

**Statins, the cholesterol-lowering drugs cause myotoxicity through
phospholipase D-mediated lipid signaling in skeletal C2C12 myoblast cells**

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By

Eric Tretter

The Ohio State University

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Project Advisor

Narasimham L. Parinandi, Ph.D.

Associate Professor of Medicine

Division of Pulmonary, Allergy, Critical Care, and Sleep Medicine,

Dorothy M. Davis Heart & Lung Research Institute,

College of Medicine & Wexner Medical Center,

The Ohio State University

ABSTRACT

Cardiovascular diseases (CVDs) are among the leading causes of mortality in the US and worldwide. Cholesterol at high levels in circulation has been established as a major risk factor for CVDs in humans. Statins, the cholesterol-lowering drugs, have been widely used for lowering and controlling the endogenous levels of cholesterol to prevent or treat CVDs. Statins lower cholesterol by inhibiting 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase), the rate-limiting enzyme in the biosynthetic pathway of endogenous cholesterol. On the other hand, statins are known to cause skeletal muscle damage (myotoxicity and myalgia) in humans while administered to lower the circulating cholesterol levels for the treatment of CVDs. However, the mechanisms and treatment of statin-induced myotoxicity and myalgia are not thoroughly established. Phospholipase D (PLD), a membrane phospholipid-hydrolyzing enzyme is ubiquitous and involved in mediating lipid signaling in mammalian cells including the skeletal muscle cells (myocytes). Therefore, we hypothesized that statins would mediate skeletal muscle myocyte damage through activation of the PLD-mediated lipid signaling, and inhibition of PLD activation would protect against the statin-induced myotoxicity. In order to test our hypothesis, we used the well-established C2C12 mouse skeletal muscle myoblast cell line and studied the PLD activation and cytotoxicity *in vitro* induced by two different widely used statins (mevastatin and simvastatin) by the CVD patients. Our studies revealed that the statins caused dose-dependent (10-100 μ M) and time-dependent (4-24 h) activation of PLD (as determined by the [³²]P-labeling of cells and thin-layer chromatography of phosphatidylbutanol formation) and cytotoxicity and mitochondrial dysfunction (as determined by the release of intracellular lactate dehydrogenase, suppression of MTT reduction, and alterations in cell morphology) in the C2C12 myoblast cells. Our results also showed that cholesterol replenishment protected against the

statin-induced toxicity to the C2C12 cells. Furthermore, our results showed that the novel PLD-specific inhibitor, 5-fluoro-2-indolyl des-chlorohalopemide (FIPI) inhibited the statin-induced PLD activation and cytotoxicity in the C2C12 myoblast cells. For the first time, our current study demonstrated the role of endogenous cellular cholesterol depletion and PLD-mediated lipid signaling in the statin-induced skeletal muscle myocyte damage and emphasized the importance of PLD inhibition in attenuating the statin-induced myotoxicity and myalgia in CVD patients consuming statins to lower the elevated levels of endogenous cholesterol.

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INTRODUCTION

Cardiovascular Diseases

In developed countries across the world, and particularly in the United States, cardiovascular diseases (CVDs) including the cerebrovascular diseases claim the spot as the number one cause of death (Stock et al., 2012; Sowers et al., 2001). CVDs are the result of restricted blood vessels that lead to chest pain, stroke, or heart attack, and can also classify other heart conditions that cause an individual pain. Pathological conditions including rheumatic heart disease, the inflammation of the heart tissue; atherosclerosis, the clogging of arteries with fat and cholesterol; and vascular inflammation serve as some of the most common cardiovascular diseases that act as a detriment to health in the American population (Essop and Peters., 2014; Granger et al., 2004; Chen et al., 2012; Varghese, 2014; Xiong et al., 2012).

CVDs have a variety of causes, both congenital and acquired. Congenital CVD means that an individual born with an abnormality of the cardiovascular system is either predisposed to or born with a cardiovascular disease (Nasr and Kussman, 2014; Singh et al., 2014). Various abnormalities of the heart chambers, septa, valves, arteries or veins, all present at birth, can serve as the cause of congenital cardiovascular disease. Acquired cardiovascular disease means that an individual's lifestyle choices or environment have contributed to a condition affecting his or her cardiovascular system that did not present itself at birth. A diet high in fat and cholesterol, lack of exercise, use of tobacco, drugs, and alcohol, and a stressful environment serve as the primary risk factors for CVDs (Lamarche and Couture, 2014; Gielen et al., 2015; Buttar et al., 2005; Salabei and Conklin, 2013; Katsiki et al., 2014; Hartley et al., 2014). High concentrations of circulating blood cholesterol can cause the deposition of this cholesterol in blood vessels and eventually lead to plaque formation in the blood vessel and ultimate obstruction of the circulation

to vital organs including the heart, brain, and kidney (Badimon L and Vilahur, 2012; Cozma et al., 2009; Sander and Giles, 2002; Madden et al., 2012). Many individuals whose lifestyles put them at risk of developing CVDs have the opportunity to modify their diets, exercise routines, and lifestyle choices; however, it has become increasingly common for medical professionals to prescribe drugs that lower endogenously synthesized cholesterol in order to lower the risk for cardiovascular disease (Ewang-Emukowhate and Wierzbicki, 2013).

Cholesterol and Cardiovascular Diseases

Cholesterol is the major component of the cell membrane lipid backbone that regulates the membrane fluidity, structure, and function (Cortes et al., 2014; Owen and Gaus, 2013). Also, cholesterol occurs in circulation in two different types, including high-density lipoproteins (HDLs) and low-density lipoproteins (LDLs) (Najam and Ray, 2015; Scanu and Wisdom, 1972). The HDLs can reduce the risk for heart disease when in high concentrations, and thus often earn the name “good cholesterol” (Rached et al., 2015) The LDLs can clog vessels when in high concentrations, and thus often earn the name “bad cholesterol” (Elshourbagy et al., 2014). Having high concentrations of LDL-cholesterol (LDL-C) is referred to as hypercholesterolemia, and can have genetic or dietary roots (Elshourbagy et al., 2014).

Familial hypercholesterolemia is an endogenous, inherited condition in which an individual has a deficiency of LDL-C receptors on the surfaces of human cells (Santos and Maranhao, 2014). These receptors would normally transport LDL-C into the cells, so a deficiency in receptors results in a high concentration of circulating LDL-C in the bloodstream that can accumulate on the vessel walls. In addition, feedback inhibition usually stops the synthesis of more cholesterol by inhibiting 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase), a rate-limiting enzyme in cholesterol synthesis (Schema-1), when high

concentrations are detected in the blood, but individuals with familial hypercholesterolemia do not have this feedback inhibition, so their cells continue to synthesize cholesterol (Trapani et al., 2011; McPherson, 2012; Salakhutdinov et al., 2011) (Schema-1). Dietary hypercholesterolemia results from a diet high in saturated fats and cholesterol, more than an individual can utilize. The excessive amount of cholesterol consumed finds its way into the blood stream and can accumulate on vessel walls leading to plaque formation and atherosclerotic lesions in the blood vessel that would obstruct blood flow leading to the CVDs and cerebrovascular disorders.

Statins as the Endogenous Cholesterol-lowering Drugs

Current therapeutic intervention for hypercholesterolemia involves the use of HMG-CoA reductase-specific inhibitors, known as statins (see the diagram of chemical structures of statins), to attenuate or suppress the synthesis of endogenous cholesterol (Trapani et al., 2011; McPherson, 2012; Salakhutdinov et al., 2011). By suppressing or inhibiting the endogenous cholesterol synthesis, there exists lowered levels of LDL-C in the blood circulation, and as a result the risk for blood vessel clogging or plaque formation decreases. Popular statins in the clinical use include atorvastatin (Lipitor), pravastatin (Pravachol), simvastatin (Zocor), mevastatin (Compactin), and lovastatin (Altoprev) (Malinowski, 1998; Moghadasian et al., 1998; Opie, 2015).

One of the downsides to statin use is the myotoxicity associated with those cholesterol-lowering drugs, called the “statin myalgia” or the “statin-induced myalgia” (Ganga et al., 2014). Studies have shown that statin use in certain individuals results in “statin intolerance” that is associated with myotoxicity, as a significant percentage of those administering statins experience painful side effects in their skeletal muscles and bones (Ahmad, 2014). Statins have pleiotropic effects once introduced into the body, but the mechanism through which they cause myalgia

remains unclear. If understood, the safety and effectiveness of statins could increase. It seems there exist multiple mechanisms through which statins cause myotoxicity, one being the mitochondrial mechanism, which holds valid to some extent (Sirvent et al., 2008). In this mechanism, statins cause damage to the mitochondria of the skeletal myocyte, which reduces the energy metabolism of the cell and can ultimately result in cell death (Dirks and Jones, 2006). Having this as the precedence, here it is hypothesized that statins cause membrane lipid signaling perturbation and mitochondrial dysfunction through cholesterol depletion, which ultimately leads to statin-induced myalgia.

Lipid Signaling and Statin-induced Myotoxicity or Myalgia

The phospholipid bilayer forms a barrier between the cell and the external environment. Membrane phospholipids, protein channels, and cholesterol compose much of the membrane bilayer of living cells including that of human (Korn, 1969; Siekevitz, 1972; Suetsugu et al., 2014). The bilayer must have the ability to interact with the environment, detect the external and internal environments of the cells, and relay information to the cell, wherein the membrane lipid signaling accomplishes these goals (Slone and Fleming, 2014). Phospholipases are the house-keeping enzymes that can hydrolyze the membrane phospholipids during the turnover of the membrane phospholipids and also form different bioactive lipid molecules, each with an associated membrane lipid signal as interpreted by the cell. Four major phospholipases exist, including the phospholipase A1, A2, C, and D, and each catalyzes a specific hydrolysis of the membrane phospholipid within the cell (Richmond and Smith, 2011; Tani et al., 2012; Ghosh et al., 2006; Krizaj, 2014; Zhang and Frohman, 2014; Follo et al., 2015). Specifically, the phospholipase D (PLD) hydrolyzes the membrane phospholipid (phosphatidylcholine, PC) releasing the phosphatidic acid (PA) which can act as a potent bioactive cell signaling mediator

(Gomez-Cambronero, 2014; Zhang and Frohman, 2014). The cell can further convert the PLD-generated PA to potent bioactive lipid signal mediators such as the lysophosphatidic acid (LPA) or diacylglycerol (DAG) upon the actions of phospholipase A1 (PLA1) or PLA2, which can cause myotoxicity (Schema-2) (Parinandi et al., 1999; Parinandi et al., 2001; Cummings et al., 2002; Patel et al., 2011; Secor et al., 2011; Varadharaj et al., 2006). It has been demonstrated that lowering of cell membrane cholesterol in the vascular endothelial cells leads to the activation of PLD and generation of PA (Kline et al., 2010). Therefore, here it is hypothesized that statins cause cholesterol depletion in the membranes of the skeletal muscle cells through the inhibition of HMG-CoA reductase, leading to the activation of PLD that generates the bioactive lipid signal mediator (PA), which in turn results in the mitochondrial damage and myotoxicity as a mechanistic basis of the statin-induced myalgia or myotoxicity. In order to test our hypothesis, in the current study, we chose the well-established skeletal muscle cell model, the C2C12 myoblast cells. Our studies revealed that the two widely used statins (HMG-CoA reductase inhibitors), mevastatin and simvastatin, to lower the elevated levels of endogenous cholesterol in circulation as the therapeutic strategy to protect against the hypercholesterolemia-induced CVDs, caused PLD activation and generation of the bioactive lipid signal mediator (PA) leading to the mitochondrial dysfunction and cytotoxicity in the C2C12 myoblast cells through cholesterol depletion.

MATERIALS & METHODS

Materials

Mouse skeletal muscle myoblast cells (C2C12s) (passage 2) were obtained from Cell Applications Inc. (San Diego, CA). Phosphate-buffered saline (PBS) was purchased from Biofluids Inc (Rockville, Maryland). Minimal Essential Medium (MEM), FBS, trypsin, nonessential amino acids, penicillin/streptomycin, Dulbecco Modified Eagle Medium (DMEM) tissue culture reagents, phosphate-free modified medium, 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide reduction kit (MTT assay kit), lactate dehydrogenase cytotoxicity assay kit (LDH release assay kit), and analytical reagents of the highest purity were all obtained from Sigma Chemical Co (St Louis, Missouri). Phosphatidylbutanol (PBt), was acquired from Avanti Polar Lipids (Alabaster, Alabama). [³²P]orthophosphate (carrier-free) was purchased from New England Nuclear (Wilmington, Delaware). Anti-rabbit AlexaFluor 488-conjugated antibody and the Amplex Red cholesterol determination kit were purchased from Molecular Probes Invitrogen Co (Carlsbad, California). 5-Fluoro-2-indolyl des-chlorohalopemide hydrochloride hydrate (FIPI) was prepared as described in earlier publications (Secor et al., 2011; Patel et al., 2011). All other reagents were acquired from the Sigma Chemical Company (St. Louis, MO).

In Vitro Cell Culture

The C2C12 myoblast cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and antibiotics up to 90-100% confluence in sterile 35-mm or 60-mm dishes and in 96-well plates under a sterile and humidified atmosphere of 95% air-5% CO₂ at 37° C. C2C12s were used up to passage 20 for experiments.

Assay of Phospholipase D (PLD) Activation

PLD activity in the C2C12 myoblast cells was determined according to our previously published procedure (Patel et. al., 2011; Secor et al., 2011). C2C12 myoblast cells cultured in 35-mm dishes were labeled with [³²P]orthophosphate (5 mCi/ml) in DMEM phosphate-free medium containing 2% (vol/vol) fetal bovine serum for 14 h. Following the experimental treatments for the chosen periods of time, [³²P]-labeled phosphatidylbutanol ([³²P]PBT) formed from the PLD activation and transphosphatidyl reaction in cellular lipid extracts, as an index of PLD activity in intact cells, was separated by thin-layer chromatography (TLC). Radioactivity associated with the [³²P]PBT was quantified by liquid scintillation counting and data were expressed as DPM normalized to the total [³²P] in the lipid extract of the cells in the dish.

Lactate Dehydrogenase (LDH) Release Assay of Cytotoxicity

The C2C12 myoblast cells were grown up to 90-100% confluence in sterile 15.5-mm dishes (24-well culture plate) and treated with DMEM alone or DMEM containing the chosen concentrations of statins and/or FIPI at designated time points. At the end of the incubation period, the supernatant was removed, and the level of lactate dehydrogenase (LDH) activity was measured spectrophotometrically according to the manufacturer's protocol (Sigma Chemical Co., St. Louis, MO).

MTT Cell Proliferation Assay

The C2C12 myoblast cells were grown up to 90-100% confluence in sterile 15.5-mm dishes (24-well culture plate) and treated with DMEM alone or DMEM containing the chosen concentrations of statins and/or FIPI at designated time points. At the end of the incubation

period, the supernatant was removed, and the extent of MTT reduction was measured spectrophotometrically according to the manufacturer's protocols (Cayman Chemical Co., Ann Arbor, MI).

Cellular Morphology

Morphological changes in the C2C12 myoblast cells grown in the sterile 35-mm dishes up to 90-100% confluence, following their exposure to the chosen concentrations of statins and/or FIPI for different time periods, were examined under light microscope as an index of cytotoxicity. Images of cell morphology were digitally captured using the Zeiss Axioskop 200 with Zen 2011 software at 20X magnification.

Cholesterol Determination

The C2C12 myoblast cells were grown up to 90-100% confluence in sterile 60-mm dishes, and treated with DMEM alone or DMEM containing the chosen concentrations of statins and/or FIPI for the chosen periods of time. Cells were then liberated from dish, reconstituted in PBS, and protein was determined following the protein determination by the BCA assay. Cholesterol contents in the samples was normalized to the total cellular protein (1 mg) according to our previously published method (Hinze et. al., 2012).

Phospholipase D1 (PLD1) Phosphorylation Visualization by Confocal Immunofluorescence Microscopy

The C2C12 myoblast cells were grown on sterile glass cover slips (~90% confluence) and treated with DMEM alone or DMEM containing chosen concentrations of statins for 12 h.

Cover slips were then rinsed three times with PBS, and fixed with 3.7% formaldehyde in PBS for 10 min. at room temperature. The cells were permeabilized in 0.25 % Triton X-100 prepared in PBS containing 0.01% Tween-20 (PBS-T) for 5 min. The cells were again washed three times with PBS-T, and treated with PBS-T containing 1 % BSA blocking buffer for 30 min. at room temperature. Cover slips were then incubated overnight at room temperature with the primary antibody [phospho-PLD1 (1:150 dilution)] in 1% BSA solution. After rinsing three times with PBS-T, the cells were labeled with secondary AlexaFluor 488 (1:100 dilution) in 1% BSA in PBS-T for 1 h. Finally, the cells were washed three times with PBS-T, mounted, and examined under Zeiss LSM 710 Confocal/Multiphoton Microscope powered by Argon-2 laser with 500-550 BP filter. The images were captured digitally and the average fluorescent intensity of triplicate samples was determined using ImageJ.

Preparation of Solutions Containing Pharmacological Agents for Treatment of Cells

All water-soluble pharmacological agent solutions were freshly formulated in DMEM for treatment of cells. The stock solutions of lipophilic pharmacological agents including all statins and FIPI were freshly assembled in DMSO and then diluted in DMEM for treatment of cells. The final DMSO concentration in the cell treatment medium did not exceed 0.1% (vol/vol) and did not appear to have any influence on experimental outcomes.

Statistical Analysis

All experiments were completed in triplicate. Results were reported as mean \pm standard deviation (SD). Statistical analysis of data was accomplished by one-way analysis of variance

(ANOVA) by use of the SigmaStat (Jandel Scientific, San Rafael, California). The statistical significance level was taken as $p \leq 0.05$.

RESULTS

Statins induce PLD activation in C2C12 myoblast cells. Statins, being the HMG-CoA reductase inhibitors are established to lower endogenous levels of cholesterol by inhibiting cellular cholesterol synthesis (Okayama et al., 2015; Bockorny and Dasanu, 2015). Also, in our earlier study, we have shown that the cholesterol-depleting agent such as the methyl- β -cyclodextrin (M β CD) lowers cholesterol levels and causes activation of PLD in the vascular endothelial cells (Kline et al., 2010; Hinzey et al., 2012). Taking these as the premise, here we hypothesized that statin-induced decrease of cellular cholesterol would lead to activation of PLD in the myocyte model, the C2C12 myoblast cells. In order to test our hypothesis, in the current study, we treated the C2C12 cells with two selected statins, mevastatin and simvastatin for different periods of time (4-24 h) and assayed the activation of PLD. Here, we determined the activity of PLD by analyzing the intracellular formation of phosphatidylbutanol as the transphosphatidylation reaction product of PLD, which is an established and widely used assay of cellular PLD activity (Natarajan et al., 2001). Both chosen statins in the current study, mevastatin and simvastatin (10 μ M) caused significant time-dependent activation of PLD in the C2C12 myoblast cells (Figs. 1A & 1B). Although the PLD activity peaked at 12 h under mevastatin treatment of cells, simvastatin caused a significant linear increase of the PLD activity from 4 h up to 24 h of treatment in a time-dependent manner as compared to the untreated control cells (Figs. 1A & 1B). At 12 h, mevastatin caused a 4.5-fold increase of PLD activation in the C2C12 cells as compared to the control untreated and mevastatin-treated cells at 4 h. However, the extent of PLD activity at 24 h although significantly increased in the mevastatin-treated cells, it dropped markedly from that at 12 h of treatment in the C2C12 myoblast cells. On the other hand, simvastatin caused a significant and \sim 7-fold increase in PLD activity at 24 h as

compared to the same in the control untreated and simvastatin-treated cells at 4 h (Fig. 1B). One noteworthy response of the control untreated cells was that the basal PLD activity (formation of PA without statin treatment) also linearly increased with time (Figs. 1A & 1B). These results revealed that both mevastatin and simvastatin caused a significant activation of PLD in the C2C12 myoblast cells and simvastatin apparently was more potent than mevastatin in inducing the activation of PLD and generating the bioactive lipid signal mediator (PA) in cells.

FIPI, the PLD-specific inhibitor attenuates statin-induced PLD activation in C2C12 cells.

FIPI is the only available PLD-specific pharmacological inhibitor and in our earlier studies we used it in our cell culture models and reported its efficacy in causing inhibition of PLD activity (Secor et al., 2011; Patel et al., 2011). Here, in this study we used FIPI (*i*) to investigate its inhibitory action on the statin-induced PLD activation and (*ii*) to confirm that statins indeed activate PLD in the C2C12 myoblast cells. By virtue of its PLD-specific inhibitory action, FIPI (0.1-1 μ M, 12 h of pretreatment) significantly and drastically inhibited the basal PLD activity in the control untreated C2C12 myoblast cells at 24 h (Figs. 2A & 2B). At 1 μ M concentration, FIPI caused ~85-92% decrease of basal PLD activation in the control untreated cells (Figs. 2A & 2B). Furthermore, FIPI, in a dose-dependent manner (0.1-1 μ M), significantly and robustly attenuated the statin-induced PLD activation in cells treated with both mevastatin (10 μ M) and simvastatin (10 μ M) for 24 h as compared with the cells treated with statins alone (Figs. 2A & 2B). FIPI (1 μ M) significantly attenuated the statin-induced PLD activation by ~90-95% in cells treated with both mevastatin (10 μ M) and simvastatin (10 μ M) for 24 h as compared to the same in the C2C12 myoblast cells treated with statins alone (Figs. 2A & 2B). These results clearly revealed that (*i*) FIPI was a potent inhibitor of the statin-induced PLD activation at doses ranging

between 0.1-1 μM and the 1 μM dose was the most efficacious in causing effective inhibition of the statin-induced PLD activation and (ii) indeed, statins induced the activation of PLD in the C2C12 myoblast cells.

Statins induce cytotoxicity in C2C12 cells. It is becoming increasingly evident that the lipid-lowering and cholesterol-depleting drugs such as the lipophilic statins cause cytotoxicity to the mammalian cells including the normal and malignant cells (Costa et al., 2013; Abdoli et al., 2013; Kotamraju et al., 2007; Kanugula et al., 2014). Also, our earlier studies have revealed that the cyclodextrin-induced cellular cholesterol depletion causes cytotoxicity in the vascular endothelial cells in culture (Kline et al., 2010; Hinzey et al., 2012). Having these reports as the basis, here we investigated whether statins (simvastatin and mevastatin) would cause cytotoxicity in the C2C12 cells as determined by the lactate dehydrogenase (LDH) release from the cells. Our results revealed that both simvastatin and mevastatin (10-100 μM) significantly induced LDH release from the cells in a dose-dependent fashion at 24 h of exposure as compared to the same in the control untreated cells (Figs. 3A & 3B). Both statins at 10 μM dose caused robust and significant increase in the LDH release from cells (2-fold increase by simvastatin; 3.7-fold increase by mevastatin) and further increase in release of the intracellular LDH was not markedly enhanced by increasing the dose of statins at 24 h of treatment of the C2C12 cells (Figs. 3A & 3B). Therefore, our current results revealed that statins (simvastatin and mevastatin) induced cytotoxicity in the C2C12 cells even at 10 μM dose as demonstrated by the release of intracellular LDH, the standard mammalian cytotoxicity assay. Furthermore, the results showed that mevastatin was more potent than simvastatin at 10 μM dose in causing cytotoxicity in the C2C12 myoblast cells (Figs. 3A & 3B).

Statins induce decrease of cholesterol in C2C12 cells. As statins are established to lower or decrease the cellular levels of cholesterol (Kotamraju et al., 2007), here we investigated whether statins would decrease/lower the cholesterol levels in the C2C12 myoblast cells. All the three tested statins (simvastatin, mevastatin, and lovastatin) at 10 μ M dose caused significant decrease of intracellular cholesterol (25%, 21%, and 46% respectively) as compared to that in the control untreated cells at 24 h of treatment (Fig. 4A). Among all the chosen statins, lovastatin was the most effective statin in decreasing the intracellular cholesterol levels in the C2C12 myoblast cells. Therefore, these results revealed that statins caused significant decrease of the intracellular levels of cholesterol in the C2C12 myoblast cells.

Cholesterol replenishment protects against statin-induced cytotoxicity in C2C12 cells. Our earlier studies have demonstrated that cholesterol replenishment offered protection against the cyclodextrin-induced cytotoxicity mediated through the intracellular cholesterol depletion in the vascular endothelial cells (Hinze et al., 2012). In the current study, we showed that statins cause both cytotoxicity (LDH release) and cholesterol depletion. Therefore, here we investigated whether cholesterol replenishment would offer protection against the statin-induced cytotoxicity (LDH release) in the C2C12 myoblast cells. Our results revealed that both mevastatin (10 μ M) and simvastatin (10 μ M) caused significant cytotoxicity as revealed by the intracellular LDH release assay at 24 h of treatment with statins as compared to the control untreated cells (Figs. 4B & 4C). Furthermore, treatment of cells with the water-soluble (methylcyclodextrin-conjugated) cholesterol (1%) offered significant protection of the statin-induced cytotoxicity (attenuation of release of intracellular LDH) (Figs. 4B & 4C). However, cholesterol at 2% dose was not effective in lowering the mevastatin-induced release of intracellular LDH and failed to protect

against the mevastatin-induced cytotoxicity in the C2C12 cells as opposed to the protective action offered by cholesterol at 1% dose (Fig. 4B). On the other hand, cholesterol at 2% dose, although effective in significantly protecting against the simvastatin-induced cytotoxicity in the C2C12 cells, its protective action was less effective than that was offered by cholesterol at 1% dose (Fig. 4C). Overall, these results revealed that (i) cholesterol replenishment offered protection against the statin-induced cytotoxicity in the C2C12 cells as revealed by the intracellular LDH assay and (ii) lower dose of cholesterol (1%) was more effective in significantly protecting against the statin-induced cytotoxicity.

FIPI, the PLD-specific inhibitor attenuates statin-induced cytotoxicity in C2C12 cells. We have earlier reported that the PLD-specific pharmacological inhibitor, FIPI protected against the oxidant- and drug-induced cytotoxicity mediated by the PLD-dependent bioactive lipid signaling in the vascular endothelial cells (Patel et al., 2011; Secor et al., 2011). Therefore, here we investigated to show whether FIPI would offer protection against the statin-induced cytotoxicity (intracellular LDH release) in the C2C12 myoblast cells to demonstrate the role of PLD therein, since in the current study it was revealed that statins caused significant activation of PLD which was significantly attenuated by FIPI in the C2C12 myoblast cells (Figs. 1 & 2). Here, our current results revealed that FIPI (1 μ M, pretreatment for 12 h) offered significant protection against simvastatin (10 μ M)- and mevastatin (10 μ M)-induced cytotoxicity at 24 h as demonstrated by the release of intracellular LDH (Fig. 5). Overall, the current study demonstrated that (i) the PLD-specific pharmacological inhibitor, FIPI offered significant protection of the statin-induced cytotoxicity and (ii) the PLD-mediated bioactive lipid signaling also was involved in the statin-induced cytotoxicity in the C2C12 myoblast cells.

Statins induce mitochondrial dysfunction in C2C12 cells. It is rapidly emerging that statins cause mitochondrial dysfunction and damage in different organs/cells including the skeletal muscle, especially during the statin-induced myopathy (Stroes et al., 2015; Abdoli et al., 2014). Although MTT reduction by the mitochondria is used as a valid indicator of cell proliferation and cytotoxicity, the assay is also utilized to ascertain the mitochondrial function (Sliman et al., 2010). Therefore, here we utilized the MTT reduction by the cellular mitochondria in the C2C12 cells to demonstrate the statin-induced mitochondrial dysfunction since MTT is reduced by a mitochondria-specific dehydrogenase (Sliman et al., 2010). Both statins, simvastatin and mevastatin, in a dose-dependent fashion (10-100 μ M) caused significant decrease of MTT reduction in the C2C12 cells at 24 h of treatment as compared to the control untreated cells (Figs. 6A & 6B). Simvastatin (10 μ M) and mevastatin (10 μ M) significantly caused 51% and 60% decrease of MTT reduction, respectively by the C2C12 myoblast cells treated for 24 h as compared to the same in the control untreated cells (Figs. 6A & 6B). Therefore, these studies demonstrated that both mevastatin and simvastatin induced the mitochondrial dysfunction as revealed by the decrease in the MTT reduction by the C2C12 cells.

FIPI, the PLD-specific inhibitor attenuates statin-induced mitochondrial dysfunction by C2C12 cells. In the earlier experiments of the current study, we had shown that (i) statins induced PLD activation, (ii) FIPI, the PLD-specific pharmacological inhibitor attenuated the statin-induced PLD activation, and (iii) FIPI offered protection against the statin-induced cytotoxicity (intracellular LDH release) in the C2C12 myoblasts. Hence, here we investigated whether FIPI would attenuate the statin-induced mitochondrial dysfunction as assayed by the decrease of MTT reduction by the C2C12 cells to establish a link between the statin-induced

PLD activation and bioactive lipid signal mediator (PA) generation and mitochondrial dysfunction. FIPI (1 μ M, 12 h of pretreatment) significantly and markedly attenuated the simvastatin (10 μ M)- and mevastatin (10 μ M)-induced decrease of MTT reduction by the C2C12 cells (Fig. 7A). Furthermore, the results showed that the protective action of FIPI against the simvastatin-induced decrease of MTT reduction by the C2C12 cells was more pronounced than that induced by the mevastatin (Fig. 7A). Overall, these studies demonstrated that FIPI significantly attenuated the statin-induced mitochondrial dysfunction in the C2C12 myoblast cells as demonstrated by the mitochondria-specific MTT reduction assay, suggesting the role of PLD-generated bioactive lipid signal mediators therein.

FIPI, the PLD-specific inhibitor attenuates statin-induced morphological alterations in C2C12 Cells. Our studies conducted so far in the current project revealed that statins (mevastatin and simvastatin) induced cytotoxicity and mitochondrial dysfunction that was mediated by the PLD-generated bioactive lipid signaling and cholesterol depletion in the C2C12 myoblast cells. Cell morphology alterations serve as an index of cytotoxicity induced by toxic stresses (Sliman et al., 2010; Patel et al., 2012). Here, we furthered to demonstrate that statins would induce the morphological alterations in the C2C12 myoblast cells, which might be protected by the PLD-specific pharmacological inhibitor, FIPI. Simvastatin, mevastatin, and lovastatin at 10 μ M dose caused severe cell morphological alteration at 24 h of treatment characterized by the light microscopic examinations including elongated myoblast cells turning into round and circular cells (Fig. 7B). This indicated the drastic changes in the morphological nature of the cells upon the statin treatment. However, FIPI pre-treatment (1 mM for 12 h) offered almost complete protection of the statin-induced morphological alterations in the C2C12 myoblast cells (Fig. 7B).

These results further confirmed that the statin-induced cell morphological alterations (cytotoxicity) was (i) protected by the PLD-specific pharmacological inhibitor, FIPI and (ii) PLD-generated bioactive lipid signaling through PA formation played a role in the statin-induced cell morphology alterations in the C2C12 cells.

Statins induce threonine phosphorylation of PLD1 in C2C12 cells. Our earlier reports revealed that oxidant stress and heavy metal toxicity cause the protein kinase-mediated serine-threonine phosphorylation of PLD isoenzymes upstream of PLD activation (Natarajan et al., 2001; Secor et al., 2011). Since the protein kinase-mediated serine-threonine phosphorylation regulates the activity of PLD isoenzymes, in this study we investigated whether statins would induce threonine phosphorylation of PLD1 isoenzyme in the C2C12 myoblast cells as examined by the confocal immunofluorescence microscopy with the aid of PLD-phosphothreonine-specific antibody immunostaining. Both mevastatin and simvastatin at 10 μ M dose induced intense phosphorylation of PLD1 isoenzyme in intact C2C12 myoblast cells at 12 h of treatment of cells with the statins (upstream of maximal PLD activation at 24 h) as compared to the same in the control untreated cells (Fig. 8). This study demonstrated that statins induce serine phosphorylation of PLD1 isoenzyme upstream of the activation of the enzyme.

DISCUSSION

High cholesterol levels (hypercholesterolemia) in circulation of humans have been considered as a risk factor for the vascular disorders, cardiovascular diseases (CVDs), cerebrovascular diseases, and chronic heart diseases (CHD). Therefore, aggressive therapy with the cholesterol-lowering drugs to lower the endogenously synthesized cholesterol have been in practice currently (Trapani et al., 2011; McPherson, 2012; Salakhutdinov et al., 2011; Malinowski, 1998; Moghadasian et al., 1998; Opie, 2015). The cholesterol-lowering drugs to combat the elevated levels of endogenously synthesized cholesterol are the statins, which are the HMG-CoA reductase-specific inhibitors since the HMG-CoA reductase is a rate-limiting enzyme in the cholesterol biosynthetic pathway (Schema-1) (Trapani et al., 2011; McPherson, 2012; Salakhutdinov et al., 2011). However, it is increasingly becoming evident that statins used for cholesterol-lowering cause adverse effects such as statin myalgia or statin myotoxicity (Ganga et al., 2014; Ahmad, 2014). Although a few mechanisms have been put forth to pinpoint the exact nature of the statin-induced myotoxicity or myalgia among patients with hypercholesterolemia and CVDs which include mitochondrial dysfunction, apoptotic pathway, and coenzyme-Q10 (CoQ10) depletion (Sirvent et al., 2008; Dirks and Jones, 2006; Stroes et al., 2015; Kotamraju et al., 2007). Nevertheless, there is still a void in understanding the exact mechanism of statin-induced myotoxicity or myalgia. Therefore, here in the current study we hypothesized that statins would cause cholesterol depletion in the membranes of the skeletal muscle cells through the inhibition of HMG-CoA reductase (Schema-1), leading to the activation of PLD that could generate the bioactive lipid signal mediator (PA) (Schema-2), which in turn might result in the mitochondrial damage and myotoxicity as a mechanistic basis of the statin-induced myalgia or myotoxicity. To test our hypothesis, we utilized the well-established skeletal muscle cell model,

the C2C12 myoblast cells. The current study demonstrated that the two widely used statins (HMG-CoA reductase inhibitors), mevastatin and simvastatin to lower the elevated circulating levels of endogenous cholesterol to combat the hypercholesterolemia-induced CVDs, induced the PLD activation and formation of the bioactive lipid signal mediator (PA) causing the mitochondrial dysfunction and cytotoxicity in the skeletal muscle cell model (C2C12 myoblast cells) through the decrease of endogenous cholesterol (Schema-3).

By virtue of their ability to selectively inhibit the rate-limiting enzyme in the cholesterol biosynthetic pathway in the mammalian cells, the potent HMG-CoA reductase inhibitors, statins drastically decrease or deplete the endogenous levels of cholesterol. On the other hand, cholesterol is an important lipidic component of the cell membranes that regulates the membrane structure and function (Cortes et al., 2014; Owen and Gaus, 2013). If the cholesterol levels and packing in the cell membranes are altered, the cell would face dire consequences such as cytotoxicity and cell death. Our earlier studies have demonstrated that the widely used experimental cholesterol-depleting agent, methyl- β -cyclodextrin (M β CD) causes cytotoxicity to the vascular endothelial cells through the cellular cholesterol depletion and cholesterol replenishment following the M β CD-mediated cholesterol depletion of the cells restores the cell viability, suggesting that the cellular cholesterol plays a crucial role in maintaining the viability of the mammalian cells (Kline et al., 2010; Hinzey et al., 2012). Along these lines, it could be surmised that the statin-induced cytotoxicity in the C2C12 myoblast cells as observed in the current study was caused by the statin-mediated depletion of cellular cholesterol in the skeletal muscle cell model. This was also confirmed by replenishing the cells with the water-soluble cholesterol which rescued the cells from statin-induced cytotoxicity. Therefore, it could be unequivocally stated that the statin-mediated cellular cholesterol depletion could be among the

prime causes of the statin-induced myotoxicity operating behind the statin-induced myotoxicity or myalgia.

Phospholipase D (PLD) is ubiquitously present in mammalian cells which belongs to the family of phospholipases that conduct the membrane phospholipid hydrolysis (Schema-2) (Parinandi et al., 1999 & 2001; Secor et al., 2011; Patel et al., 2011; Cummings et al., 2002; Varadharaj et al., 2006). Although PLD is a house-keeping enzyme involved in the metabolism of membrane phospholipids, the enzyme is known to be activated by a variety of agonists including hormones, reactive oxygen species, heavy metals, toxins, and metabolic stresses (Parinandi et al., 1999 & 2001; Secor et al., 2011; Patel et al., 2011; Cummings et al., 2002; Varadharaj et al., 2006). PLD exists in the cells as two isoforms such as PLD1 and PLD2 (Parinandi et al., 1999 & 2001; Secor et al., 2011; Patel et al., 2011; Cummings et al., 2002; Varadharaj et al., 2006) which are localized in different cellular compartments such as the cytosol and nucleus. These isoforms can be translocated and activated by the agonists. Protein kinases such as mitogen-activated protein kinases (MAPKs), protein tyrosine kinases, protein kinase C, G-protein coupled receptors, and receptor-mediated kinases phosphorylate the PLD isoforms at the corresponding amino acid residues (serine or threonine or tyrosine), cause the translocation of these isoforms and activate them (Parinandi et al., 1999 & 2001; Secor et al., 2011; Patel et al., 2011; Cummings et al., 2002; Varadharaj et al., 2006). Thus the PLD isoforms in the cells upon activation by the agonists hydrolyze the cell membrane phospholipids (e.g. phosphatidylcholine) and form the most potent bioactive signal lipid, the PA (Secor et al., 2011; Patel et al., 2011). PA is known to be capable of causing cytotoxicity and cellular cytoskeletal alterations that could lead to the dysfunction of the cells and even cell death (Secor et al., 2011; Patel et al., 2011). The PLD-generated PA can further be transformed by either PLA1 or PLA2

into LPA or by lipid phosphate phosphohydrolase into DAG (Parinandi et al., 1999 & 2001; Secor et al., 2011; Patel et al., 2011; Cummings et al., 2002; Varadharaj et al., 2006). Both LPA and DAG are potent bioactive signal mediators which are involved in invoking the adverse cellular responses. Our earlier studies have demonstrated that the cholesterol depletion mediated by M β CD in the vascular endothelial cells leads to the activation of PLD causing cytotoxicity through the generation of the lipid signal mediator, PA (Kline et al., 2010). Along these lines, in the current study it was demonstrated for the first time that statins induced the activation of PLD in the C2C12 cells which was attenuated by the PLD-specific inhibitor, FIPI. Furthermore, the current study also showed that the PLD-specific inhibitor, FIPI effectively protected against the statin-induced cytotoxicity. The results of the current study also revealed that statins induced the upstream threonine phosphorylation of PLD1 isoenzyme in the C2C12 cells that could have been mediated by the MAPKs or PKC leading to the translocation and activation of the enzyme as previously observed in other cellular models. Thus, a connection between statin-induced PLD activation and statin cytotoxicity in the C2C12 muscle cell model offers a PLD-dependent mechanism of statin myotoxicity and myalgia suggesting a role of the PLD-generated bioactive lipids such as PA, LPA, and DAG therein.

In the current study, it was shown that statins caused the mitochondrial dysfunction as determined by the MTT reduction ability in the C2C12 cells (Sliman et al., 2010). The statin-induced mitochondrial dysfunction (decrease in MTT reduction catalyzed by the mitochondrial dehydrogenase) and cytotoxic cell morphology alterations were attenuated by the PLD-specific inhibitor. This suggested a reasonable connection or association with the PLD activation and subsequent generation of the bioactive signal lipid mediators (PA, LPA, and DAG) and the mitochondrial dysfunction and cytotoxicity in the C2C12 myoblast cells. However, other

mitochondria-driven mechanisms of the statin-induced myotoxicity such as apoptosis, CoQ10 loss, decline of ATP production, and reactive oxygen species generation are not ruled out in mediating the statin-induced cytotoxicity in the C2C12 cells.

Overall the current study demonstrated that the cholesterol-lowering HMG-CoA inhibitor drugs, statins, caused the cellular cholesterol depletion leading to the activation of PLD which in turn caused the mitochondrial dysfunction and cytotoxicity in the skeletal muscle cell model, C2C12 cells (Schema-3). Thus, it is highly reasonable to ascertain that PLD activation and formation of the PLD-generated bioactive lipids could act as potential players in the statin-induced myalgia and myotoxicity. Therefore, PLD could be a pharmacological target for combating the statin myotoxicity.

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LIST OF ABBREVIATIONS

ATP: adenosine 5-phosphate

BCA: bicinchoninic acid

BSA: bovine serum albumin

CoQ10: coenzyme Q10

CVD: cardiovascular disease

CHD: chronic heart disease

DAG: diacylglycerol

DMEM: Dulbecco's modified Eagle medium

DMSO: dimethylsulfoxide

EDTA: ethylenediaminetetraacetic acid

FBS: fetal bovine serum

FIPI: 5-fluoro-2-indolyl des-chlorohalopemide hydrochloride hydrate

HDL: high-density lipoprotein

HMG-CoA reductase: 3-hydroxy-3-methyl-glutaryl-CoA reductase

LDH: lactate dehydrogenase

LDL-C: low-density lipoprotein-cholesterol

LPA: lysophosphatidic acid

MAPK: mitogen-activated protein kinase

M β CD: methyl- β -cyclodextrin

MEM: minimal essential medium

MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide

PA: phosphatidic acid

PBS: phosphate-buffered saline

PBS-T: phosphate-buffered saline-Tween-20

PBt: phosphatidylbutanol

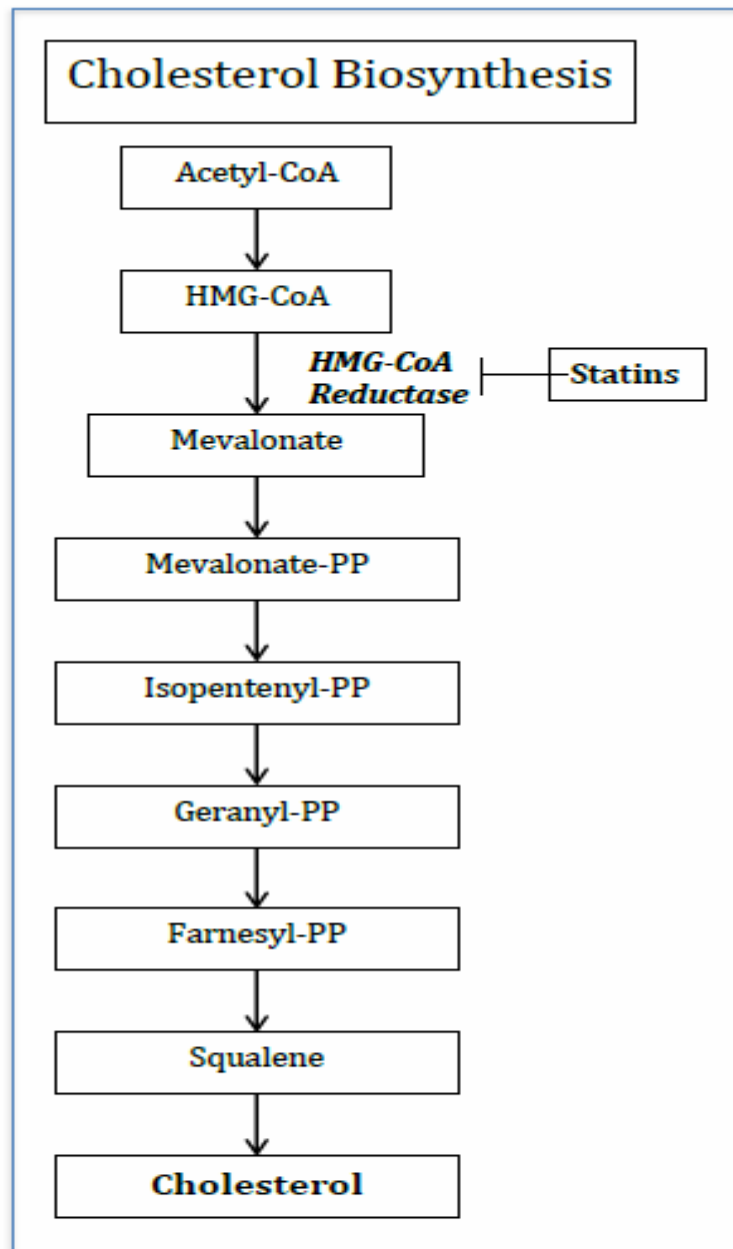
PKC: protein kinase C

PLA₂: phospholipase A₂

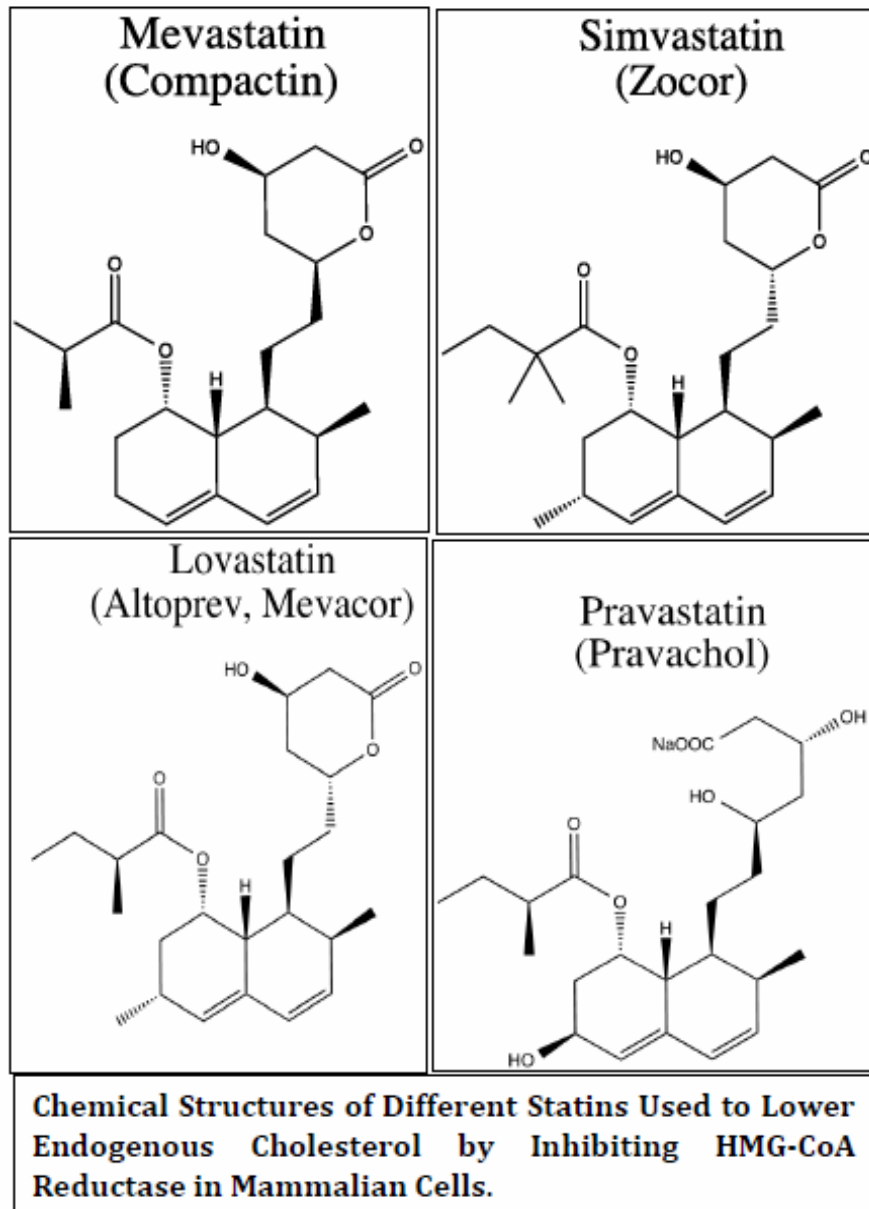
PLD: phospholipase D

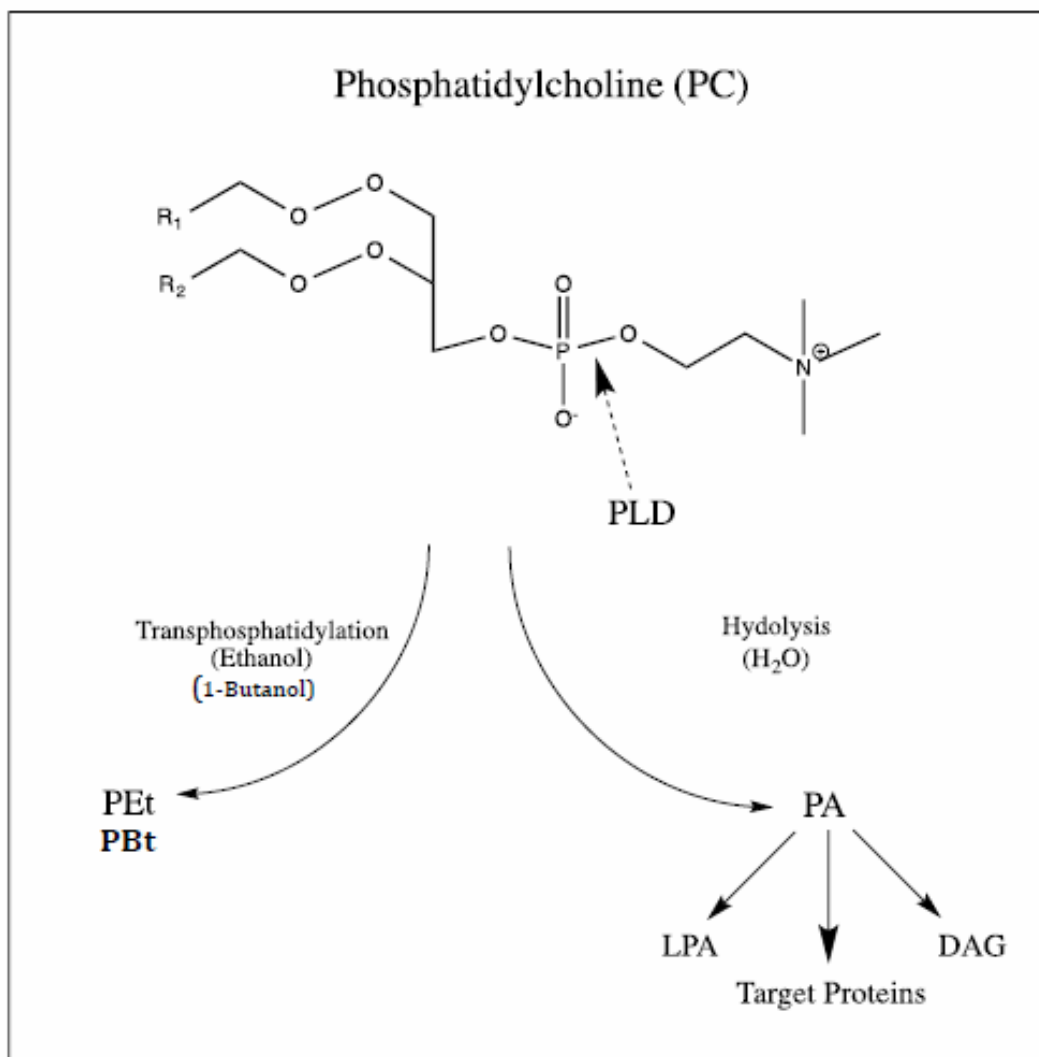
ROS: reactive oxygen species

TLC: thin-layer chromatography



Schema-1: *Pathway of cellular cholesterol synthesis in mammalian cells.* HMG-CoA reductase is the rate limiting enzyme that converts HMG-CoA to mevalonate. HMG-CoA reductase is inhibited by the statins to cause decrease/reduction in the de novo synthesized cellular cholesterol. [PP = pyrophosphate]





Schema-2: Mechanism of the phospholipase D (PLD)-mediated hydrolysis of the membrane phospholipids (e.g. phosphatidylcholine, PC) in mammalian cells. PLD hydrolyzes PC at the head group and forms the bioactive lipid signal mediator, phosphatidic acid (PA). PA can further be converted to potent bioactive lipid signal mediators such as the lysophosphatidic acid (LPA) by the action of phospholipases A1/A2 (PLA1 or PLA2) and diacylglycerol (DAG) by the action of lipid phosphate phosphatase. Both LPA and DAG are potent lipid mediators of cell signaling. PA can also directly modulate cellular proteins and cause functional alterations in the cells. One salient feature of PLD is that the enzyme is capable of using stereospecifically a primary alcohol instead of water such as ethanol, 1-butanol, and 1-propanol during the hydrolysis of the membrane phospholipid to convert the PLD-generated PA into the corresponding phosphatidylalcohols (e.g. phosphatidylethanol - PEt, phosphatidylbutanol - PBt, and phosphatidylpropanol - PProp). Hence, the phosphatidylalcohols serve as the indices of PLD activity in cells *in situ*.

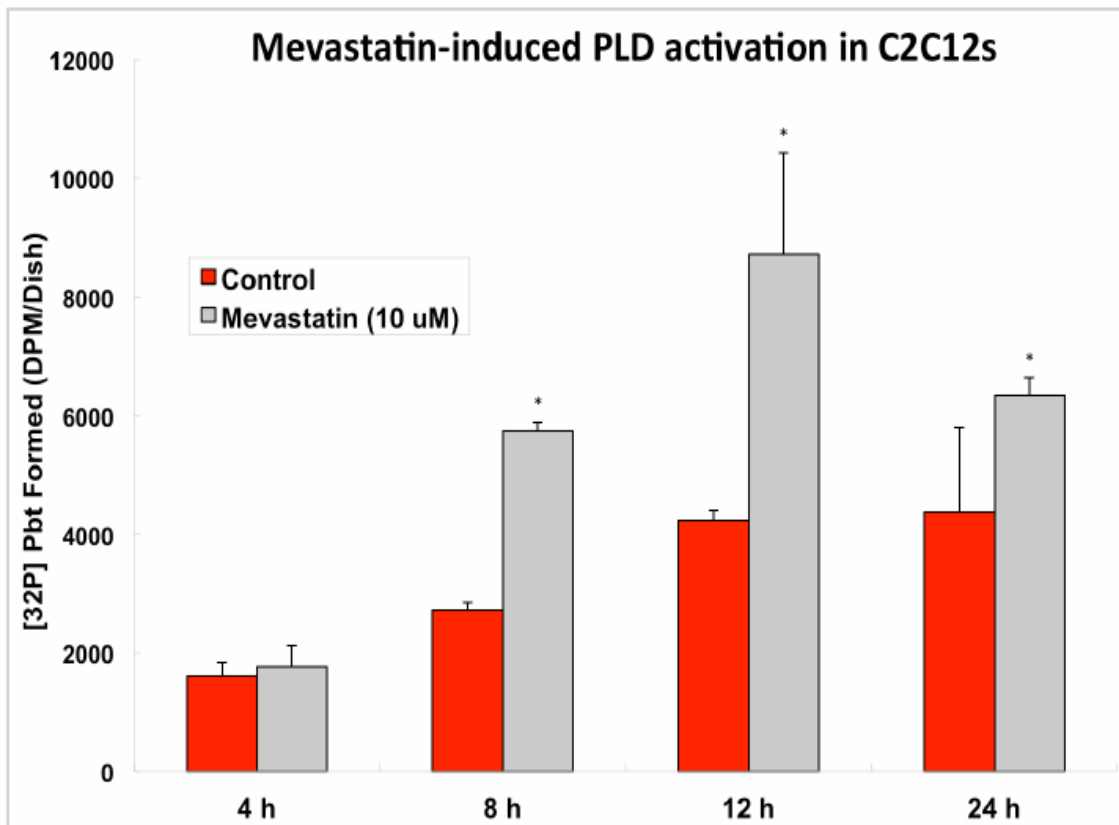


Figure-1A: Mevastatin induces PLD activation in C2C12 cells. C2C12 myoblast cells (2×10^5 cells) were pre-labeled for 12 h with carrier free $[^{32}\text{P}]$ orthophosphate in complete medium, following which cells were treated with mevastatin ($10 \mu\text{M}$) for different time periods (4-24 h) in 95% air-5% CO_2 under humidified sterile environment at 37°C . Under identical conditions, appropriate controls were established without the statin treatment. At the end of the experiment for the designated time, lipids were extracted under acidic conditions with 2:1 chloroform-methanol (vol/vol) and separated by thin-layer chromatography (TLC) as described in the Materials & Methods. Phosphatidylbutanol (Pbt) as the product of PLD activity generated by the transphosphatidyl reaction was identified by iodination on the TLC plate with authentic Pbt standard as described in the Materials & Methods. The Pbt spots on the TLC plates were scrapped and $[^{32}\text{P}]$ radioactivity was determined on a liquid scintillation counter and normalized to the total lipid phosphorus $[^{32}\text{P}]$ of the cells and expressed as DPM/cells in the dish. Each histogram is an average of results obtained from 3 independent experiments under identical conditions with \pm S.D. *Significantly different from the untreated control cells at $p \leq 0.05$.

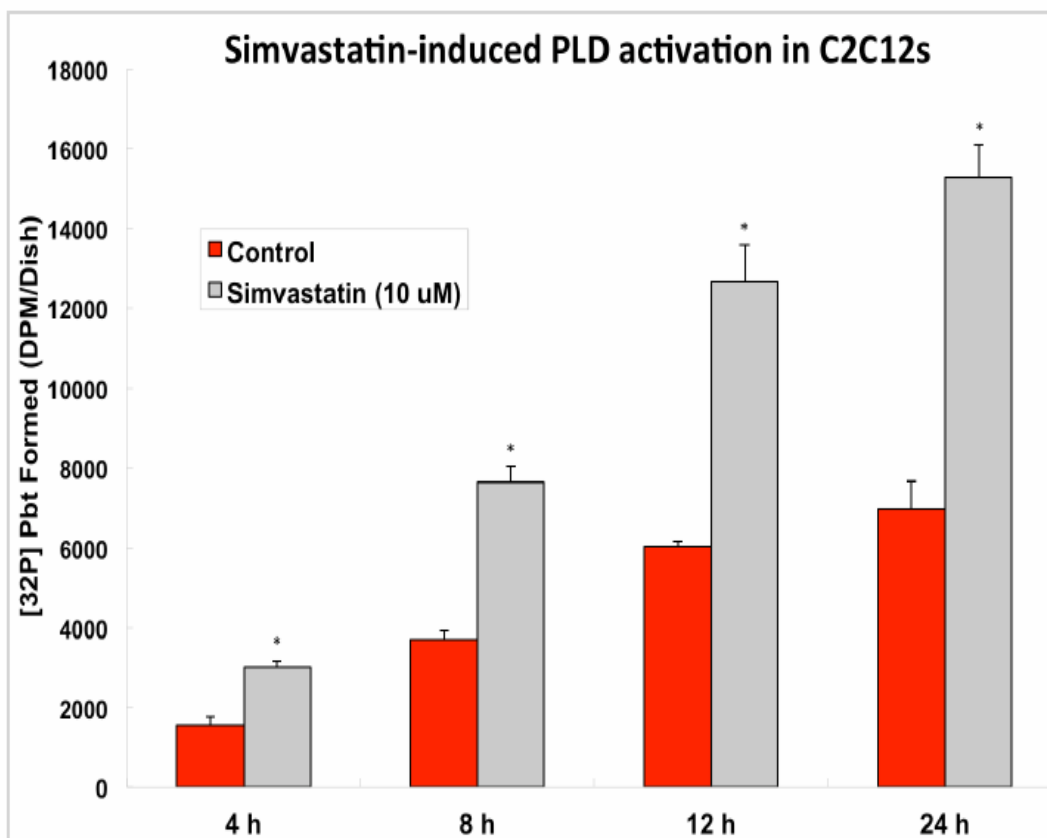


Figure-1B: *Simvastatin induces PLD activation in C2C12 cells.* C2C12 myoblast cells (2×10^5 cells) were pre-labeled for 12 h with carrier free [^{32}P] orthophosphate in complete medium, following which cells were treated with simvastatin (10 μM) for different time periods (4-24 h) under a humidified sterile atmosphere of 95% air-5% CO_2 at 37° C. Under identical conditions, appropriate controls were established without the statin treatment. At the end of the experiment for the designated time, lipids were extracted under acidic conditions with 2:1 chloroform-methanol (vol/vol) and separated by thin-layer chromatography (TLC) as described in the Materials & Methods. Phosphatidylbutanol (Pbt) as the product of PLD activity generated by the transphosphatidylation reaction was identified by iodination on the TLC plate with authentic Pbt standard as described in the Materials & Methods. The Pbt spots on the TLC plates were scrapped and [^{32}P] radioactivity was determined on a liquid scintillation counter and normalized to the total lipid phosphorus [^{32}P] of the cells and expressed as DPM/cells in the dish. Each histogram is an average of results obtained from 3 independent experiments under identical conditions with \pm S.D. *Significantly different from the untreated control cells at $p \leq 0.05$.

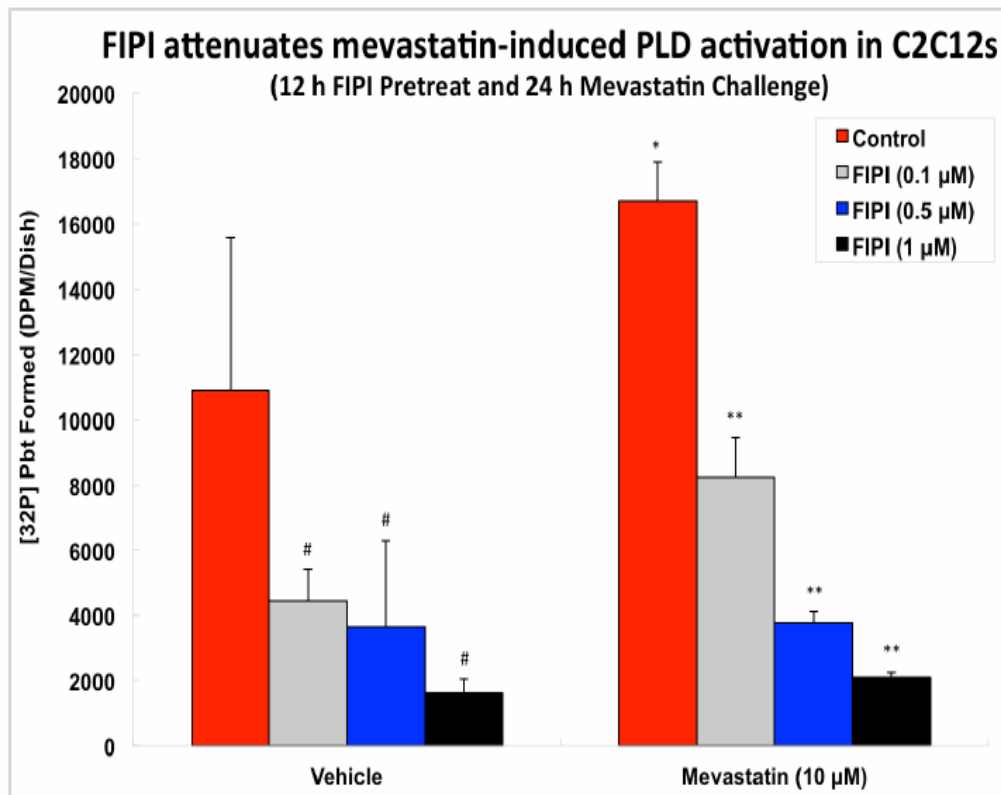


Figure-2A: *FIPI, the PLD-specific inhibitor attenuates mevastatin-induced PLD activation in C2C12 cells.* C2C12 myoblast cells (2×10^5 cells) were pre-labeled for 12 h with carrier free [^{32}P] orthophosphate in complete medium, following which cells were first treated with the PLD-specific pharmacological inhibitor, FIPI (0.1-1 μM) for 12 h and then treated with mevastatin (10 μM) for 24 h in absence and presence of FIPI under a humidified sterile atmosphere of 95% air-5% CO_2 at 37 $^\circ\text{C}$. Under identical conditions, appropriate controls were established without FIPI and the statin treatment and with FIPI treatments alone. At the end of the experiment for the designated time, lipids were extracted under acidic conditions with 2:1 chloroform-methanol (vol/vol) and separated by thin-layer chromatography (TLC) as described in the Materials & Methods. Phosphatidylbutanol (Pbt) as the product of PLD activity generated by the transphosphatidylation reaction was identified by iodination on the TLC plate with authentic Pbt standard as described in the Materials & Methods. The Pbt spots on the TLC plates were scrapped and [^{32}P] radioactivity was determined on a liquid scintillation counter and normalized to the total lipid phosphorus [^{32}P] of the cells and expressed as DPM/cells in the dish. Each histogram is an average of results obtained from 3 independent experiments under identical conditions with \pm S.D. *Significantly different from the untreated control cells at $p \leq 0.05$. **Significantly different from the statin-treated cells at $p \leq 0.05$.

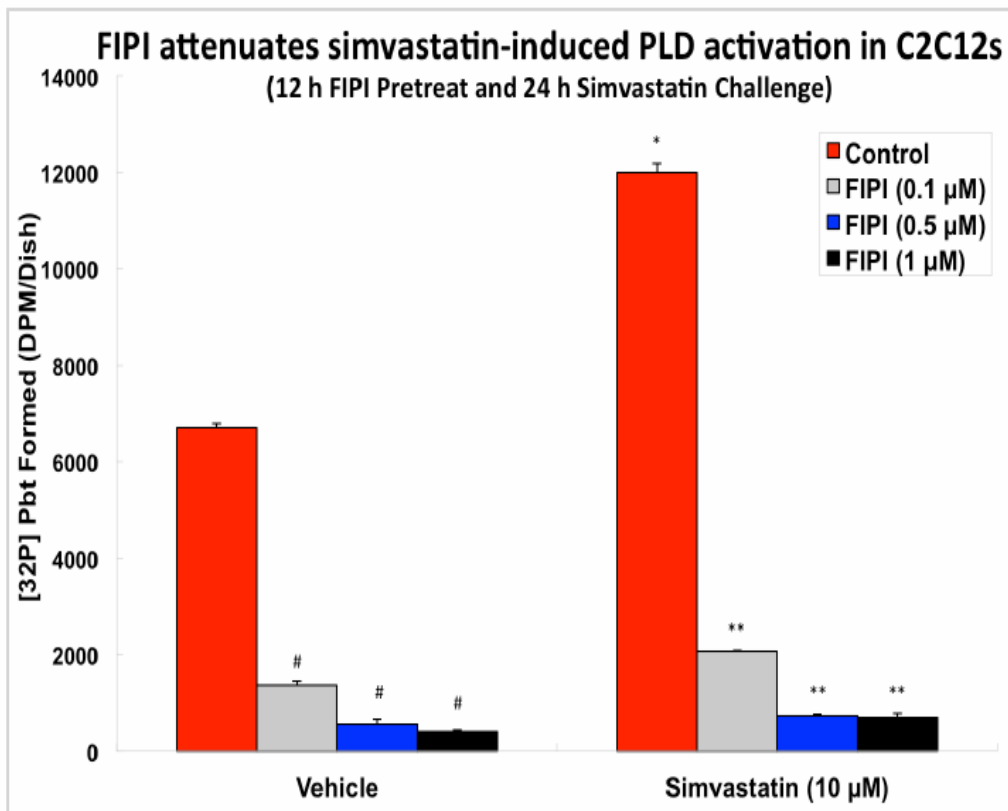


Figure-2B: FIPI, the PLD-specific inhibitor attenuates simvastatin-induced PLD activation in C2C12 cells. C2C12 myoblast cells (2×10^5 cells) were pre-labeled for 12 h with carrier free [^{32}P] orthophosphate in complete medium, following which cells were first treated with the PLD-specific pharmacological inhibitor, FIPI (0.1-1 μM) for 12 h and then treated with simvastatin (10 μM) for 24 h in absence and presence of FIPI under a humidified sterile atmosphere of 95% air-5% CO_2 at 37° C. Under identical conditions, appropriate controls were established without FIPI and the statin treatment and with FIPI treatments alone. At the end of the experiment for the designated time, lipids were extracted under acidic conditions with 2:1 chloroform-methanol (vol/vol) and separated by thin-layer chromatography (TLC) as described in the Materials & Methods. Phosphatidylbutanol (Pbt) as the product of PLD activity generated by the transphosphatidyltransferase reaction was identified by iodination on the TLC plate with authentic Pbt standard as described in the Materials & Methods. The Pbt spots on the TLC plates were scrapped and [^{32}P] radioactivity was determined on a liquid scintillation counter and normalized to the total lipid phosphorus [^{32}P] of the cells and expressed as DPM/cells in the dish. Each histogram is an average of results obtained from 3 independent experiments under identical conditions with \pm S.D. *Significantly different from the untreated control cells at $p \leq 0.05$. **Significantly different from the statin-treated cells at $p \leq 0.05$.

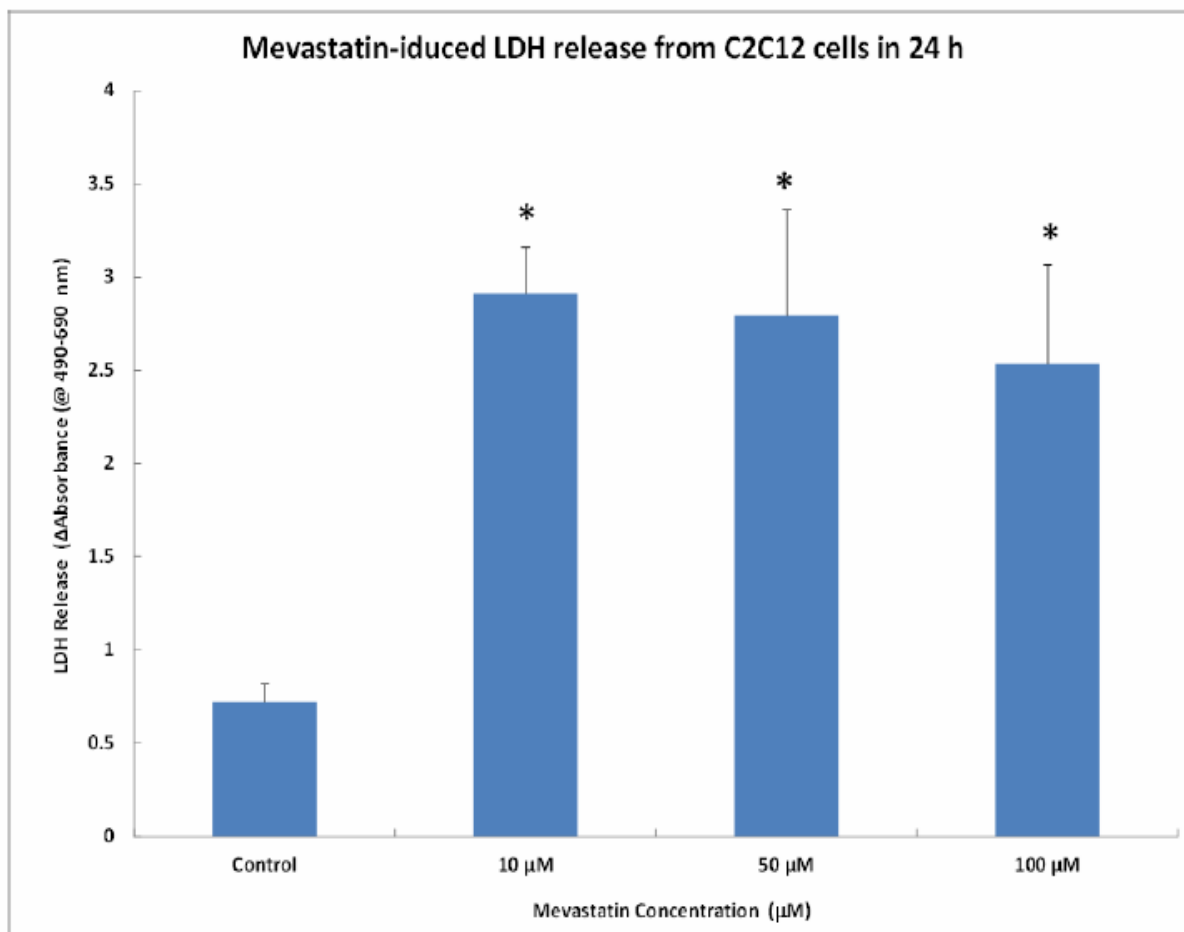


Figure-3B: Mevastatin induces lactate dehydrogenase (LDH) release from C2C12 cells. C2C12 myoblast cells were cultured up to ~90-100% confluence in 15.5-mm sterile dishes (24-well cell culture plate) in complete medium and then treated with complete medium or medium containing different concentrations of mevastatin (10-100 μM) for 24 h under a humidified sterile atmosphere of 95% air-5% CO₂ at 37° C. Appropriate controls without the statin treatments were established simultaneously under identical conditions. At the end of the treatment, the supernatant was removed, and the extent of release of LDH was determined on a plate reader (visible) by the commercially available LDH spectrophotometric assay kit (Sigma Chemical Co., St. Louis, MO) according to the manufacturer's recommendations as described in the Materials & Methods. Each histogram is an average of results obtained from 3 independent experiments under identical conditions with ± S.D. *Significantly different from the untreated control cells at $p \leq 0.05$.

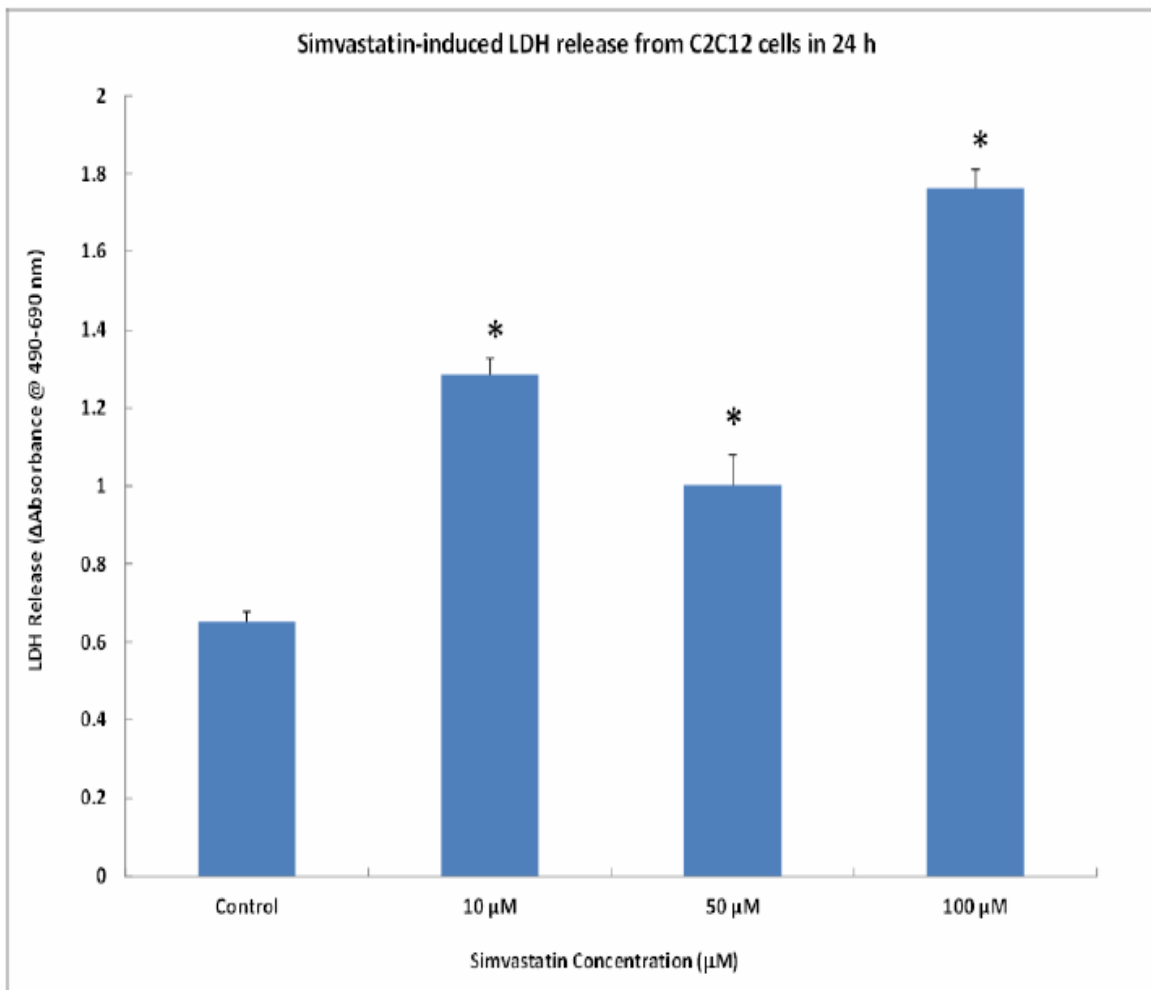


Figure-3A: Simvastatin induces lactate dehydrogenase (LDH) release from C2C12 cells. C2C12 myoblast cells were cultured up to ~90-100% confluence in 15.5-mm sterile dishes (24-well cell culture plate) in complete medium and then treated with complete medium or medium containing different concentrations of simvastatin (10-100 μM) for 24 h under a humidified sterile atmosphere of 95% air-5% CO₂ at 37° C. Appropriate controls without the statin treatments were established simultaneously under identical conditions. At the end of the treatment, the supernatant was removed, and the extent of release of LDH was determined on a plate reader (visible) by the commercially available LDH spectrophotometric assay kit (Sigma Chemical Co., St. Louis, MO) according to the manufacturer's recommendations as described in the Materials & Methods. Each histogram is an average of results obtained from 3 independent experiments under identical conditions with ± S.D. *Significantly different from the untreated control cells at $p \leq 0.05$.

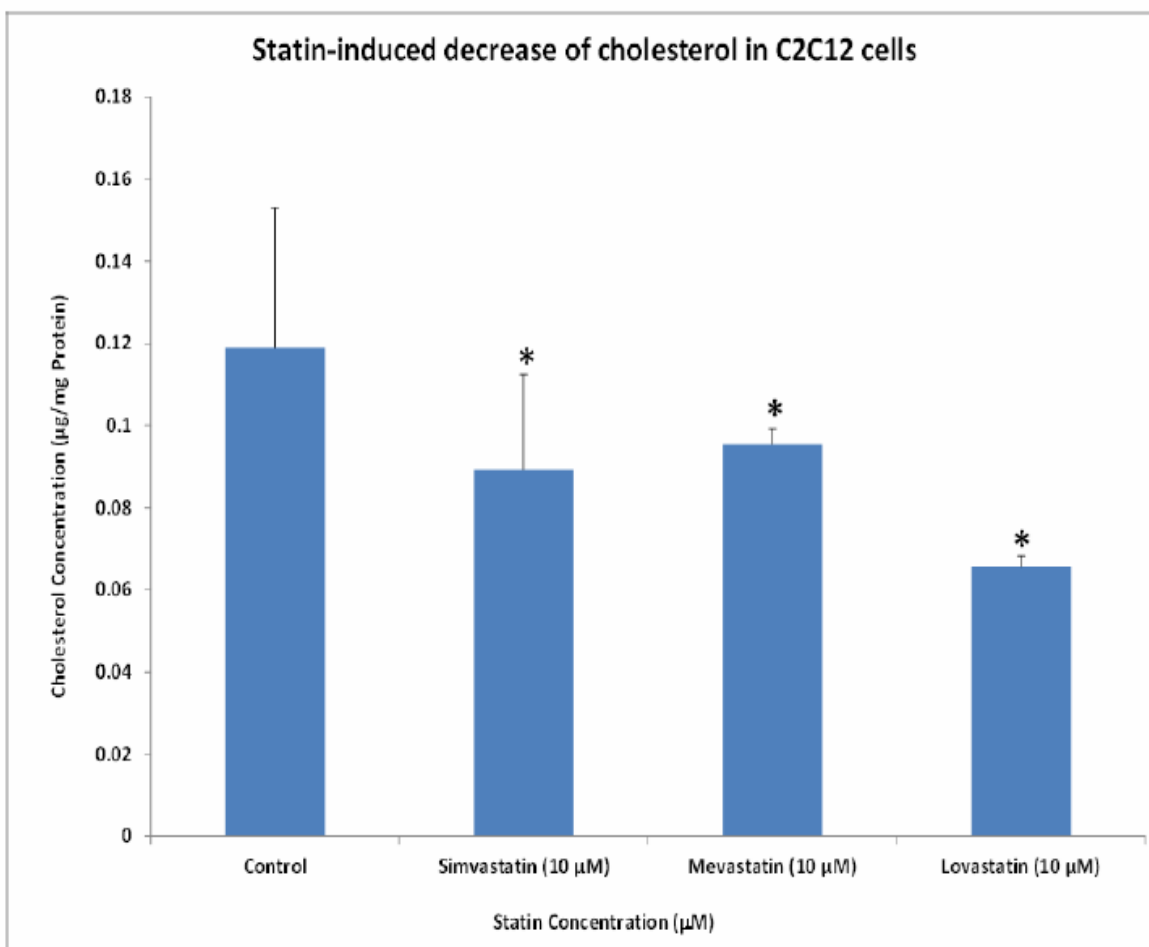


Figure-4A: Statins induce decrease of cholesterol in C2C12 cells. C2C12 myoblast cells were cultured in complete medium up to ~90-100% confluence in 60-mm sterile culture dishes and treated with different statins at 10 µM concentration for 24 h under a humidified sterile atmosphere of 95% air-5% CO₂ at 37° C. Appropriate controls were established simultaneously under identical condition without statin treatments. At the end of the treatment, the cholesterol content in the cells was then determined spectrofluorometrically on a plate reader (fluorescence) with the commercially available cholesterol determination kit according to the manufacturer's recommendation (Molecular Probes – Invitrogen Detection Technologies, Grand Island, NY) as described in the Materials & Methods. The cellular levels of cholesterol were normalized to protein and expressed as µg/mg protein. Each histogram is an average of results obtained from 3 independent experiments under identical conditions with ± S.D. *Significantly different from the untreated control cells at $p \leq 0.05$.

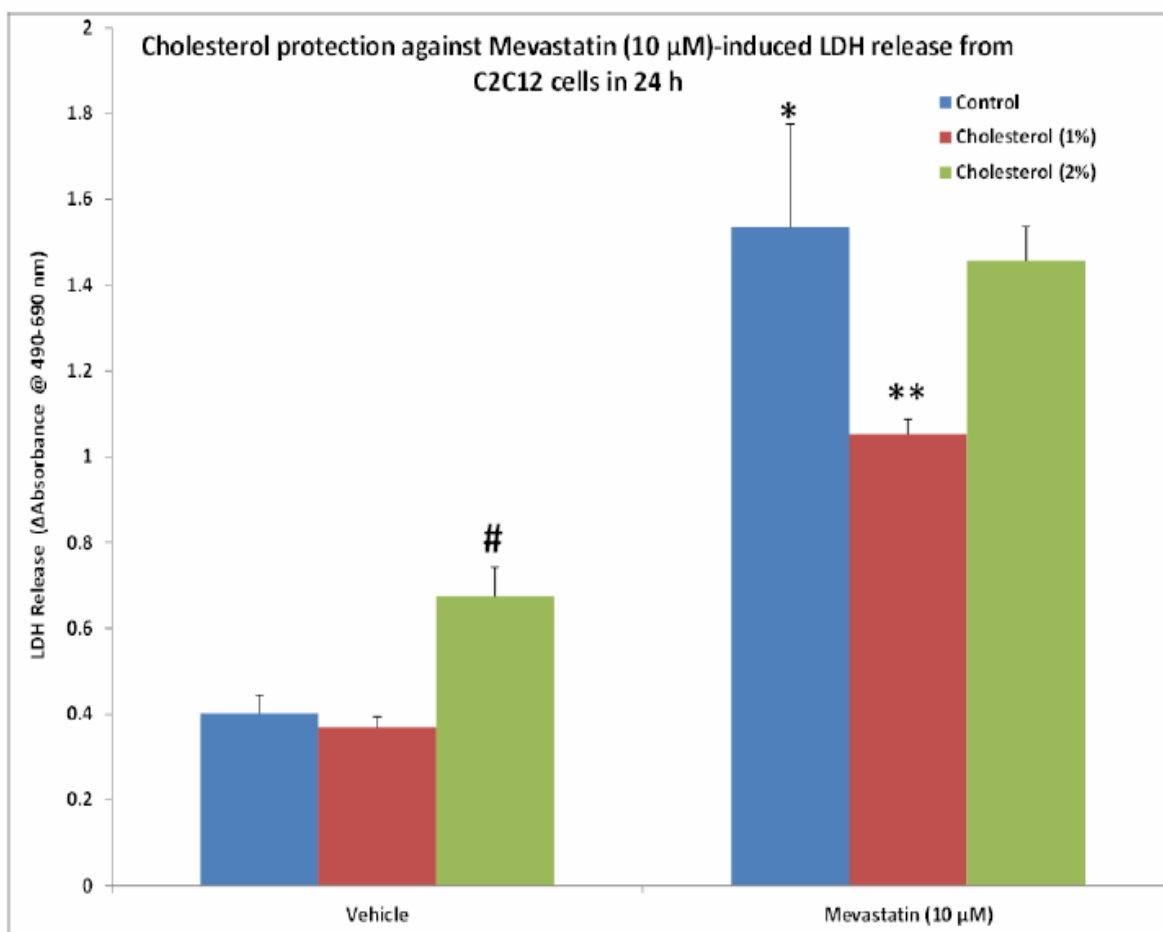


Figure-4B: *Cholesterol replenishment protects against mevastatin-induced lactate dehydrogenase (LDH) release from C2C12 Cells.* C2C12 myoblast cells were cultured up to ~90-100% confluence in 15.5-mm sterile dishes (24-well cell culture plate) in complete medium and then treated with complete medium or medium containing different concentrations of water-soluble cholesterol alone (1-2%) or mevastatin (10 μM) alone or water-soluble cholesterol (1-2%) + mevastatin (10 μM) for 24 h under a humidified sterile atmosphere of 95% air-5% CO₂ at 37° C. Appropriate controls without the water-soluble cholesterol and statin treatments were established simultaneously under identical conditions. At the end of the treatment, the supernatant was removed, and the extent of release of LDH was determined on a plate reader (visible) by the commercially available LDH spectrophotometric assay kit (Sigma Chemical Co., St. Louis, MO) according to the manufacturer's recommendations as described in the Materials & Methods. Each histogram is an average of results obtained from 3 independent experiments under identical conditions with ± S.D. *Significantly different from the untreated control cells at $p \leq 0.05$. **Significantly different from the statin-treated cells at $p \leq 0.05$. # Significantly different from the untreated control cells at $p \leq 0.05$.

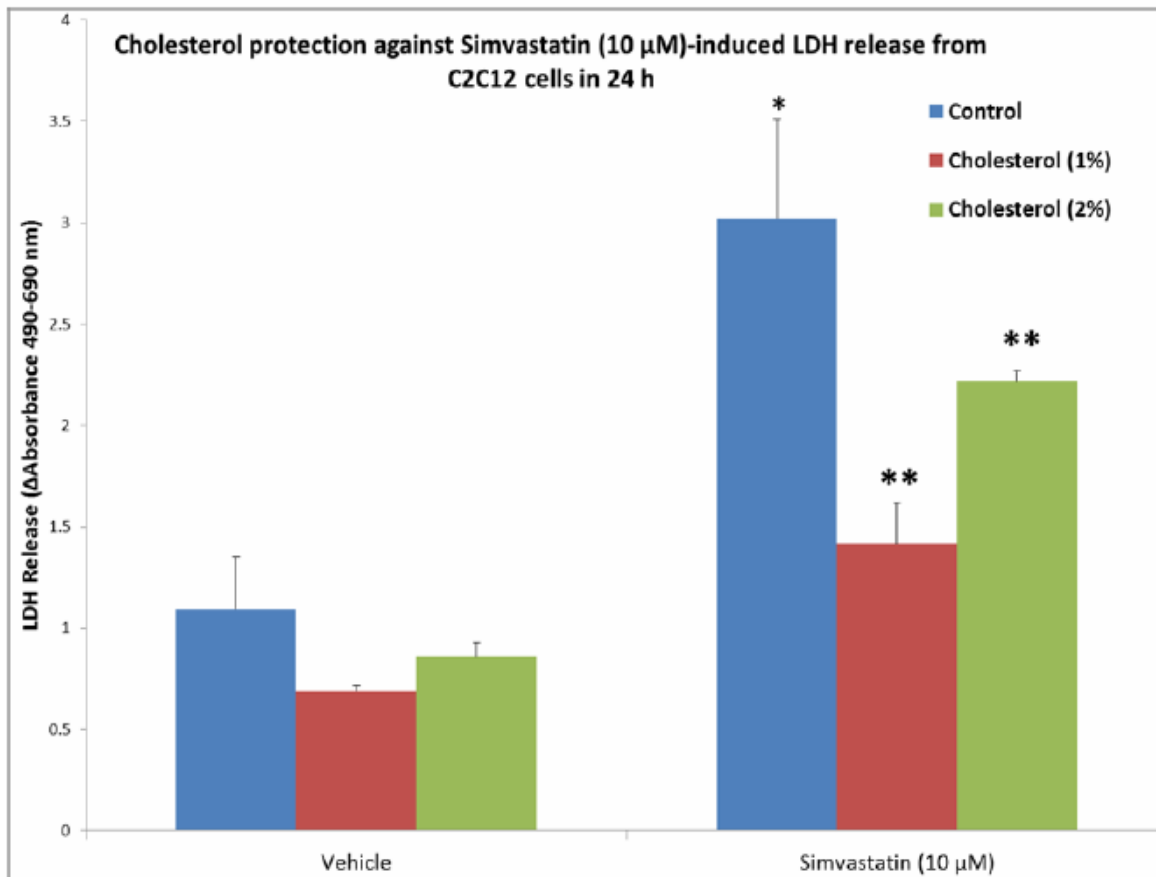


Figure-4C: Cholesterol replenishment protects against simvastatin-induced lactate dehydrogenase (LDH) release from C2C12 Cells. C2C12 myoblast cells were cultured up to ~90-100% confluence in 15.5-mm sterile dishes (24-well cell culture plate) in complete medium and then treated with complete medium or medium containing different concentrations of water-soluble cholesterol alone (1-2%) or simvastatin (10 μM) alone or water-soluble cholesterol (1-2%) + simvastatin (10 μM) for 24 h under a humidified sterile atmosphere of 95% air-5% CO₂ at 37° C. Appropriate controls without the water-soluble cholesterol and statin treatments were established simultaneously under identical conditions. At the end of the treatment, the supernatant was removed, and the extent of release of LDH was determined on a plate reader (visible) by the commercially available LDH spectrophotometric assay kit (Sigma Chemical Co., St. Louis, MO) according to the manufacturer’s recommendations as described in the Materials & Methods. Each histogram is an average of results obtained from 3 independent experiments under identical conditions with ± S.D. *Significantly different from the untreated control cells at $p \leq 0.05$. **Significantly different from the statin-treated cells at $p \leq 0.05$.

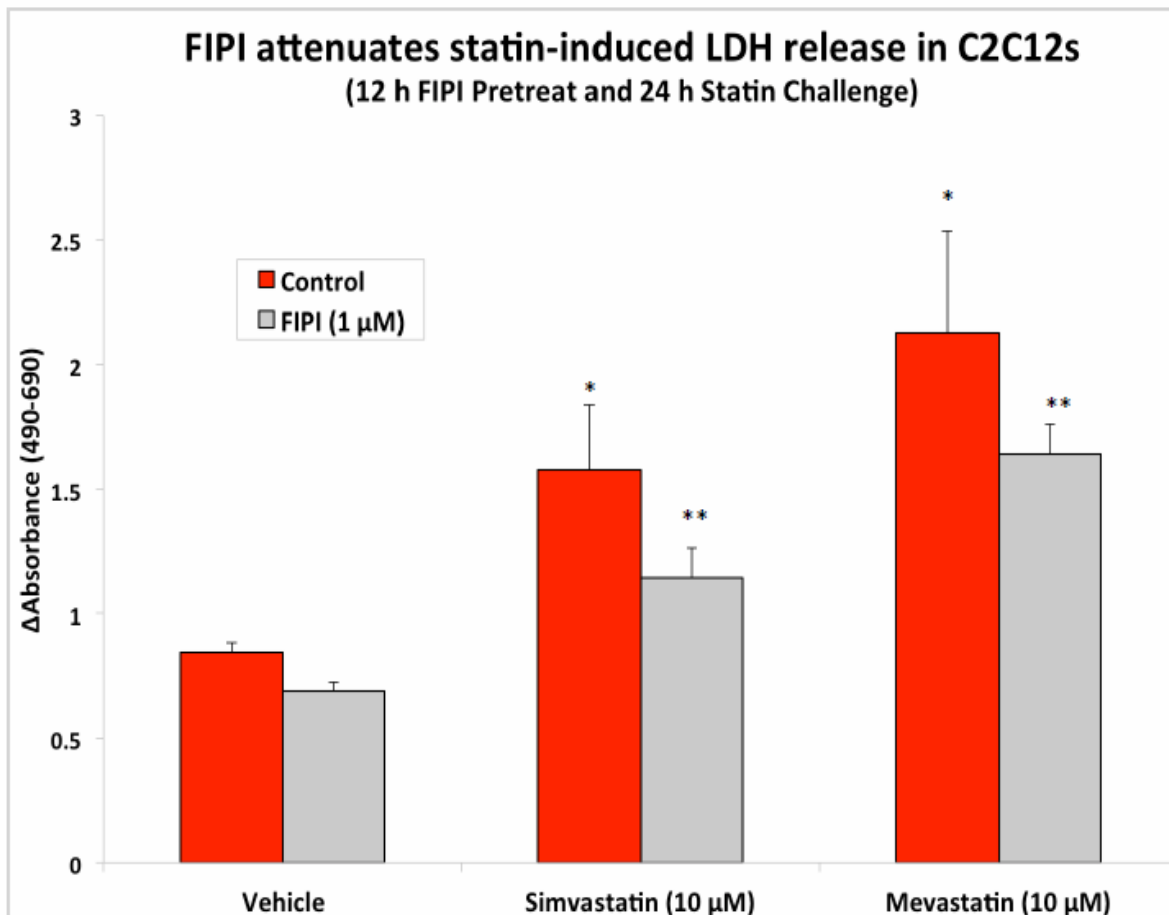


Figure-5: *FIPI, the PLD-specific inhibitor attenuates statin-induced lactate dehydrogenase (LDH) release from C2C12 cells.* C2C12 myoblast cells were cultured up to ~90-100% confluence in 15.5-mm sterile dishes (24-well cell culture plate) in complete medium and then pretreated for 12 h with FIPI, and then treated with simvastatin (10 μM) and mevastatin (10 μM) for 24 h under a humidified sterile atmosphere of 95% air-5% CO₂ at 37° C. Appropriate controls without and with FIPI and statins alone were established simultaneously under identical conditions. At the end of the treatment, the supernatant was removed, and the extent of release of LDH was determined on a plate reader (visible) by the commercially available LDH spectrophotometric assay kit (Sigma Chemical Co., St. Louis, MO) according to the manufacturer's recommendations as described in the Materials & Methods. Each histogram is an average of results obtained from 3 independent experiments under identical conditions with ± S.D. *Significantly different from the untreated control cells at $p \leq 0.05$. **Significantly different from the statin-treated cells at $p \leq 0.05$.

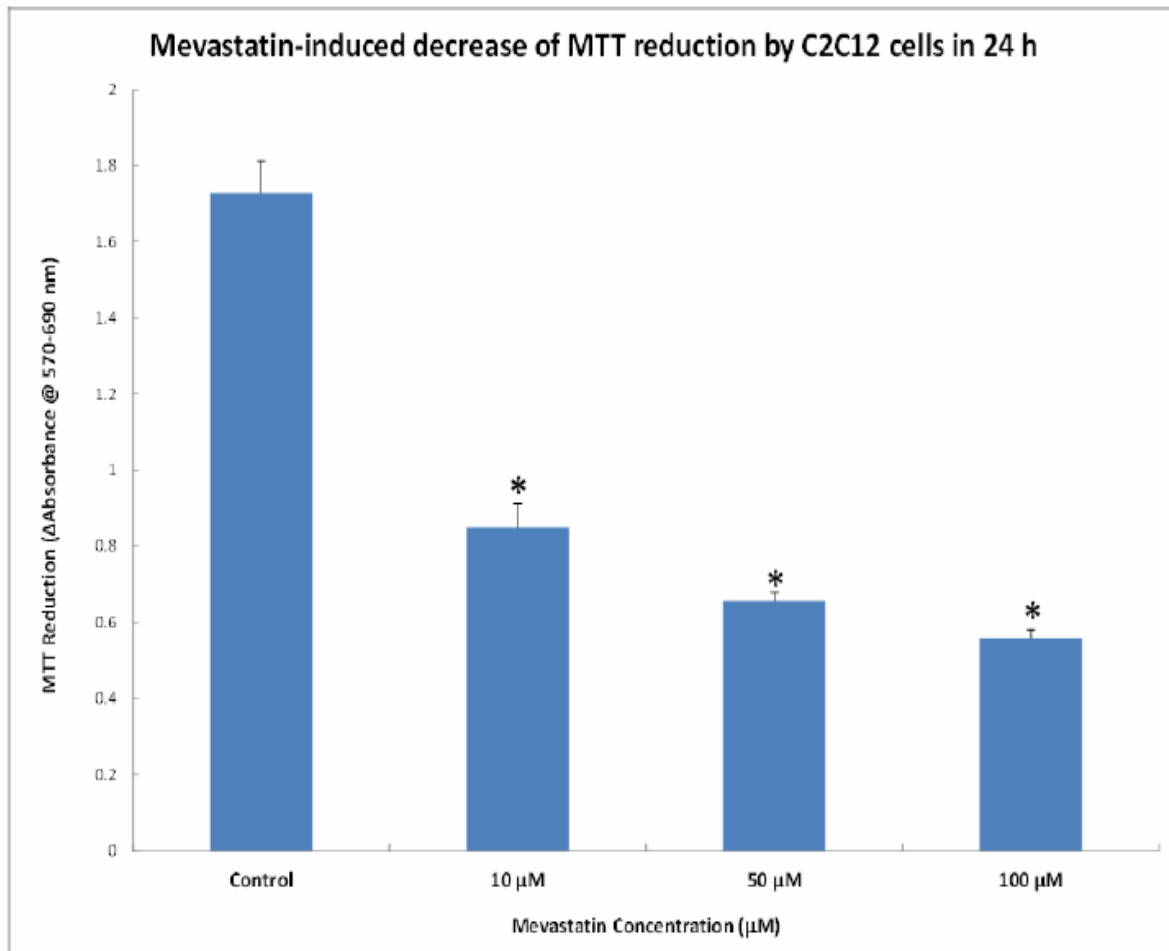


Figure-6B: *Mevastatin induces decrease of MTT reduction by C2C12 cells.* C2C12 myoblast cells were cultured up to ~90-100% confluence in 15.5-mm sterile dishes (24-well cell culture plate) in complete medium and then treated with complete medium or medium containing different concentrations of mevastatin (10-100 μM) for 24 h under a humidified sterile atmosphere of 95% air-5% CO₂ at 37° C. Appropriate controls without the statin treatments were established simultaneously under identical conditions. At the end of the treatment, the supernatant was removed, and the extent of MTT reduction by the cells was determined on a plate reader (visible) by the commercially available MTT reduction spectrophotometric assay kit (Cayman Chemical Co., Ann Arbor, MI) according to the manufacturer's recommendations as described in the Materials & Methods. Each histogram is an average of results obtained from 3 independent experiments under identical conditions with ± S.D. *Significantly different from the untreated control cells at $p \leq 0.05$.

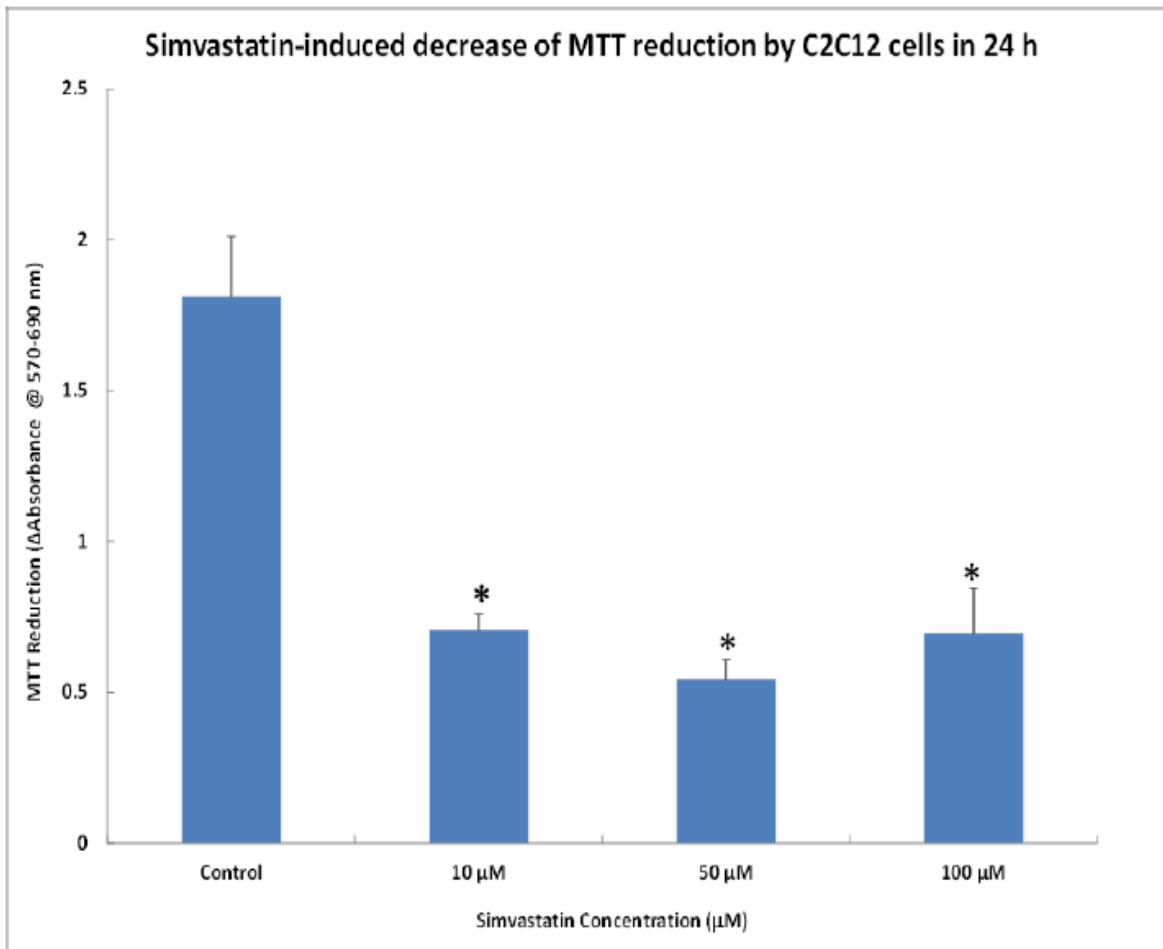


Figure-6A: *Simvastatin induces decrease of MTT reduction by C2C12 cells.* C2C12 myoblast cells were cultured up to ~90-100% confluence in 15.5-mm sterile dishes (24-well cell culture plate) in complete medium and then treated with complete medium or medium containing different concentrations of simvastatin (10-100 μM) for 24 h under a humidified sterile atmosphere of 95% air-5% CO₂ at 37° C. Appropriate controls without the statin treatments were established simultaneously under identical conditions. At the end of the treatment, the supernatant was removed, and the extent of MTT reduction by the cells was determined on a plate reader (visible) by the commercially available MTT reduction spectrophotometric assay kit (Cayman Chemical Co., Ann Arbor, MI) according to the manufacturer's recommendations as described in the Materials & Methods. Each histogram is an average of results obtained from 3 independent experiments under identical conditions with ± S.D. *Significantly different from the untreated control cells at $p \leq 0.05$.

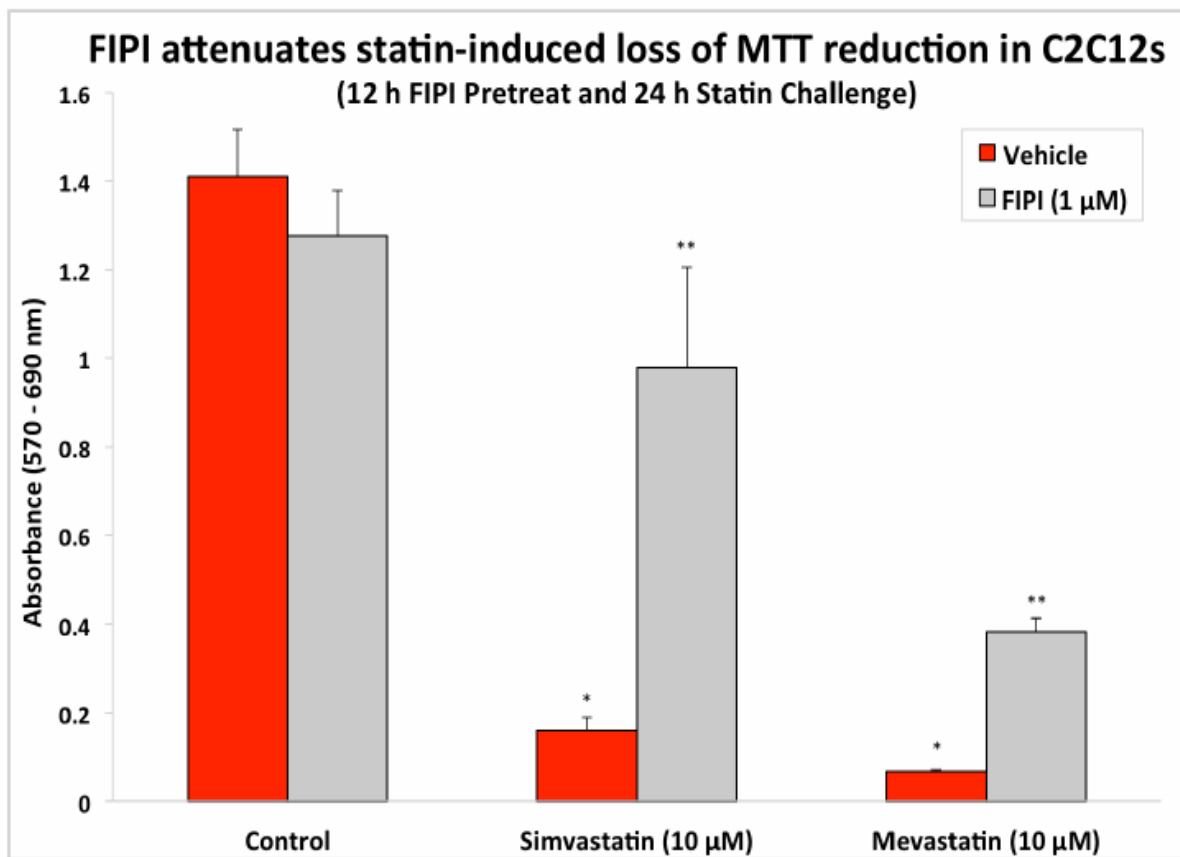


Figure-7A: *FIPI, the PLD-specific inhibitor attenuates statin-induced decrease of MTT reduction by C2C12 cells.* C2C12 myoblast cells were cultured up to ~90-100% confluence in 15.5-mm sterile dishes (24-well cell culture plate) in complete medium, pre-treated with the PLD-specific pharmacological inhibitor (FIPI, 1 μM) for 12 h, and then treated with complete medium alone or medium containing FIPI alone or medium containing simvastatin (10 μM) and mevastatin (10 μM) for 24 h under a humidified sterile atmosphere of 95% air-5% CO₂ at 37° C. Appropriate controls without FIPI treatment and the statin treatments were established simultaneously under identical conditions. At the end of the treatment, the supernatant was removed, and the extent of MTT reduction by the cells was determined on a plate reader (visible) by the commercially available MTT reduction spectrophotometric assay kit (Cayman Chemical Co., Ann Arbor, MI) according to the manufacturer's recommendations as described in the Materials & Methods. Each histogram is an average of results obtained from 3 independent experiments under identical conditions with ± S.D. *Significantly different from the untreated control cells at $p \leq 0.05$. **Significantly different from the statin-treated cells at $p \leq 0.05$.

FIPI attenuates statin-induced morphological alterations in C2C12s (12 h FIPI Pretreat and 24 h Statin Challenge)

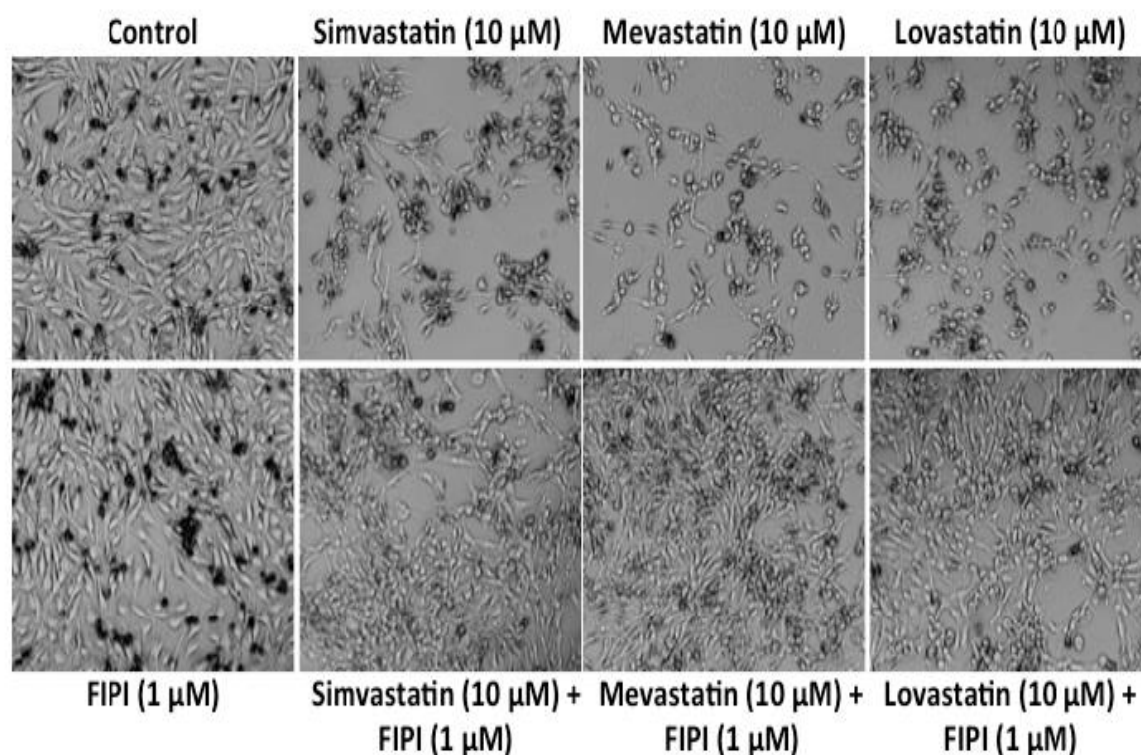
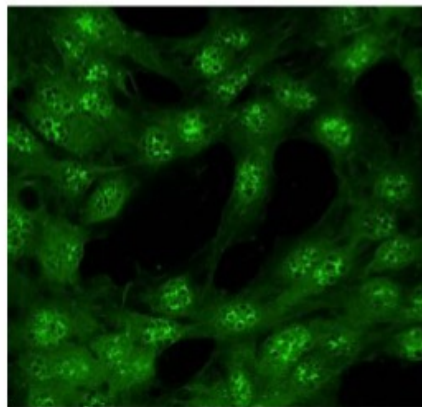
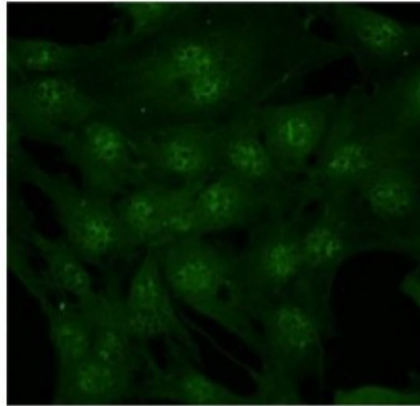


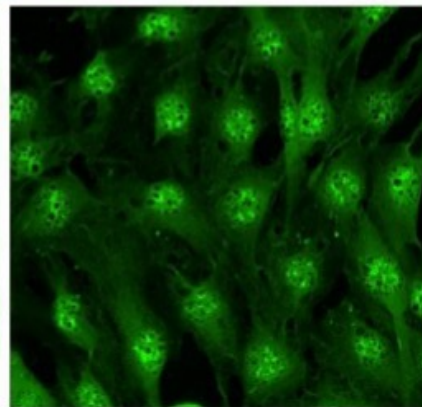
Figure-7B: *FIPI, the PLD-specific inhibitor attenuates statin-induced morphological alterations in C2C12 cells.* C2C12 myoblast cells were cultured in sterile 35-mm dishes up to ~90-100% confluence in complete medium under a humidified atmosphere of 95% air-5% CO₂ at 37° C, and then pre-treated with the PLD-specific pharmacological inhibitor, FIPI (1 μ M) alone for 12 h, following which the cells were treated with medium alone or medium containing simvastatin (10 μ M), mevastatin (10 μ M), and lovastatin (10 μ M) or FIPI (1 μ M) + statin (10 μ M) for 24 h. Appropriate controls were established with cells treated with medium alone or FIPI alone under identical conditions. At the end of the experiment, the images of cellular morphology were digitally captured using the Zeiss Axioskop 200 with Zen 2011 software at 20 X magnification. Each photomicrograph is a typical representative of at least 3 independent observations from 3 different experiments conducted under identical conditions.

Statin-induced PLD1 phosphorylation in C2C12s
(12 h Statin Treatment)

Control

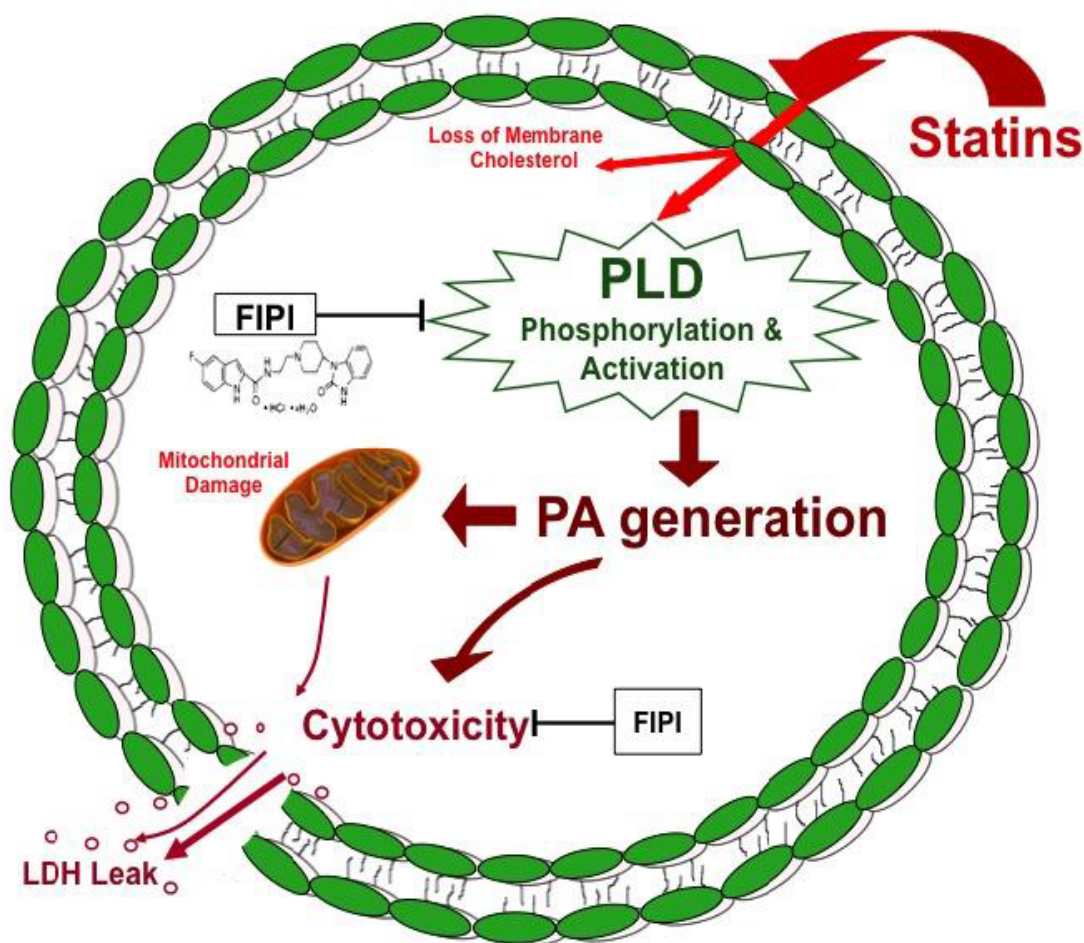


Mevastatin (10 μM)



Simvastatin (10 μM)

Figure-8: *Statins induce threonine phosphorylation of PLD1 in C2C12 cells.* C2C12s were cultured on sterile glass cover slips (90% confluence) in complete medium under a humidified atmosphere of 95% air-5% CO₂ at 37° C and then treated with complete medium alone or medium containing chosen concentrations of statins for 12 h. As described in the Materials & Methods, the cells on the cover slips were treated overnight at room temperature with the primary antibody [phosphothreonine-PLD1 (1:150 dilution)] in 1% BSA solution. Following that, the cells were labeled with the secondary AlexaFluor 488 (1:100 dilution) for 1 h and then the cells were examined under the Zeiss LSM 710 Confocal/Multiphoton Microscope powered by Argon-2 laser with 500-550 BP filter. The images were captured digitally. Each confocal fluorescence micrograph is a typical representative of 3 independent observations from 3 different experiments conducted under identical conditions. The bright green fluorescence in situ depicts the threonine phosphorylation of PLD1 isoform.



Schema-3: Proposed mechanism of statin-induced PLD activation leading to cytotoxicity through depletion of endogenous cholesterol in C2C12 myoblast cells. In addition to loss of cellular cholesterol, the PLD-generated lipid signal mediator, phosphatidic acid (PA) apparently is responsible for causing the statin-induced myotoxicity in the cells. Also, mitochondrial dysfunction, as observed by the loss of MTT reduction appears to be a critical player in statin-induced cytotoxicity in the C2C12 myoblast cells. Statin-induced PLD activation appears to be mediated by the upstream threonine phosphorylation of PLDs that may be regulated by the statin-mediated loss of membrane cholesterol and subsequent activation of the serine-threonine protein kinases. Both cholesterol replenishment and the pharmacological inhibition of PLD by the PLD-specific inhibitor, FIPI offer protection against the statin-induced cytotoxicity in C2C12 myoblast cells. Overall, the results of the present study suggest that the statin-induced myotoxicity is mediated by the upstream cholesterol loss and the associated PLD activation leading to generation of the bioactive lipid signal mediator (PA) and also the mitochondrial dysfunction.