between the non-cancerous and cancer muscle cell lines.

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FIGURE LEGENDS:

Figure 1: Simvastatin induces cell death in RH30 and C2C12 cells. (A-D). RH30 cells were treated with simvastatin (0.5, 1, 2.5, 5, 10, 20 μM) and cell viability was assessed 24, 48, 72 and 96 hrs after that by MTT assay. Control cells for each time point were treated with the solvent control (DMSO). Results are expressed as a percentage of corresponding time point control and represent the means ± SD of 15 replicates in three independent experiments (*, $p<0.05$; ****, $p<0.0001$). (E-H). C2C12 cells were treated with simvastatin (0.5, 1, 2.5, 5, 10, 20 μM) and cell viability was assessed 24, 48, 72 and 96 hrs after that by MTT assay. Control cells for each time point were treated with the solvent control (DMSO). Results are expressed as a percentage of corresponding time point control and represent the means ± SD of 15 replicates in three independent experiments (****, $p<0.0001$). (I&J). **Simvastatin significantly decreased cell viability in C2C12 compared to RH30 cells.** RH30 and C2C12 cells were treated with simvastatin (0.5, 1, 2.5, 5, 10, 20 μM) and cell viability was assessed 24, 48, 72 and 96 hrs after that by MTT assay. Control cells for each time point were treated with the solvent control (DMSO). Results are expressed as a percentage of corresponding time point control and represent the means ± SD of 15 replicates in three independent experiments (****, $p<0.0001$). (K&L). Phase contrast microscopy showed that simvastatin (10 μM, 48 hrs) induces morphological changes (cellular shrinkage) and decrease in the number of cells in RH30 cells.

Figure 2: Simvastatin induces cell death in RH30 and C2C12 cells is dependent on mevalonate cascade isoprenoid mediators. (A-H) 5 mM MA, 30 μM GGPP, 30 μM FPP, or 50 μM cholesterol, were added to the cells 4 hrs prior to treatment with simvastatin (10 μM, 96

hrs). Cell death was measured by MTT assay in C2C12 (A-D), and RH30 cells. For each experiment control cells were treated with simvastatin solvent (DMSO) alone (control) or with both DMSO and the appropriate solvent (i.e. ethanol for "mevalonate control). Mevalonate (A, E) and GGPP (B, F) significantly inhibited simvastatin induced cell death in both C2C12 and RH30 cells while FPP (C, G) only inhibited simvastatin-induced cell death in RH30 cells. Our results also showed that cholesterol (D, H) is not involved in simvastatin induced cell death in C2C12 and RH30 cells. Results are expressed as mean ± SD of 15 replicate in 3 independent experiments (* $p< 0.05$, *** $p< 0.001$, and ****$p < 0.0001$). (I&J) Our results also showed that FPP (I) and GGPP (J) significantly rescues simvastatin induced cell death in RH30 cells compared to C2C12 cells.

Figure 3: Simvastatin induces dose and time depended apoptosis cell death in C2C12 and RH30 cells. Percent sub-G1 (A-C) C2C12, (D-F) RH30, abundance induced by simvastatin (5, and 10 μM) or DMSO solvent control after 48 and 72 hrs. Results represent the means ± SD of 9 replicates in three independent experiments. ****$p< 0.0001$; and ***$p< 0.001$ compared to time-matched control. Representative figures of the flow cytometry histogram for C2C12 and RH30 are shown (A and D). Our results showed that simvastatin (10 μM) induced significant more apoptosis in RH30 compared to C2C12 cells in 48 (G) and 72 (H) hours (** $p< 0.01$, and **** $p < 0.0001$).

Figure 4: Simvastatin inhibits autophagy flux inhibition in C2C12 and RH30 cells. (A) C2C12 and RH30 cells were treated with simvastatin (10 μM, 0-72 hours) and cell lysates were

collected. Immunoblotting for LC3β and p62 were performed. The results showed that simvastatin induced accumulation of LC3β II and inhibits p62 degradation in both C2C12 and RH30 cells. C2C12 and C2C12 cells were treated with simvastatin (10 μM, 24 h). (B-D) using immunocytochemistry LC3 puncta and changes in lysosomal activity (LysoTracker red staining) has been investigated. The results showed that simvastatin increased LC3 puncta in both cell lines. Our results also showed that simvastatin (10 μM, 24 hours) increase lysosomal acidity in RH30 while inhibits lysosomal acidification in C2C12 cells (B). C2C12 (E) and RH30 (F) cells were treated with simvastatin (10 μM, 24h) and Baf-A1 (100 nM, +3 hours) followed by immunocytochemistry to evaluate LC3 puncta and changes in lysosomal activity (LysoTracker red staining). The results showed that simvastatin increased LC3 puncta and decreased LysoTracker red fluorescence intensity in C2C12 cells while increased LC3 puncta and increased LysoTracker red fluorescence intensity in RH30 cells. On the other hand, Baf-A1 and simvastatin + Baf-A1 did not significantly change LC3 puncta in both C2C12 and RH30 cells (G, H) showing that simvastatin inhibited autophagy flux like Baf-A1. Transmission electron microscopy showed that in treated C2C12 (I) and RH30 (J) cells there are accumulated autophagosome-like structures compared to control and normal cells after 72 hours treatment. Arrows show the autophagolysosomes containing the cargo (magnification ×11,600). Autophagy inhibition (Baf-A1, 4 nM, 24 hours) significantly increased simvastatin-induced cell death (10 μM, 24 hours) in RH30 (L) and C2C12 (M) cell lines (** $p < 0.01$, Results represent the means ± SD of 15 replicates in three independent experiments). Baf-A1 (4 nM) and simvastatin (10 μM) combination did not increase accumulation of LC3β-II and p62 in both RH30 and C2C12 cells (K).

Figure 5: Simvastatin induces apoptosis and autophagy in C2C12 and RH30 3D culture.

(A&B). Bright field image of C2C12 (A) and RH30 (B) 3D culture which shows the morphology of untreated and simvastatin treated cells (5, 10 µM, 96 hours) in 3D culture. (C-H). Viability assay was done by adding the live/dead solution to cells 48 and 96 hours after treatment with simvastatin (0–20 µM). Cells were incubated for 2 hours in the dark at room temperature, rinsed three times with DPBS, and confocal microscopy was used to capture live/dead cell images in C2C12 (C) and RH30 (D) cells. Quantification of live/dead assay was measured by calculating the ratio of live: total cells which showed a significant decrease in viability of C2C12 (E&F) and RH30 (G&H) cells treated with different concentrations of simvastatin. The data showed simvastatin significantly induces cell death in both C2C12 and RH30 cells ($P < 0.0001$) while simvastatin induces more cell death in RH30 compared to C2C12 cells. (I&J) IF labeling of C2C12 cells (I) and RH30 cells (J) by cleaved PARP following treatment with simvastatin (10 µM, 48 hours) increased number of cells with cleaved PARP in simvastatin treated cells in comparison to control cells which is the hallmark of increase of apoptosis in these cells. (K&L) After treatment of C2C12 (K) and RH30 (L) cells with simvastatin (10 µM, 48 h), cells were IF labeled with autophagosome markers, LC3 and P62. Data showed that simvastatin increases LC3 puncta (green) which is not localized with p62 compared to corresponding time-matched control, a hallmark of autophagy flux inhibition in C2C12 and RH30 3D culture

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