Chapter 15

Mitochondrial Oxidative Stress and Calcium-Dependent Permeability Transition are Key Players in the Mechanisms of Statins-Associated Side Effects

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Abstract

Statins are cholesterol-lowering medicines utilized worldwide and are associated with reduced risk of cardiovascular mortality and events. However, 0.5–10% of patients suffer from adverse effects especially on skeletal muscle. Recently, new onset of diabetes has been reported in subjects on statin therapy. Pro- and anti-oxidant effects of statins have been reported, thus fostering a debate. Previously reported data provide evidence that statins induce alterations in intracellular calcium homeostasis and mitochondrial dysfunctions that can be counteracted by antioxidants (e.g., CoQ10, creatine, and L-carnitine). Therefore, we have proposed that statin-induced inhibition of mitochondrial respiration leads to oxidative stress that opens a calcium-dependent permeability transition pore, an event that may lead to cell death. In addition, mitochondrial oxidative stress caused by statin treatment may be a signal for cellular antioxidant system responses such as catalase upregulation, possibly explaining the alleged statins' antioxidant properties. Muscle mitochondrial dysfunction induced by statin treatment may be associated with the peripheral insulin resistance and may explain statins-induced new onset of diabetes. Together, the data presented in this review suggest that the statins' detrimental effects can be prevented by co-administration of antioxidants.

Keywords: statins adverse effects, statins pleiotropic effects, reactive oxygen species (ROS), mitochondrial permeability transition, antioxidants

1. Introduction

Familial hypercholesterolemia (FH) is an autosomal dominant disorder characterized by the presence of very high levels of low-density lipoprotein cholesterol (LDLc) in the blood stream

since birth. This cholesterol disorder was first described in the 1960s, and the existence of a mutated LDL receptor (LDLr) in FH patients was later discovered by Brown and Goldstein [1]. They observed that FH fibroblasts did not specifically bind and internalize LDL when compared with normal fibroblasts; that finding was the beginning of decades of work and discoveries concerning cholesterol metabolism regulation that led the pair to Nobel Prize award in 1985. Although the homozygous mutants for LDLr have an early cardiac death in the first or second decade of life, heterozygous FH patients usually do not present any early severe symptoms. The lack of diagnosis and treatment may have severe consequences considering the lifetime exposure to high LDLc concentrations. Increased LDLc levels are a well-established independent risk factor for cardiovascular diseases [2], and lowering LDL serum levels remains the primary treatment target in hypercholesterolemia [3, 4] that is undertaken in order to prevent and reduce cardiovascular and coronary heart diseases [5, 6].

Cholesterol is synthesized from acetyl-CoA by a 30-step pathway, in which 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase is the rate-limiting enzyme, converting HMG-CoA into mevalonate. However, besides being involved in cholesterol synthesis, mevalonate is also a precursor for isoprenoids farnesyl diphosphate. Geranyl- (GPP), farnesyl- (FPP) and geranylgeranyl-pyrophosphate (GGPP) are precursors of sterols, dolichols, CoQ10, isoprenoids, and carotenoids. These important metabolites are involved in membrane structures, protein glycosylation and prenylation, electron transport in mitochondrial respiratory chain, and scavenging of ROS [7].

The first cholesterol-lowering agent, citrinin, was discovered in the 1970s. It was derived from fungal cultures, but this product was discontinued due to its hepatotoxicity [8, 9]. After this, another fungal-derived compound called compactin was purified and tested in rats; however, it failed to reduce plasma cholesterol because it had the rebound effect of inducing HMG-CoA reductase activity a few hours after administration [10]. At the end of the 1970s, a very potent compound chemically similar to compactin was synthesized based on independent studies from Endo and Alberts [11, 12], and after several trials, this potent compound, lovastatin, was approved and commercially available in 1986 [13]. Presently, there are seven natural (fungal-derived) or synthetic statins that are commercially available; this group consists of three hydrophilic (pravastatin, rosuvastatin, and pitavastatin) and four lipophilic (lovastatin, simvastatin, fluvastatin, and atorvastatin) [14–16]. Cerivastatin was approved by the Food and Drug Administration in 1998, but it was removed from the market in 2001 after reports of fatal rhabdomyolysis [17].

Statins are one of the most successful drugs for reducing cardiovascular diseases. High-intensity statins treatment is associated with the greatest reduction in mortality [18]. In addition to lowering plasma cholesterol, various studies have reported that statins have pleiotropic effects such as antioxidant, anti-inflammatory, and anti-tumorigenesis. Regarding statins redox effects, some groups have demonstrated protective roles of these compounds against cell oxidative damage [19, 20], whereas others have reinforced their toxic effects [21, 22]. Despite these discrepancies in these results over the last decade, accumulated data have indicated that alterations in mitochondrial energy-linked functions such as respiration, oxidative phosphorylation, redox state,

Ca^{2+}-dependent permeability transition underlie statins toxicity. The impact on cell or tissue pathophysiology will depend on the intensity of statins' effects on mitochondria. In this chapter, we review the literature data on the statins effects on mitochondrial functions and consequent toxic tissue events.

1.1. Mitochondrial energy-linked functions and reactive oxygen generation

Mitochondria participation in the process of statin toxicity adds to the numerous roles of these organelles in cell pathophysiology [23, 24]. Considering that statin-mediated mitochondrial dysfunctions include many aspects of mitochondrial physiology such as inhibition of respiration, depletion of ubiquinone, redox imbalance, opening of the mitochondrial permeability transition pore (PTP) and disruption of energy conservation, we next outline some of these mitochondrial properties in the following sections.

During the last several decades, mitochondria have emerged as the center of attention in processes of cell signaling, cell injury, and cell death [25, 26]. According to the concept of coupling between respiration and oxidative phosphorylation through a transmembrane proton electrochemical potential that was introduced by Peter Mitchell [27], it is not difficult to understand that any condition that interferes with the ability to sustain the inner membrane proton potential leads to mitochondrial dysfunction [28]. In addition, the continuous oxygen reduction by the mitochondrial electron transport chain to build up the transmembrane proton gradient also generates a well-regulated amount of superoxide [23, 29]. Therefore, mitochondria have developed a complex antioxidant defense system composed of Mn-superoxide dismutase that converts the superoxide radical generated during respiration into hydrogen peroxide (H_2O_2). H_2O_2 is then reduced to water by glutathione and thioredoxin peroxidase or catalase [30]. Oxidized glutathione (GSSG) and thioredoxin (TSST) generated by peroxidases are converted to their reduced forms by glutathione and thioredoxin reductases, using NADPH as reducing power. NADH then reduces $NADP^+$, in a reaction catalyzed by NADP transhydrogenase that is present in the inner mitochondrial membrane [31–33]. Therefore mitochondria redox state is tightly regulated and connected with whole cell redox balance [34–36]. Furthermore, it is now generally accepted that superoxide as well as other forms of ROS can function as a signal for either adaptation or maladaptation to stress conditions [35]. In this regard, mitochondrial ROS generation leads to a nonlinear dose-response relationship called mitohormesis. In mitohormesis, high reactive oxygen concentrations exert devastating and irreversible effects on cell function and structures, whereas low concentrations may be associated with protective effects due to activation of cellular defense mechanisms [37, 38]. In fact, at progressively increasing physiological levels, ROS may successively regulate cellular processes such as proliferation and differentiation, activate adaptive programs such as transcriptional upregulation of antioxidant genes, and at higher levels, ROS may be a signal for senescence and regulated cell death [35]. In addition to the physiological processes, it seems that mitochondrial oxidative stress is responsible for the development and progression of a series of diseases such as cancer, diabetes, inflammatory diseases, hypertension, neurodegenerative and ischemia-related diseases, and aging [39–46]. Statin toxicity may also include the participation of mitochondrial generated ROS [47–49].

1.2. Mitochondrial Ca^{2+} transport and mitochondrial membrane permeability transition (MPT)

Ca^{2+} modulates several metabolic pathways through transient changes in its free concentrations in different cell compartments [50, 51]. In order to fulfill these physiological roles, Ca^{2+} movements across cell membranes are driven directly or indirectly by ATP hydrolysis. Therefore, defects in processes that supply cellular ATP may lead to deregulation in Ca^{2+} signaling that may compromise cell functioning, redox balance, and mitochondrial membrane permeability transition (MPT) [51, 52]. In this review, we briefly describe how mitochondrial Ca^{2+} load promotes MPT [53].

MPT is characterized by the opening of a high conductance, nonspecific proteinaceous pore, the PTP. It was first described by Hunter and collaborators [54] and then demonstrated by Vercesi's group to be dependent on redox imbalance promoted either by thiol oxidants or oxidative stress [55]. Matrix Ca^{2+} participates in at least two steps in the process of PTP opening: (a) stimulates superoxide generation by mitochondria and (b) binds to membrane sites exposing specific buried thiols to the oxidants (**Figure 1**) [55]. Accordingly, Ca^{2+} binding to cardiolipin alters mitochondria inner membrane lipid organization characterized by increased lipid packing and domain formation. As a consequence, the electron transfer along the respiratory complexes is impaired favoring superoxide generation [56].

Robust data has provided evidence that PTP opening is a main step in the mitochondrial pathway leading to cell death either by apoptosis or necrosis [57, 58], and is a major cause of cell death under a variety of pathophysiological conditions, including ischemia/reperfusion injury, traumatic brain injury, neurodegenerative diseases, metabolic diseases, muscular dystrophy, and drug toxicity [59–67].

Since mitochondrial Ca^{2+} overload stimulates superoxide generation and MPT, the mechanisms of Ca^{2+} transport by mitochondria will be outlined next. The inner mitochondrial membrane possesses three different carriers for Ca^{2+} influx and efflux [68]. A mitochondrial calcium uniporter (MCU) located in the inner membrane mediates the influx of Ca^{2+} down its electrochemical gradient without coupling Ca^{2+} transport to the flux of another ion. This mechanism was discovered in the 1960s [69, 70], but the molecular nature of the channel was only recently identified [71, 72]. Ca^{2+} release from mitochondria occurs via $Ca^{2+}/3Na^{+}$ or a $Ca^{2+}/2H^{+}$ exchangers [73–75] depending on the tissue [68, 76].

The high loads of matrix Ca^{2+} that stimulate ROS production in mitochondria [55] appear to be associated with either dysregulation of cellular Ca^{2+} homeostasis or regulated release from endo(sarco)plasmic reticulum [77–79] (**Figure 1**). Under both conditions, the opening of the PTP can occur allowing for the movements of molecules up to 1.5 KDa. The entry of solutes and water to the matrix causes large amplitude mitochondrial swelling. These conditions disrupt both the electrochemical proton potential and oxidative phosphorylation [23, 55]. When PTP opens in a large number of mitochondria, cell death occurs by necrosis due to the lack of ATP, and when PTP is limited to a small number of mitochondria, apoptosis is triggered by the release of cytochrome c [80]. Anti-apoptotic proteins (members of Bcl-2 family) or cyclosporine A inhibits the opening of PTP [81, 82]. Evidence has been provided that high intracellular Ca^{2+} levels and ROS have additive effects in the process of PTP opening [23, 53, 55, 83–88].

It is well recognized that mitochondrial Ca^{2+} is essential for PTP opening [54, 55, 89, 90], whereas oxidative modifications of inner membrane protein thiols, oxidative stress, presence

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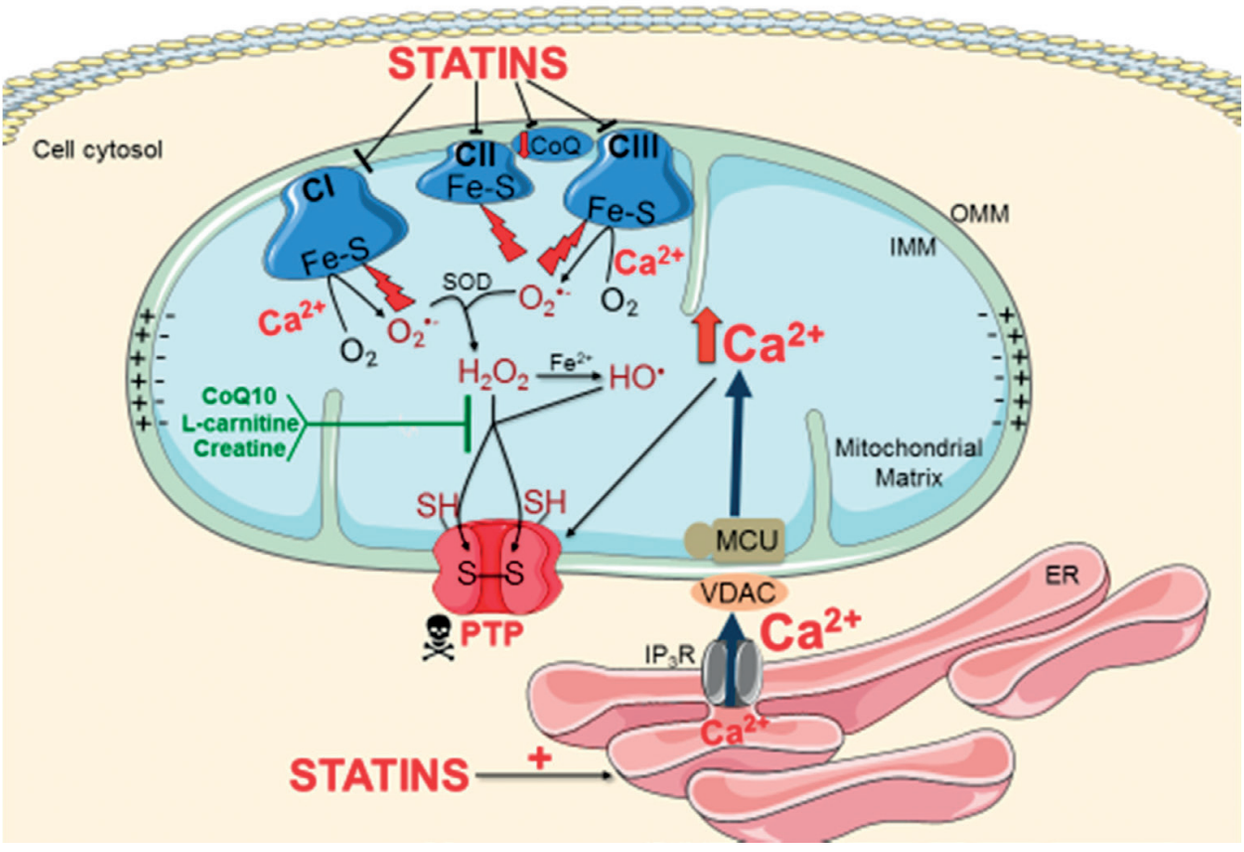

Figure 1. Statins triggers mitochondrial oxidative stress and calcium-dependent permeability transition. Statins diminishes the respiratory capacity at the level of complexes I, II and III of the respiratory chain, increasing superoxide generation ($O_2^{\bullet-}$). The Fe-S clusters present in these respiratory complexes are vulnerable to superoxide attack, thus inhibiting their activity and diminishing their resistance to Ca^{2+} induced MPT. Superoxide is dismutated in hydrogen peroxide (H_2O_2). When not metabolized by mitochondrial antioxidant systems, H_2O_2 can induce (directly or indirectly) membrane protein sulfhydryl-disulfide transitions, a process involved in PTP opening. Statins also impair cellular Ca^{2+} homeostasis, inducing Ca^{2+} release from the ER via IP_3R and increasing cytosolic Ca^{2+} levels. Thus, mitochondria uptake the excessive cytosolic Ca^{2+} via VDAC and MCU channels, leading to its accumulation in mitochondrial matrix. Ca^{2+} binds to membrane sites exposing specific buried thiols to the oxidants and also impairs mitochondrial respiration, increasing $O_2^{\bullet-}$ formation. The association of ROS and mitochondrial Ca^{2+} overload, PTP may open and trigger cell death. In addition, a decrease in the levels of CoQ10 that acts as an electron carrier and antioxidant also occurs due to inhibition of the mevalonate pathway by statins. The antioxidants CoQ10, L-carnitine and creatine prevent PTP opening induced by statins.

of inorganic phosphate [53, 55, 83, 85, 91], and Bcl-2 family proteins [81, 82] participate in PTP modulation. The close location of mitochondria and the endoplasmic reticulum (ER) [75] permits mitochondria to take up large amounts of Ca^{2+} that are released from the ER. This process seems to be controlled via a redox-regulated cross talk between mitochondria and ER that is mediated by NADPH oxidases [36]. Such redox interactions may link PTP opening to the induction of Ca^{2+} signals specifically for cell death [26]. Considering the understanding on how Ca^{2+} and ROS act synergistically in the mechanism of PTP opening, it should be emphasized that mitochondria are more susceptible to MPT when their antioxidant systems are exhausted, especially due to an oxidized state of NADPH and GSH [55]. Accordingly, mitochondria isolated from mice deficient in nicotinamide nucleotide transhydrogenase (NNT), which cannot sustain NADPH in the reduced state, present defective antioxidant capacity and increased susceptibility to MPT [92, 93]. Thus, MPT can be induced by pro-oxidants and prevented or even reversed by antioxidants [85, 86, 94, 95].

2. Statins pleiotropic effects

Statins are among the most commonly prescribed medicines worldwide. They are safe and well-tolerated and seem to present a range of cholesterol-independent protective actions called pleiotropic effects. Indeed, several studies claim that statins act as antioxidants [19, 96], anti-inflammatory agents [97], and can increase stability of the atherosclerotic plaque [98], improve endothelial function [99], and induce cancer cell death [100].

2.1. Antioxidant responses triggered by statins

Extensive literature reports have indicated that antioxidant effects can be attributed to statins. It has been postulated that statins decrease systemic or local oxidative stress and this appears to confer additional vascular protection. The first possible mechanism for this protective effect could be secondary to statins' main target effect, which is to decrease the concentration of the oxidizable substrate, LDLc. This decrease may lead to a reduction in oxidized-LDL, which constitutes a very early step involved in atherosclerosis development [101–103].

Another antioxidant mechanism frequently attributed to statins is the upregulation of cellular antioxidant defenses. For instance, atorvastatin treatment decreased the expression of essential NAD(P)H oxidase subunits and upregulated catalase expression in cultured rat vascular smooth muscle cells and in the vasculature of spontaneous hypertensive rats (SHR) [104]. Simvastatin treatment restored endothelial function in SHR by increasing superoxide dismutase and glutathione peroxidase activities [105].

Other studies have demonstrated a protective effect by statins against oxidative damage of biomolecules. In whole blood leukocytes of non-treated dyslipidemic diabetic type 2 patients, simvastatin treatment [19] protected against DNA oxidative damage. Similarly, rosuvastatin inhibited lipid peroxidation and attenuated the oxidative damage to DNA in treated rat liver [106]. Rosuvastatin-treated HL-60 cells exhibited a glutathione-dependent protective mechanism against DNA oxidation [107]. In addition, simvastatin or fluvastatin administration prevented lipid peroxidation, superoxide generation, cytokine production, and neutrophil accumulation in a rat colitis model [108].

With respect to statins' effects on specific mitochondrial redox homeostasis, literature reports are more controversial. It was shown that atorvastatin and simvastatin reduced oxidative stress triggered by Ca^{2+} and prevented MPT and cytochrome c release in rat liver mitochondria [96]. On the other hand, results from our group and others suggest that statins, when administered to mitochondria, muscle biopsies, or *in vivo* exert pro-oxidant activities (this will be discussed in more detail in the next section) [47, 49, 109]. Thus, our hypothesis for the alleged statin antioxidant effects is based on the mitohormesis concept [37, 38]: mild mitochondrial oxidative stress caused by statins may function as a signal that leads to a cellular adaptive response such as increasing the expression and activity of cellular antioxidant systems in order to overcome this stress.

2.2. Statins and cancer

Statins have been proposed as adjuvant in cancer therapy since the 1990s and, until then, several mechanisms have been proposed for this specific function depending on the type of cancer and

statins lipophilicity [100, 110–112]. In this regard, literature reports suggest that the mevalonate pathway inhibition is associated with anti-proliferative, pro-apoptotic, and anti-metastatic statins effects [113]. In addition, statins may impair cell membrane function, due to the lowering of cholesterol levels and inhibition of the tumor cell cycle, and may lead to cell death by distinct pathways, including the mitochondrial pathway (for more details, see Ref. [114] and other reviews).

Prostate cancer is one of the most commonly diagnosed cancer in men and is a significant cause of male morbidity and mortality [115]. Literature reports have shown that statins protect against prostate cancer in human patients [116, 117], and some of these effects may be attributed to a decreased isoprenoid synthesis due to mevalonate pathway inhibition. As a consequence, Ras proteins that regulate signaling pathways of cell proliferation, angiogenesis, and metastasis are not able to be isoprenylated, thus reducing their function and triggering apoptosis [118]. Statins also stimulate the mitochondrial apoptosis pathway [119, 120] via an increase in pro- and decrease in anti-apoptotic Bcl-2 proteins [121], activation of caspases 3, 7, 8, and 9 [122–124], and decrease in the formation of lipid rafts, membrane microdomains involved in several regulatory functions, including cell survival [125, 126]. In addition, statins have a dose-dependent effect on cell death. For instance, simvastatin at concentrations below 10 μM induced PC3 prostate cancer cells apoptosis [21] via a mechanism sensitive to mevalonate but not to cyclosporin A (CysA), an MPT inhibitor. On the other hand, necrosis is stimulated by higher doses of simvastatin (≥60 μM) and is preceded by an increase in free cytosolic Ca^{2+} concentration and PTP opening, sensitive to CysA, but not to mevalonate [21]. Both MPT and necrosis induced by simvastatin (60 μM) are sensitive to L-carnitine (antioxidant) and piracetam (membrane stabilizer) in an additive manner. When combined, these compounds act at lower doses than when each compound is used separately [22]. These data provide evidence that statin toxicity to tumor cells is not only the result of HMG-CoA reductase inhibition but also is mediated by the increase in free cytosolic Ca^{2+} concentration, stimulation of ROS generation, and PTP opening [21, 22]. Although many studies show that statins which are efficient in inducing tumor cell death claim their potential use as adjuvant therapy, there are no robust data that non-tumor cells are less affected by statins' toxic effects than tumor cells. Therefore, it is still premature to conclude that statins are anti-tumorigenic agent.

3. Statins adverse effects

After decades of statins' use, some side effects have been consistently described in a minority of patients, particularly regarding muscle function. Adverse effects other than muscle symptoms such as headache, digestive problems, liver enzymes abnormalities, and neurological dysfunction may occur in some patients [127, 128]. The side effects are often the decisive factor for the noncompliance to statins treatment [129, 130] and its discontinuation usually makes the side effect symptoms disappear [131].

The precise mechanisms involved in statins toxicity and the reasons why only a few subjects are affected remain unclear. Several groups, including ours, have proposed that mitochondria are the main players in statin-induced toxicity.

3.1. Mitochondrial dysfunction caused by statins treatment

Mitochondrial redox imbalance is associated with aging, degenerative disorders, and drug-induced toxicity [26, 132]. Several reports concerning statin *in vitro* effects on isolated tissues or mitochondria from experimental models demonstrated that statins promote inhibition of mitochondrial respiration, mitochondrial oxidative stress, and cell death [47, 49, 109, 133]. It has been previously shown that lipophilic (cerivastatin, fluvastatin, atorvastatin, and simvastatin) and hydrophilic (pravastatin) statins-induced mitochondrial membrane potential decrease in rat skeletal muscle cell line (L6) [133]. The four lipophilic statins also induced mitochondrial swelling, cytochrome c release, and DNA fragmentation in these L6 cells. Mitochondrial β-oxidation enzymes activities were strongly impaired by all lipophilic statins, but in the case of pravastatin, it occurred only at high concentrations. In isolated rat skeletal muscle mitochondria, glutamate-supported state 3 respiration and respiratory control ratios were decreased by all lipophilic statins, but not by pravastatin [133]. According to the authors, this mitochondrial dysfunction caused by lipophilic statins in skeletal muscle might partially explain the muscle symptoms presented by some patients. Abdoli and coworkers demonstrated in isolated rat liver mitochondria that atorvastatin, simvastatin, and lovastatin increased ROS formation followed by lipid peroxidation, inner mitochondrial membrane depolarization, and a decreased GSH/GSSG ratio [47].

More recently, mitochondrial redox imbalance [67, 134] was observed in a genetic human familial hypercholesterolemia mouse model, the LDL receptor knockout mouse (*LDLr*$^{-/-}$) [135]. Mitochondria isolated from several tissues of these mice (liver, heart, and brain) and intact spleen mononuclear cells presented higher ROS production and higher susceptibility to MPT. In addition, these mitochondria showed lower capacity to sustain reduced NADPH [67, 134], which is the most important reducing power involved in reconstituting mitochondrial antioxidant systems [132]. As a consequence, H_2O_2 accumulates and PTP opens [67, 134]. Since cholesterol synthesis consumes a large amount of NADPH, we have proposed that the increased steroidogenesis observed in these mice would be partially responsible for the lower mitochondrial content of NADPH and Krebs cycle intermediates observed in their liver mitochondria [67, 134]. Therefore, we hypothesized that inhibition of cholesterol synthesis by statins treatment could prevent the decrease in NADPH oxidation in *LDLr*$^{-/-}$ mice mitochondria. Unexpectedly, liver mitochondria from wild type and *LDLr*$^{-/-}$ mice treated with lovastatin presented a higher susceptibility to PTP opening, and *in vitro* experiments revealed a drug dose- and class-dependence of this effect [109]. Statin induced PTP opening was shown to be Ca^{2+}-dependent and associated with oxidation of protein thiol groups. Thus, statins induced a direct oxidative damage in mitochondrial proteins [109].

3.2. Ca^{2+} and statins toxicity

It has been proposed by our group and others that statins impair cellular Ca^{2+} homeostasis, leading to mitochondrial dysfunction. Increased cytosolic Ca^{2+} levels were observed after simvastatin treatment of myoblasts culture [136], rat skeletal muscle [137], and human skeletal muscle fibers, and this was followed by mitochondrial Ca^{2+} accumulation [138]. Indeed, Hattori and coworkers [139] proposed that statins induced Ca^{2+} release from the endoplasmic reticulum to the cytosol in human CD19+ primary lymphocytes. As a consequence of high Ca^{2+} levels in the cytosol, Ca^{2+} enters the mitochondria and induces MPT as demonstrated by our group in PC3 cells after simvastatin treatment [21, 22].

3.3. Statins effects on respiratory chain complexes

It is well known that enzymes containing 4Fe-4S clusters are particularly vulnerable to damage by superoxide or peroxynitrite radicals [140–145]. Complexes I and II present six and one of these 4Fe-4S clusters, respectively, thus showing a high superoxide-sensitivity. Some studies have demonstrated that superoxide generation inhibits respiration at complex I and II levels as a result of 4Fe-4S clusters damage. These alterations diminish resistance to Ca^{2+}-induced MPT and induce necrotic cell death [65, 145]. As mentioned before, our group demonstrated that mitochondrial dysfunction caused by simvastatin incubation in permeabilized skeletal muscle was L-carnitine and CoQ10 sensitive [49]. L-carnitine did not protect against CoQ10 depletion, indicating that both CoQ10 and L-carnitine are protecting mitochondrial respiration due to its ROS scavenging properties. Since L-carnitine also binds Fe^{2+} [146], it is feasible that this antioxidant molecule interacts with 4Fe-4S clusters in complexes I and II of the respiratory chain, protecting these sites against superoxide attack. Simvastatin lowered the ADP-stimulated respiration supported by substrates of complexes I and II in primary human skeletal myotubes and increased susceptibility to MPT, mitochondrial oxidative stress, and apoptosis [48]. These results are in agreement with a decrease in complex I activity in muscle of patients undergoing statin treatment [147].

Another study performed in myoblasts culture (C2C12) incubated with several statins (atorvastatin, cerivastatin, fluvastatin, lovastatin, pitavastatin, pravastatin, rosuvastatin, and simvastatin) showed that the respiratory capacity is reduced not only at the levels of respiratory chain complexes I and II but also in complex III [148]. In this case, it was suggested that statins in the lactone form binds to Q_o site of complex III, inhibiting its activity. Similarly, complex III activity of muscle from patients presenting myopathies induced by statins was also reduced [148]. On the other hand, statins do not seem to affect the complex IV-supported respiration [49, 148].

4. Muscle sensitivity to statins

It is well known that about 10% of patients undergoing statin treatment develop mild myopathic symptoms such as weakness, muscle pain, exercise intolerance, and other symptoms that are usually with normal or minimally elevated creatine kinase (CK) serum levels [149, 150]. Moreover, myositis, defined as muscle symptoms associated with increased CK, is usually present [151, 152]. Rhabdomyolysis, the most severe adverse effect of statins, is a very rare condition affecting 1.6/100,000 patients-years. It may result in acute renal failure and disseminated intravascular coagulation, leading to death. This condition is frequently related to drug interactions and occurs with CK levels 10-fold higher than the normal limit and elevated levels of creatinine [153, 154]. Increased intracellular lipid stores, cytochrome oxidase-negative myofibers, ragged red fibers, and subsarcolemmal accumulation of mitochondria were found in patients with muscle symptoms during statin therapy [155, 156]. Schick and colleagues also observed reduced mitochondrial DNA levels in patients treated with simvastatin [157]. Muscle-associated statin toxicity seems to be more severe with increasing lipophilicity, whereas more hydrophilic statins exert only mild or no toxicity [133, 153]. The myotoxic effect is attributed to their ability to penetrate and accumulate in cell membranes and alter their structural conformation [158–160].

On the other hand, high statin sensitivity may also be related to genetic factors; for instance, the activity of specific liver transporters may be impaired, thus reducing statins hepatic uptake and increasing its plasma concentrations that may potentially affect muscles [161–163].

Skeletal muscles are highly heterogeneous and present distinct fiber types classified as I or II and their respective subtype spectrum as determined by the myosin heavy chain isoforms. Type I and II fibers present relatively distinct metabolic, contractile, and motor properties in addition to antioxidant defense capacity. Thus, type I fibers appear red due to high myoglobin content, extensive mitochondrial content, and oxidative capacity, whereas type II fibers have relatively low myoglobin and mitochondrial content that depends mostly on glycolytic activity [164, 165]. In this regard, our group observed that respiratory rates were inhibited in the presence of Ca^{2+} in permeabilized plantaris muscle (predominantly type II fibers) in *LDLr*$^{-/-}$ mice chronically treated with pravastatin and catalase activity increased. In contrast, no alterations were observed in soleus muscle (predominantly type I fibers) [166]. Similarly, previous studies reported a distinct sensitivity of different muscle fiber types to lovastatin [162]. After 10 days of lovastatin administration, rat gastrocnemius muscles showed organelle degeneration, microvacuolization, and 20–50% necrosis, whereas soleus muscle was spared, suggesting that type II fibers are more vulnerable to lovastatin-induced myopathy [167]. In line with this finding, Westwood and colleagues characterized time-dependent muscle necrosis triggered by simvastatin or cerivastatin in rats after 10 days of treatment. The authors demonstrated that glycolytic fibers were more prone to necrosis than oxidative fibers, which in turn were consistently spared even when myotoxicity was severe. Since these fibers present distinct metabolism and MPT may precede necrosis, it is conceivable that mitochondria exert a central role in this process. In fact, it was observed that the first subcellular alterations were found in mitochondria of type II fibers, characterized by vacuolization as well as myeloid and vesicular body accumulation in sarcolemma areas [168]. Later, the same group performed a similar study using rosuvastatin in rats. Although a much higher statin dose was required to achieve muscle necrosis in comparison to the earlier study, the same pattern of muscle damage was observed and the soleus muscle remained unaffected [169]. Specific soleus-insensitivity to statin toxicity has also been demonstrated by other groups. Schaefer and coworkers demonstrated necrosis and inflammation in muscles with predominance of type II fibers in rats after 15 days of cerivastatin administration. Sarcomere disruption and altered mitochondria was also found in degenerated fibers, while these alterations were not found in type I fibers [170]. Similarly, cerivastatin-induced degeneration was evident in several muscles but not in the soleus muscle of female rats after the same treatment time (15 days). After 15 days of treadmill exercises, the severity of muscle damage had increased, but the soleus remained unaltered. Degenerated mitochondria were also observed with no changes in contractile elements such as endoplasmic reticulum and other subcellular compartments [171]. Although the role of mitochondria in myotoxicity in type II fibers is well established, there is no consensus as to whether this involvement precedes myofiber degeneration, thus justifying further studies to clarify this matter [170, 171]. In addition, MPT is associated with apoptosis or necrosis in several diseases [172] and is probably an important statin-induced event in muscle necrosis.

5. Statins toxicity to liver

Although rare, the main liver injury studies have reported statins toxicity alone [173–176] or in combination with other drugs with variable patterns of injury [177–181]. Some cases exhibited autoimmune features [180, 182, 183] and a range of latencies to onset [184] and progression was also observed [182, 185]. Liver adverse symptoms are unspecific and most patients remain asymptomatic [186]. A 3-fold increase in serum aspartate (AST) and alanine (ALT) aminotransferases activities have been described in less than 1% of patients receiving starting and intermediate statins doses [187–191] and this alteration may be accompanied by bilirubin elevation [192]. Two factors are frequently related to the hepatotoxic effects of statins: (a) the lipophilicity of these medicines and (b) alterations in cytochrome P450 system [193–195]. Accordingly, lipophilic statins (atorvastatin and simvastatin) are associated with more than 130 cases of liver injury, and a few cases progress to liver transplantation and death [173, 174, 178]. Rare cases of portal inflammation or fibrosis and mild necrosis were also described in patients undergoing lovastatin treatment [196] or atorvastatin treatment [197]. On the other hand, hydrophilic statins are minimally metabolized by the cytochrome P450 pathway [193–195] and are generally less toxic [109, 198]. A multicenter report also showed that pravastatin was well-tolerated in patients with compensated chronic liver disease [199]. Our group also attributes statin-induced liver toxicity to mitochondrial dysfunction associated with oxidative stress and MPT [193].

6. Statins and new onset of diabetes

Recent studies suggest that chronic use of statins is associated with risk of developing type 2 diabetes [200–202]. Meta-analyses of large-scale statin trials support the concept of the diabetogenic effect of statins, but the precise mechanisms have not yet been identified [203, 204]. We have recently revealed diabetes-related mechanisms induced by statin treatment in a familial hypercholesterolemia animal model, the *LDLr*$^{-/-}$. We demonstrated that pravastatin-treated *LDLr*$^{-/-}$ mice exhibit marked reductions of insulinemia and of glucose-stimulated insulin secretion by isolated pancreatic islets. These effects were associated with increased oxidative stress and apoptosis [205] and were counteracted by co-treatment with CoQ10 (Lorza-Gil et al., unpublished data). Therefore, we have proposed that pancreatic toxic effects of pravastatin could be caused by statin inhibition of CoQ10 biosynthesis. On the other hand, we and others have hypothesized that insulin signaling in their target tissues (such as muscle) could also be impaired by chronic statin treatment. However, studies relating statins therapy and insulin sensitivity are controversial [206–208]. A meta-analysis by Baker and colleagues shows that while pravastatin improved insulin sensitivity, atorvastatin, simvastatin, and rosuvastatin worsened it [209]. Experimental studies suggest that atorvastatin leads to reduced expression of GLUT4 in adipocytes *in vivo* and *in vitro* [210] and that simvastatin decreases IGF-1 signaling (pAKT, pERK) in muscle cells [211]. Kain et al. [212] showed that myotubes treated with simvastatin and atorvastatin presented impaired insulin signaling pathway and glucose uptake. We have evidence that long-term pravastatin treatment of hypercholesterolemic mice also induces

marked insulin resistance and increased muscle protein degradation (Lorza-Gil et al., unpublished data). Therefore, toxic effects on insulin secreting cells in conjunction with impaired muscle insulin signaling may explain the new onset of diabetes reported in statin-treated subjects.

7. Antioxidant supplement and statins toxicity

The cholesterol biosynthesis pathway generates several products including CoQ10 [213]. CoQ10 is an essential component of the electron transport chain where it acts as an electron carrier [214]. Ubiquinol, the reduced form of ubiquinone, when associated with proteins in the inner mitochondrial membrane, has an important function as a lipophilic antioxidant [215, 216]. CoQ10 also has additional functions such as regeneration of reduced intra- and extracellular forms of ascorbic acid and tocopherol (vitamin E) [217, 218], participation in redox processes associated with PTP opening [219], and regulation of muscle uncoupling proteins [220]. It is also known that the reduced form of ubiquinone occurs in all cellular membranes [221–223] as well as in serum lipoproteins and DNA, protecting them from oxidative damage [224]. CoQ10 content is larger in tissues such as cardiac and skeletal muscles that have high energy demand [223]. Therefore, decreased synthesis of ubiquinone may result in two harmful conditions: (a) insufficient rates of mitochondrial ATP synthesis [225] and (b) decreased mitochondrial antioxidant capacity [49].

Some studies have proposed that statin-induced mitotoxicity may be mediated by diminished CoQ10 content with consequent impairment of mitochondrial respiration [111, 226–234]. On the other hand, our group has provided evidence that under our experimental conditions, the reduction of mitochondrial respiration associated with CoQ10 depletion was mainly due to its free radical scavenging action rather than its electron carrier function. Indeed, it has been demonstrated that incubation of permeabilized rat soleus muscle with simvastatin inhibited both ADP and FCCP-stimulated oxygen consumption supported by complex I or II substrates. Additionally, ubiquinone content was diminished by 40% and the H_2O_2 content was significantly increased. Under these conditions, all of the following compounds, including mevalonate, CoQ10, or L-carnitine protected against H_2O_2 generation but only mevalonate prevented CoQ10 depletion. Thus, independent of CoQ10 levels, L-carnitine prevented the toxic effects of simvastatin. This allows for the conclusion that L-carnitine antioxidant action prevailed in the protection against simvastatin-induced respiratory inhibition [49]. Therefore, it can be concluded that CoQ10 also acted as a free radical scavenger in this mechanism. Accordingly, Kettawan and coworkers previously demonstrated that a decrease in ubiquinone levels in serum, liver, and heart in mice undergoing simvastatin treatment increased lipoperoxidation. Simvastatin also reduced NADPH-CoQ reductase activity, whereas the co-administration of CoQ10 and simvastatin to mice diminished these deleterious effects [235]. Another study revealed that simvastatin reduced mitochondrial CoQ10 levels associated with DNA oxidative damage and reduced ATP synthesis followed by cell death in hepatocytes (HepG2). All of these alterations were reversed by CoQ10 supplementation [236]. Furthermore, it was recently shown that CoQ10 supplementation improved respiratory control in liver mitochondria isolated from rats treated with high

doses of atorvastatin and/or a cholesterol-rich diet [237]. Despite all data correlating CoQ10 depletion with statin toxicity, the efficacy of ubiquinone supplementation in patients with side effects is still under debate [231, 238–240].

Creatine is a guanidine compound synthesized endogenously [241] and widely and safely used as supplement by athletes to increase their performance [242]. The role of creatine on the maintenance of ATP/ADP ratio by activating CK is very well known, but it also exerts other actions. Creatine participates on a protein complex involved in MPT regulation [55, 243, 244] and was firstly mentioned as antioxidant in 1998 [245]. A few years later, Lawler and coworkers showed that this compound was capable of scavenging radicals such as superoxide and peroxynitrite [246]. In our recent work, we showed that diet supplementation with creatine protected *LDLr*$^{-/-}$ mice against pravastatin sensitization to Ca^{2+}-induced MPT [166].

L-carnitine stimulates β-oxidation by increasing carnitine palmitoyltransferase 1A mRNA expression. This action prevents mitochondrial oxidative stress induced by free fatty acids, increasing mitochondrial function [22, 247]. Another property of L-carnitine is to bind Fe^{2+} [248] that participates in the mitochondrial oxidative stress involved in MPT [249]. Thus, it is feasible to propose that L-carnitine protects complexes I and II of the respiratory chain against superoxide attack by interacting with 4Fe-4S clusters in these sites. In a previous work performed in PC3 prostate cancer, we showed that L-carnitine and piracetam (a membrane stabilizer) prevented MPT and necrosis induced by simvastatin (60 μM) [22].

Taken together, these experimental results suggest that ROS generation and mitochondrial oxidative stress play an important role on statins toxicity.

8. Conclusions

Cardiovascular benefits of statins therapy are undoubted and appear to be present across diverse demographic and clinical groups. However, the side effects may affect a minority of patients. In this review, we addressed the cellular and molecular mechanisms related to statin side effects. Mitochondrial oxidative stress seems to be the main cause of toxicity in statin sensitive tissues (**Figure 1**). The levels and consequences of mitochondrial oxidative stress seem to be more deleterious in skeletal muscle. This effect is secondary to: (a) inhibition of electrons flow at the levels of respiratory complexes I, II, and III, and (b) decrease in the levels of CoQ10 due to inhibition of the mevalonate pathway. In association with mitochondrial Ca^{2+} overload due to increased cytosolic free Ca^{2+} concentrations, the PTP may open and trigger cell death. *In vitro* experiments provide evidence that this can be blocked in a concerted manner by L-carnitine plus the membrane stabilizer piracetam. Experiments performed with muscle biopsies taken from hypercholesterolemic mice, chronically treated with pravastatin, show that either CoQ10 or creatine can protect against statin-induced mitochondrial muscle toxicity both *in vitro* and *in vivo*. Statin treatment may also result in pro- or antioxidant actions depending on statin class (lipophilicity), dose, and patient's background. We suggest that mitochondrial oxidative stress caused by statin treatment may be a signal for cellular antioxidant system response (such as

catalase upregulation) possibly explaining the alleged statin antioxidant properties. Together, the experimental evidence presented in this review suggests that statins' detrimental effects could be prevented by antioxidants administration such as CoQ10, L-carnitine, and creatine.

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Conflict of interest statement

The authors declare no conflicts of interest.

Abbreviations

ALT	Alanine transaminase
AST	Aspartate transaminase
CK	Creatine kinase
CoQ10	Coenzyme Q10
Cys A	Cyclosporin A
ER	Endoplasmic reticulum
FCCP	Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
FH	Familial hypercholesterolemia
FPP	Farnesyl-pyrophosphate
GGPP	Geranylgeranyl-pyrophosphate
GPP	Geranyl-pyrophosphate
GSH	Glutathione
GSSG	Glutathione oxidized
HMG-CoA	3-Hydroxy-3-methylglutaryl coenzyme-A
IMM	Inner mitochondria membrane

IP_3R	Inositol 1,4,5-trisphosphate receptor
MCU	Mitochondrial calcium uniporter
MPT	Mitochondrial permeability transition
NNT	Nicotinamide nucleotide transhydrogenase
OMM	Outer mitochondria membrane
PTP	Permeability transition pore
ROS	Reactive oxygen species
SHR	Spontaneous hypertensive rats
TSST	Thioredoxin
VDAC	Voltage-dependent anion-selective channel

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