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Statins induce calcium-dependent mitochondrial permeability transition

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Abstract

Statins (3-hydroxy-3-methylglutaryl-CoA reductase inhibitors) are used in the treatment of hypercholesterolemic patients to reduce risk of cardiovascular diseases because of their cholesterol lowering action. Other lipid independent protective actions of statins have been reported. However, some adverse side effects have, also, been described. We report, here, that liver mitochondria isolated from hypercholesterolemic LDL receptor knockout mice treated during 15 days with therapeutic doses (100 mg/kg, p.o.) of lovastatin presented a higher susceptibility to develop membrane permeability transition (MPT). In experiments in vitro, lovastatin-induced MPT in a dose-dependent manner (10–80 μM) by a mechanism sensitive to cyclosporin A (cyclophilin sequestrant), dithiothreitol (reducing agent), adenine nucleotide carrier inhibitor (ADP), catalase (H_2O_2 reductant) and EGTA (calcium chelator). In agreement with the inhibition of the mitochondrial swelling by dithiothreitol, lovastatin, also, decreased the content of total mitochondrial membrane protein thiol groups. Simvastatin had similar effects on mitochondria; however, pravastatin, a hydrophilic statin, had a weaker effect in inducing MPT. In conclusion, statins can act directly on mitochondria either in vivo or in vitro inducing permeability transition, which is a process involved in cell death.

Keywords: Statins; Mitochondrial permeability transition; Hypercholesterolemic LDL receptor knockout mice

1. Introduction

Mitochondria use electron transport to generate an H^+ electrochemical gradient across the inner mitochondrial membrane. This electrochemical gradient is, then, used by the ATP synthase (F_1–F_0 ATPase) to phosphorylate ADP to ATP. To sustain this electrochemical potential the inner mitochondrial membrane should remain impermeable to H^+ (Mitchell, 1961). Loss of membrane impermeability to protons can be associated with a mitochondrial

Abbreviations: CsA, cyclosporin A; DTNB, 5,5′-dithiobis(2-nitrobenzoic) acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Hepes, 2-hydroxyethyl-1-piperazinethanesulfonic acid; MLM, mice liver mitochondria; MMM, mice muscle mitochondria; MPT, mitochondrial permeability transition; RLM, rat liver mitochondria; ROS, reactive oxygen species; $\Delta\Psi$, transmembrane electrical potential

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pathway of cell death, and membrane permeability transition (MPT) may be a key event in this process (Castilho et al., 1995; Kowaltowski et al., 2001). MPT is characterized by a Ca^{2+} promoted opening of a non selective proteinaceous inner membrane pore, sensitive to the immune suppressor cyclosporin A (Kowaltowski et al., 2001), associated with oxidative modifications of inner membrane protein thiol groups (Fagian et al., 1990).

Ca^{2+}-induced MPT is enhanced by a variety of compounds called inducers (for a list see Zoratti and Szabo, 1995) including inorganic phosphate (Kowaltowski et al., 1996), oxidants of pyridine nucleotides (Lehninger et al., 1978; Zago et al., 2000), and thyroid hormones (Castilho et al., 1998). Most of these inducers are compounds capable of enhancing Ca^{2+}-induced mitochondrial oxidative stress or to react with membrane protein thiol groups (Castilho et al., 1995; Kowaltowski et al., 2001). MPT has been directly implicated in either necrosis or apoptosis in a variety of pathological situations (Argaud et al., 2005; Kim et al., 2003; Polyak et al., 1997; Petit et al., 1996).

Recent results from our group demonstrated that mitochondria from atherosclerosis-prone, hypercholesterolemic, LDL receptor knockout mice have a higher susceptibility to develop mitochondrial membrane permeability transition (MPT). This was related to a lower mitochondria NADPH pool. Experiments in vivo provided direct evidence of higher cholesterol de novo synthesis, which decreases NADPH reducing equivalents in tissues from hypercholesterolemic mice. These findings could explain the reduced antioxidant capacity of mitochondria from hypercholesterolemic mice, leading to the greater susceptibility to MPT (Oliveira et al., 2005). Therefore, it is conceivable to hypothesize that inhibition of cholesterol synthesis by treatment with statins could correct the decreased mitochondria antioxidant defenses in hypercholesterolemic mice.

Statins are inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, which catalyzes the rate-limiting step in cholesterol synthesis. Statins are frequently prescribed to effectively prevent and reduce the risk of coronary artery disease through lowering serum cholesterol levels (Shepherd et al., 1995). Other lipid independent protective effects, collectively called pleiotropic, have been described. They include improvement of endothelial cell dysfunction, anti-inflammatory and anti-proliferative actions (Futterman and Lemberg, 2004; Liao, 2002). Although large clinical trials revealed the efficacy and relative safety of statins, adverse effects may, also, arise. Complications chiefly concern the hepatic function, skeletal muscle and peripheral nerves (Muscari et al., 2002). Myopathies are the most common reported side effect (Rosenson, 2004; Anon., 2004; Ucar et al., 2000).

In this work, we investigated whether the treatment of LDL receptor knockout mice with lovastatin influences the susceptibility to develop MPT and whether statins could exert direct effects on isolated mitochondria.

2. Materials and methods

2.1. Isolation of muscle and liver mitochondria

Muscle mitochondria were isolated from mice hind limb skeletal muscle by homogenization in ice-cold medium containing 100 mM sucrose, 100 mM KCl, 50 mM Tris–HCl, 1 mM K_2HPO_4, 0.1 mM EGTA and 0.2% BSA, pH 7.4, followed by differential centrifugation (Tonkonogi and Salhin, 1997). The final mitochondrial pellet was resuspended in ice-cold storage buffer containing 0.2 M mannitol, 0.1 M sucrose, 10 mM Tris–HCl, pH 7.4. Addition of 0.2% fatty acid-free BSA in the buffers throughout the isolation procedure depleted mitochondria from endogenous FFA.

Liver mitochondria were isolated by conventional differential centrifugation (Kaplan and Pedersen, 1983) from adult mice or rats fasted overnight. The livers were homogenized in 250 mM sucrose, 1 mM EGTA, and 10 mM Hepes buffer (pH 7.2). The mitochondrial suspension was washed twice in the same medium containing 0.1 mM EGTA and the final pellet was resuspended in 250 mM sucrose to a final protein concentration of 80–100 mg/ml.

2.2. Standard incubation procedure

The experiments were carried out at 30 °C, with continuous magnetic stirring, in standard medium containing 125 mM sucrose, 65 mM KCl, 5 mM potassium succinate, 2 mM inorganic phosphate, 1 mM magnesium chloride, 2 μM rotenone, 10 mM Hepes buffer (pH 7.2), and 30 μM $CaCl_2$. Other additions are indicated in the figure legends.

2.3. Mitochondrial swelling

Mitochondrial swelling was determined as the decrease in the turbidity of the mitochondrial suspension measured at 520 nm in a Hitachi U-3000 spectrophotometer.

2.4. Determination of protein thiol groups content

The mitochondrial suspension incubated in standard reaction medium was submitted to three subsequent freeze–thawing cycles to release matrix proteins and centrifuged at 10,000 rpm for 2 min. The pellet was treated twice with 200 μl of 6.5% trichloracetic acid and centrifuged 2 min at 10,000 rpm in order to precipitate proteins. The final pellet was resuspended in 1 ml of medium containing 100 μM of DTNB, 0.5 mM EGTA, and 0.5 M Tris–HCl, pH 8.3. Absorption was measured at 412 nm, using cysteine for calibration.

2.5. Measurements of mitochondrial transmembrane electrical potential ($\Delta\Psi$) using safranine

The mitochondrial membrane potential was estimated as fluorescence changes of safranine O, recorded on a model F-4010 Hitachi fluorescence spectrophotometer operating at excitation and emission wavelengths of 495 and 586 nm, respectively, with a slit width of 5 nm. $\Delta\Psi$ was calculated according to Akerman and Wikstron (1976) using a calibration curve obtained when mitochondria were incubated in a K^+-free reaction medium containing 250 mM sucrose, 10 mM Na-Hepes buffer, pH 7.2, and 0.5 mM EGTA.

2.6. Measurements of mitochondrial transmembrane electrical potential ($\Delta\Psi$) using TPP^+

Mitochondria were incubated in the standard medium containing 3 μM tetraphenylphosphonium (TPP^+). The concentration of TPP^+ in the extra mitochondrial medium was continuously monitored with a TPP^+ selective electrode prepared in our laboratory according to Kamo et al. (1979). The membrane potential was, then, calculated assuming that the TPP^+ distribution between mitochondria and medium follows the Nernst equation (Muratsugu et al., 1977).

2.7. Animals and lovastatin treatment

The experiments were approved by the university's Ethic Committee and were in accordance with the Guidelines on the Handling and Training of Laboratory Animals published by the Universities Federation for Animal Welfare (1992). LDL receptor knockout mice (Ishibashi et al., 1993) had access to standard laboratory rodent chow diet (Nuvital CR1, PR, Brazil) and water ad libitum and were housed at 22 ± 2 °C on a 12 h light–dark cycle. Male and female, aged 4–6 months, were used in this study. Lovastatin (100 mg/kg) or placebo (2% arabic gum) were given daily through gavage, during 15 days.

2.8. Statistical analyses

The results of experiments performed in at least three independent experiments are displayed as means ± S.D. and significance was assessed by ANOVA, followed by the Tukey post test, with significance level set at $p < 0.05$ using InStat software (InStat Software Inc., Richmond, CA, USA).

2.9. Chemicals

Cyclosporin A, Oligomycin, DTNB, EGTA, FCCP, ADP, and DTT were purchased from Sigma (St. Louis, USA), Lovastatin, Pravastatin and Simvastatin were obtained from Galena Química e Farmacêutica Ltda (SP, Brazil). All other reagents were products of the highest purity grade available.

3. Results

3.1. Plasma cholesterol levels and mitochondrial respiration in LDL receptor knockout hypercholesterolemic mice treated with lovastatin

Two-week treatment of LDL receptor knockout mice with lovastatin (100 mg/kg BW) reduced plasma total cholesterol concentration by 20% (390 ± 22 versus 308 ± 19 mg/dl, $n = 10$, $p < 0.05$). Liver mitochondria respiratory control (phosphorylating/resting respiration ratio) measured in Ca^{2+} free medium were essentially similar in lovastatin treated as compared to untreated LDL receptor knockout mice (4.45 ± 0.64 and 4.34 ± 0.79). Phosphorylation efficiency (ADP/O consumption ratio) was similar in both mitochondria: 2.52 ± 0.25 and 2.51 ± 0.34.

3.2. Membrane potential disruption and swelling of mitochondria isolated from hypercholesterolemic mice treated with lovastatin

Mitochondria membrane transport and permeability properties were investigated in control and in lovastatin treated or untreated hypercholesterolemic mice. The experiments depicted in Fig. 1 show that control mitochondria (line a) restores $\Delta\Psi$ after four sequential additions of Ca^{2+} (25 nmol/mg protein). However,

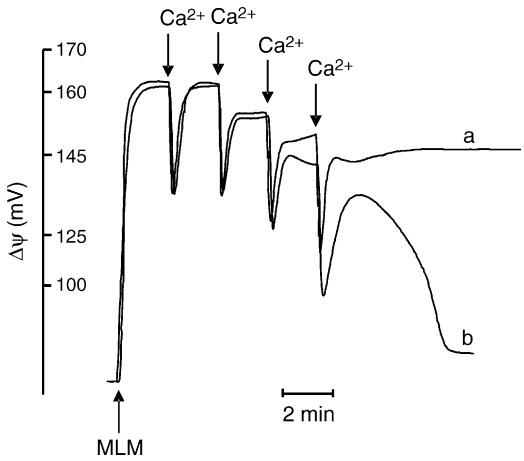

Fig. 1. Dose-response curve of mitochondrial membrane potential after sequential additions of Ca^{2+}. MLM (0.5 mg/ml) were incubated in the standard medium containing 3 μM TPP^+. Ca^{2+} (25 nmol/mg protein) was added as indicated. Traces represent liver mitochondria isolated from: control C57Bl6 mice (line a) and hypercholesterolemic mice (line b). The results are representative of three independent experiments performed in duplicates. $\Delta\Psi$ at 12 min was 146.0 mV ± 2.8 for control mice (line a) vs. totally collapsed $\Delta\Psi$ for hypercholesterolemic mice (line b), $p < 0.001$.

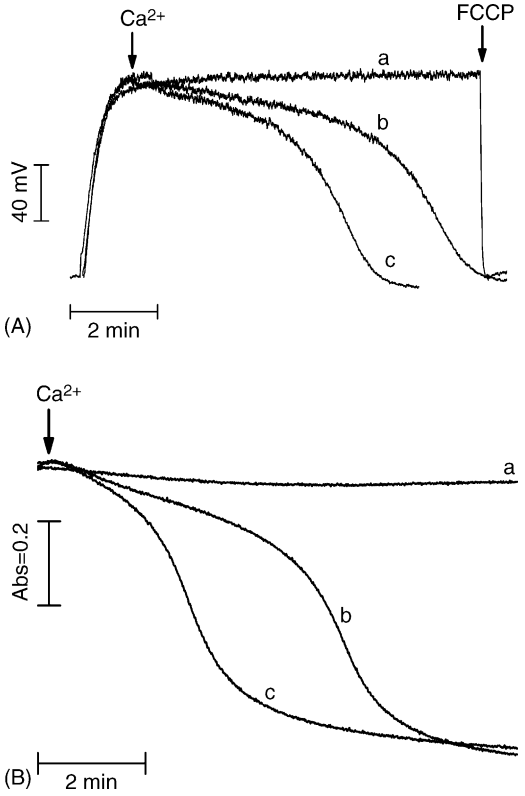

Fig. 2. Effect of in vivo lovastatin treatment on Ca^{2+}-induced mitochondrial membrane potential disruption (A) and mitochondrial swelling (B). Panel A: MLM (0.5 mg/ml) were incubated in the standard medium containing 5 μM safranine plus 100 μM Ca^{2+}. Traces represent liver mitochondria isolated from: control mice (line a), hypercholesterolemic mice (line b) and hypercholesterolemic mice treated with lovastatin (line c). The results are representative of six independent experiments performed in duplicate. $\Delta\Psi$ at 5 min were: 169.0 mV ± 2.2 for control mice (line a) vs. 141.0 mV ± 1.8 for hypercholesterolemic mice (line b), $p < 0.01$, and totally collapsed $\Delta\Psi$ for hypercholesterolemic treated lovastatin (line c), $p < 0.001$. Panel B: MLM (0.5 mg/ml) were incubated in the standard medium containing 100 μM Ca^{2+}. Traces represent liver mitochondria isolated from: control mice (line a), hypercholesterolemic mice (line b) and hypercholesterolemic treated lovastatin (line c). The results are representative of six independent experiments performed in duplicates. The absorbances at 5 min were: control mice (line a: 1.481 ± 0.028), hypercholesterolemic (line b: 1.255 ± 0.046) and hypercholesterolemic mice treated with lovastatin (line c: 0.953 ± 0.063), $p < 0.001$.

hypercholesterolemic mice mitochondria (line b) showed decreased capacity to sustain $\Delta\Psi$ after the third addition of calcium (75 nmol) and lost $\Delta\Psi$ irreversibly after the fourth addition of calcium (100 nmol). Fig. 2 shows the effect of a single Ca^{2+} addition (100 nmol/mg protein) on mitochondria isolated from control mice (line a), hypercholesterolemic mice (line b) and hypercholesterolemic mice treated with lovastatin (line c). In agreement with Fig. 1, while the mitochondria from control mice maintained $\Delta\Psi$ after Ca^{2+} accumulation (Fig. 2A, line a), hypercholesterolemic mice mitochondria underwent $\Delta\Psi$ decrease (Fig. 2A, line b). Lovastatin treatment enhanced mitochondria sensitivity to Ca^{2+}-induced loss of $\Delta\Psi$ in hypercholesterolemic mice, as shown by the earlier disruption of $\Delta\Psi$ (Fig. 2A, line c). Accordingly, liver mitochondria from hypercholesterolemic mice treated with lovastatin (Fig. 2B, line c) incubated in standard medium containing 100 μM calcium underwent swelling faster than untreated hypercholesterolemic mice mitochondria (Fig. 2B, line b) and controls (Fig. 2B, line a).

3.3. Mitochondrial membrane potential disruption and swelling induced by lovastatin in vitro

In order to check if the lovastatin in vivo effects described above could be a direct action of lovastatin on mitochondria, acute in vitro experiments were performed with isolated mice mitochondria. The addition of lovastatin in the incubation medium promoted $\Delta\Psi$ disruption in mitochondria from muscle (Fig. 3A, line b) and liver (Fig. 3B, line b) of control C57Bl6 mice. The same effect observed in mice mitochondria was verified in rat liver mitochondria: lovastatin promoted $\Delta\Psi$ disruption (Fig. 4, line b) and mitochondrial swelling (Fig. 5, lines b–f) when compared to the respective controls (lines a). These lovastatin effects were inhibited by cyclosporin A (Figs. 3 and 4: line c; Fig. 5: line g), a cyclophilin sequestrant, which prevents MPT pore opening. Mitochondrial swelling experiment shown in Fig. 5 shows that the lag time between drug addition and the onset of mitochondrial swelling was decreased with increasing lovastatin concentrations in the range of 10–80 μM (lines b–f).

Since mitochondria from mice and rats responded to lovastatin in a similar manner, we performed the following experiments on rat liver mitochondria.

3.4. Dithiothreitol, ADP, catalase and EGTA inhibited mitochondrial swelling induced by lovastatin

The mechanisms by which lovastatin-induced mitochondrial permeability transition (MPT) were additionally investigated. Fig. 6 shows that Ca^{2+} chelation by EGTA (line c) totally prevented the mitochondrial swelling that occurred in the presence of lovastatin (line b), indicating that the mitochondrial permeabilization is Ca^{2+} dependent. Membrane permeabilization was, also, prevented by the presence of ADP/Oligomycin (line

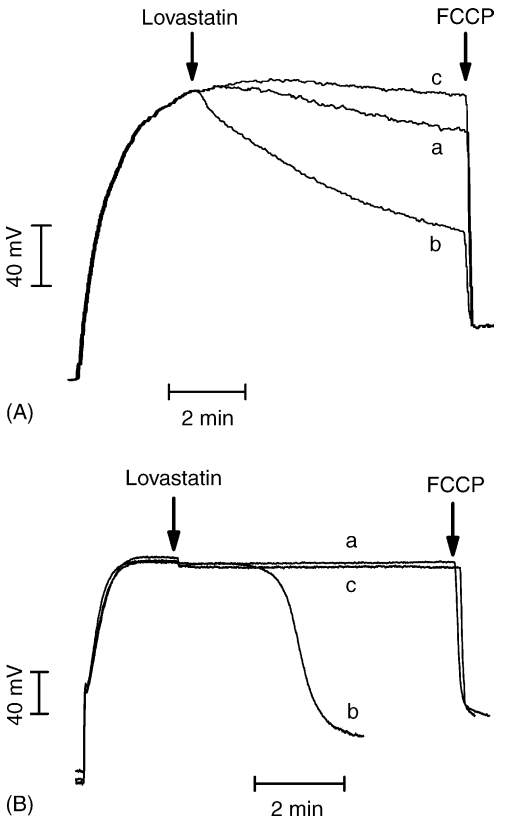

Fig. 3. Mitochondrial membrane potential disruption in muscle (A) and liver (B) mitochondria from mice induced by lovastatin in vitro. Panel A: MMM (0.5 mg/ml) were incubated in medium containing NAD^+-linked substrates (α-ketoglutarete, pyruvate, malate, and glutamate) and 5 μM safranine (line a), plus 40 μM lovastatin (line b) and plus 1 μM cyclosporin A (line c). FCCP (1 μM) was added where indicated. The results are representative of three independent experiments performed in duplicates. $\Delta\Psi$ at 7 min were: 154.0 mV ± 3.1 (line a) vs. 99.0 ± 5.0 (line b), or 166.0 mV ± 4.1 (line c), $p < 0.001$. Panel B: MLM (0.5 mg/ml) were incubated in a standard medium containing 5 μM safranine (line a) and 40 μM lovastatin (line b) plus 1 μM cyclosporin A (line c). FCCP (1 μM) was added where indicated. The result is representative of three independent experiments performed in duplicate. $\Delta\Psi$ at 7 min was 169.0 mV ± 3.0 (line a) vs. totally collapsed (line b), or 170.0 mV ± 2.7 (line c), $p < 0.001$.

d). Oligomycin was used to prevent ADP phosphorylation allowing ADP to bind to and block the adenine nucleotide carrier, a putative component of the permeability transition pore (Halestrap et al., 1997). Addition of catalase (line e) partially inhibited lovastatin-induced mitochondrial swelling, suggesting the participation of H_2O_2 in this process. Dithiothreitol (DTT, line f), a disulfide reductant, also, blocked the lovastatin-induced MPT effect. The DTT effect suggests that the oxidation of protein thiol groups is involved in this permeabilization.

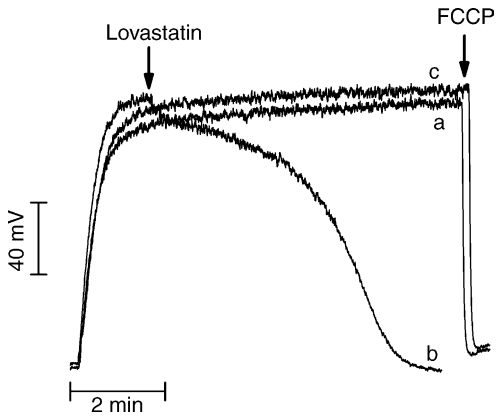

Fig. 4. Lovastatin-induced mitochondrial membrane potential disruption in rat liver mitochondria. RLM (0.5 mg/ml) were incubated in the standard medium containing 5 μM safranine (line a), plus 40 μM lovastatin (line b), plus 1 μM cyclosporin A (line c). FCCP (1 μM) was added where indicated. The results are representative of 10 independent experiments performed in duplicates. $\Delta\Psi$ at 7 min were 164.0 mV ± 3.9 (line a) vs. totally collapsed (line b), or 166.3 mV ± 4.1 (line c), $p < 0.001$.

3.5. Oxidation of mitochondrial membrane protein thiol groups is promoted by lovastatin in the presence of Ca^{2+}

The participation of protein thiol groups' oxidation in the mechanism of MPT induction by lovastatin was examined by determining the content of reduced thiol groups in the mitochondrial membrane. Results presented in Table 1 indicate that there is a significant decrease in the protein thiol content, due to thiol oxidation, when mitochondria were incubated with lovastatin. Both MPT inhibitors, ADP and dithiothreitol, prevented

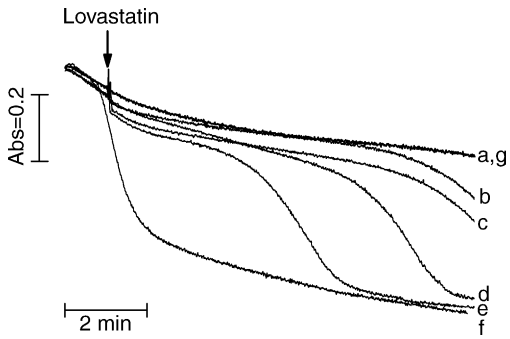

Fig. 5. Dose-dependent effect of lovastatin on mitochondrial swelling. RLM (0.5 mg/ml) were incubated in the standard medium containing: 0, 10, 20, 30, 40 and 80 μM lovastatin, respectively (lines a–f) or 80 μM lovastatin plus 1 μM CsA (line g). The results are representative of six independent experiments performed in duplicate. The absorbance at 8 min was: 1.453 ± 0.040 (line b), 1.330 ± 0.040 (line c), 1.172 ± 0.042 (line d), 1.119 ± 0.018 (line e), 1.074 ± 0.049 (line f) vs. 1.529 ± 0.035 (line a), $p < 0.001$.

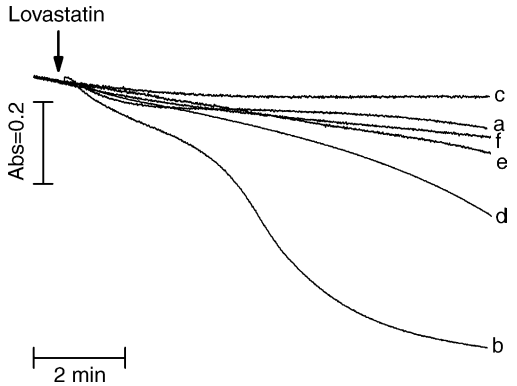

Fig. 6. Inhibition by EGTA, dithiothreitol, catalase and ADP on lovastatin-induced mitochondrial swelling. RLM (0.5 mg/ml) were incubated in the standard medium containing 40 μM lovastatin (line b) in the presence of: 500 μM EGTA (line c), 2 μM catalase (line d), 500 μM ADP plus 1 μg/ml oligomycin (line e) or 500 μM dithiothreitol (line f). Line a represents the control experiment (no additions). The results are representative of five independent experiments performed in duplicates. The absorbances at 7 min were 1.573 ± 0.043 (line a), 1.122 ± 0.033 (line b), 1.625 ± 0.063 (line c), 1.470 ± 0.038 (line d), 1.539 ± 0.033 (line e), 1.566 ± 0.038 (line f). Line b (lovastatin) differed significantly at $p < 0.001$ from all other treatments (a, c–f).

the thiol groups' oxidation. As expected, the presence of cyclosporin A did not inhibit the oxidation of the protein thiol groups.

3.6. Comparative effects of hydrophobic and hydrophilic statins on mitochondrial swelling

Fig. 7 shows that simvastatin and lovastatin (hydrophobic statins) presented similar effects of inducing mitochondrial swelling (lines b and c). However, the effect of pravastatin (hydrophilic statin) was significantly milder. Doubling pravastatin medium concentration (80 μM) promoted mitochondria swelling similarly to 40 μM lovastatin (data not shown).

Table 1
Oxidation of mitochondrial membrane protein thiol groups promoted by lovastatin and Ca^{2+}: prevention by ADP and DTT

Conditions	–SH (nmol/mg protein)
100 μM EGTA	46 ± 0.7
No addition	40 ± 0.5
40 μM lovastatin	$18 \pm 0.2^{*}$
40 μM lovastatin + 0.5 mM DTT	44 ± 0.4
40 μM lovastatin + 0.5 mM ADP	38 ± 0.6
40 μM lovastatin + 1 μM CsA	$19 \pm 0.4^{\#}$

Experiments were conducted under the same conditions to those of Fig. 1. The reactions were stopped at 10 min. Values represent average of three independent experiments ± S.D. $^{*}p < 0.05$ when comparing lovastatin with other conditions, except lovastatin plus CsA ($^{\#}p > 0.05$) by ANOVA.

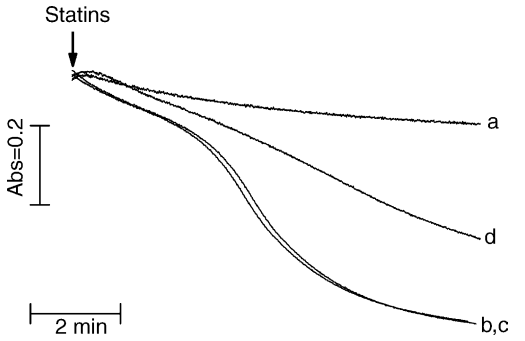

Fig. 7. Comparison of lovastatin, simvastatin, and pravastatin effects on mitochondrial swelling. RLM (0.5 mg/ml) were incubated in the standard medium containing 40 μM lovastatin (line b), simvastatin (line c), or pravastatin (line d). Line a represents control (no additions). The results are representative of six independent experiments. The absorbances at 7 min were: 1.555 ± 0.042 (line a), 1.270 ± 0.020 (line b), 1.017 ± 0.055 (line c) and 1.026 ± 0.060 (line d). Line a is significantly different at $p < 0.001$ from others (b–d).

4. Discussion

The efficacy of statins in lipid lowering and reducing the risk of coronary heart disease is well established. New cholesterol guidelines (Stein, 2002) may lead to the use of statins more frequently and at higher doses. Myopathy, the most common adverse effect of statins is dose-related (Anon., 2004). Although strong epidemiology data are lacking, the incidence of muscular toxicity occurs in 1–7% of statin treated patients (Ucar et al., 2000) and severe myopathy is in the range of 0.1–0.5% with monotherapy (Hodel, 2002); however, signs of systemic oxidation injury (isoprostene levels) can be detected in ~10% of the treated patients (Sinzinger et al., 2002). Statins inhibit synthesis of mevalonate, a precursor of ubiquinone, which functions as an electron carrier in the mitochondrial respiratory chain and act as an important intracellular and systemic antioxidant as well (Hargreaves, 2003). Some studies have reported lowered plasma Coenzyme Q10 levels, the predominant form of ubiquinone in man, in statin treated than in untreated hypercholesterolemic patients (Ghirlanda et al., 1993; De Pinieux et al., 1996). Lower ubiquinone levels have, also, been reported in tissues (liver and heart) of statin treated hamsters (Belichard et al., 1993).

In this work, we used statins in an attempt to correct increased susceptibility to mitochondrial permeability transition observed in hypercholesterolemic mice (Oliveira et al., 2005), but we found that the drug treat-

ment worsened this defect (Fig. 2). The doses of statins used in the present study were just the sufficient to reduce cholesterol in the hypercholesterolemic mice (Bisgaier et al., 1997). However, it should be emphasized that these doses were much higher than those used in humans. In vitro doses were similar to those used by others in isolated cell models (Sirvent et al., 2005; Cafforio et al., 2005; Kaneta et al., 2003; Zhong et al., 2003).

It has been previously reported that statins-induced apoptosis in cultured cells and, in some cases, the apoptosis occurred via the mitochondria pathway (Mutoh et al., 1999; Kaneta et al., 2003; Johnson et al., 2004; Werner et al., 2004). Although this property of statin has been considered as potential anti-cancer therapy (Cafforio et al., 2005), one should consider its actual citotoxic action.

The mechanisms by which mitochondria would be involved in statin-induced cell death are not well clarified. Statin effects in vivo and in situ may be secondary to several metabolic changes in response to the HMG-CoA reductase inhibition. By using isolated mice and rat mitochondria preparations, we demonstrated direct effects of statins on muscle and liver mitochondria. Lovastatin-induced Ca^{2+}-dependent mitochondrial permeability transition in a dose-dependent manner, and this is associated with the oxidation of membrane protein thiol groups, but the exact mechanism of thiol group oxidation is unknown. In experiments using the probe H_2DCF-DA, we did not observe an increase in formation of oxidized DCF in the presence of statin (data not shown). However, the involvement of ROS in lovastatin-induced MPT is clearly demonstrated by the partial inhibition of mitochondrial swelling in presence of catalase. Lovastatin could act indirectly by inhibiting one of the mitochondrial thiol reductase enzymes. Alternatively, lovastatin could stimulate mitochondria associated nitric oxide synthase (NOS), as previously shown for the endothelial NOS (Castro et al., 2004; Yokoyama et al., 2005). In the absence/deficiency of substrates/cofactors, NOS generates superoxide instead of NO (Xia et al., 1996). Hydrophobic statins were more potent than hydrophilic statins in inducing MPT, probably due to different affinities of these compounds to critical binding sites on the inner mitochondrial membrane.

It has been proposed that Ca^{2+} either alters the reactivity of mitochondrial membrane protein thiol groups in the presence of thiol reagents (Bernardes et al., 1994) or directly regulates the MPT pore opening (Zoratti and Szabo, 1995). The central role of calcium is, further, supported by studies showing that statins induce alterations in cellular Ca^{2+} homeostasis. Increased calcium cytosolic concentration has been shown in myoblasts cultured with simvastatin (Nakahara et al., 1994), skeletal muscle of simvastatin treated rats (Pierno et al., 1999) and by acute application of simvastatin on human skeletal muscle fibers (Sirvent et al., 2005). In the latter study, the authors showed that a mitochondrial depolarization and Ca^{2+} efflux preceded a large sarcoplasmic reticulum Ca^{2+} release.

In summary, we demonstrated that statins directly induce mitochondrial membrane permeability transition through promoting calcium dependent oxidation of protein thiol groups. This process is dose-dependent and is more potently triggered by hydrophobic statins. These effects on mitochondria might lead to cell injury or death contributing to the deleterious side effects reported in statin treated patients.

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