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Protective effects of l-carnitine and piracetam against mitochondrial permeability transition and PC3 cell necrosis induced by simvastatin

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1. Introduction

Statins are natural or synthetic compounds which inhibit the conversion of 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) into mevalonic acid, the rate-limiting step in cholesterol synthesis (Endo, 1992). These compounds are widely used in the clinic for the treatment of hypercholesterolemia (Shepherd et al., 1995; Collins et al., 2003) and many studies suggest an antioxidant effect of these compounds when used in therapeutic doses (Cook et al., 1988; Guzman et al., 1993; Bhuiyan and Seccombe, 1995; Hsu et al., 2006). In addition, literature data indicate that statins cause a series of adverse (Sirvent et al., 2005; Kwak et al., 2012) or beneficial (Manfredini et al., 2010; Li et al., 2010) effects that include protection against prostate cancer (Oliveira et al., 2008; Marcella et al., 2011; Mondal et al., 2011).

We have previously reported that, at 10 μM, simvastatin induced mainly apoptosis in PC3 cells, which was prevented by mevalonic acid but not by Cyclosporin A, an inhibitor of the mitochondrial membrane permeability transition (MPT) (Oliveira et al., 2008). At higher concentrations (60 μM), simvastatin induced necrosis, which was prevented by Cyclosporin A but not by mevalonic acid. Cell necrosis was preceded by a threefold increase in cytosolic free Ca2+ and a significant decrease in both respiratory rate and mitochondrial membrane potential. Both mitochondrial dysfunction and necrosis were prevented by Cyclosporin A, indicating that necrosis, in this model, is dependent on MPT (Oliveira et al., 2008).

MPT is a nonselective permeabilization of the inner mitochondrial membrane, promoted by mitochondrial Ca2+ loading (Hunter et al., 1976), changes in membrane structure (Ricchelli et al., 1999; Grijalba et al., 1999) and oxidative stress (Vercesi, 1984; Fagian et al., 1990; Kowaltowski et al., 2001; Figueira et al., in press). A recent study (Vaseva et al., 2012) demonstrated that inhibition of MPT by Cyclosporin A is mediated by the blockade of a p53-Cyclophilin D (CypD) complex formation under oxidative stress-induced necrosis in brain ischemia/reperfusion injury. The authors provided evidence that p53 accumulates in the mitochondrial matrix in response to oxidative stress and triggers permeability transition pore (PTP) opening and necrosis by physical interaction with the PTP regulator CypD. This study confirms the participation of MPT in oxidative stress-induced necrosis in cerebral stroke and other vascular disorders that are leading causes of death (Vaseva et al., 2012). Therefore, targeting PTP may provide avenues for therapeutic protection and/or prevention against cell death that occurs in these pathologies.

A large body of evidences indicate that either l-carnitine or piracetam prevent and/or ameliorate mitochondrial dysfunction caused by a series of conditions either in vivo or in vitro (Moretti et al., 2002; Virmani et al., 2005; Silva-Adaya et al., 2008; Elinos-Calderon et al., 2009; Zhang et al., 2010). Therefore, the aims of the present paper were two: first, to dissect the events leading to simvastatin induced MPT and cell death and second, to better understanding the mechanisms underlie the protection by l-carnitine and piracetam. The results indicate that l-carnitine

1.1. Cell necrosis

Mitochondrial oxidative stress followed by membrane permeability transition (MPT) has been considered as a possible mechanism for statins cytoxicity. Statins use has been associated with reduced risk of cancer incidence, especially prostate cancer. Here we investigated the pathways leading to simvastatin-induced prostate cancer cell death as well as the mechanisms of cell death protection by l-carnitine or piracetam. These compounds are known to prevent and/or protect against cell death mediated by oxidative mitochondrial damage induced by a variety of conditions, either in vivo or in vitro. The results provide evidence that simvastatin induced MPT and cell necrosis were sensitive to either l-carnitine or piracetam in a dose-dependent fashion and mediated by additive mechanisms. When combined, l-carnitine and piracetam acted at concentrations significantly lower than they act individually. These results shed new light into both the cytotoxic mechanisms of statins and the mechanisms underlying the protection against MPT and cell death by the compounds l-carnitine and piracetam.

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2. Material and methods

2.1. Chemicals

Simvastatin (99.5% purity) was purchased from Galena Química e Farmacêutica Ltda (Campinas, SP, Brazil). Piracetam was obtained from BioLab Farmacêutica (São Paulo, SP, Brazil). RPMI 1640 medium and fetal bovine serum (FBS) were obtained from Cultilab (Campinas, SP, Brazil). l-carnitine, Cyclosporin A, carbonyl cyanide p-trifluoromethoxyphenylhydrazone (CCCP), adenosine 5'-diphosphate (ADP), dimethyl sulfoxide (DMSO), propidium iodide and digitonin were obtained from Sigma (ST. Louis, MO, USA). MitoSox was obtained from Life Technologies. All other chemicals were standard commercial products of reagent-grade quality.

2.2. Cell culture and simvastatin treatment

The PC3 human prostate cancer cell line was obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained in RPMI 1640 medium supplemented with 10% FBS and 10 mM (N-[2-hydroxyethyl]piperezine-N-2-[2-ethanesulfonic acid]; HEPES). The cells were maintained at 37 °C in air atmosphere with 5% CO₂. The cultures were maintained below 80% confluence. To evaluate the effects of simvastatin the cells were plated in growth medium containing either 0.1% DMSO or simvastatin dissolved in DMSO (stock solution of 100 mM) and diluted in the culture medium immediately prior to use. When indicated, cyclosporin A, l-carnitine and piracetam were added during the treatments.

2.3. Flow cytometry analysis

Cell death was analyzed by flow cytometry in a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with an argon laser and CellQuest software (version 4.1). Ten thousand events were acquired for each sample. The PC3 population was identified by their light-scattering characteristics, enclosed in electronic gates, and analyzed for the intensity of the fluorescent probe signal, as described below.

2.4. Analysis of cell viability by propidium iodide staining

PC3 were incubated with propidium iodide (PI, 1:50) immediately before analyzing the samples in FACS. Necrosis was quantified as the number of PI positive cells divided by the total number of cells.

2.5. Mitochondrial superoxide generation

For the quantification of mitochondrial superoxide generation, PC3 cells were treated with 6 μM l-carnitine, 6 μM piracetam or 4 μM l-carnitine + 4 μM piracetam in the presence or absence of 60 μM simvastatin for 2 h. Then they were harvested, washed with cold PBS and trypsinized, followed by staining with 3 μM MitoSox at 37 °C for 10 min. The samples were analyzed in the FL-2 channel of the flow cytometer (Payne et al., 2007).

2.6. Determination of mitochondrial membrane potential (ΔΨₘ) in digitonin-permeabilized PC3 Cells

Mitochondrial membrane potential (ΔΨₘ) in digitonin-permeabilized cells was estimated as changes in the fluorescence of safranine O (Figueira et al., 2012), recorded using a spectrofluorometer (Hitachi, model F4500, Tokyo, Japan), operating at excitation and emission wavelengths of 495 and 586 nm, respectively, with slit width of 5 nm. After treatment with simvastatin, PC3 cells (10⁶ cells) were permeabilized with 20 μM digitonin (Campos et al., 2004) in 0.5 ml of standard reaction medium (125 mM sucrose, 65 mM KCl, 10 mM Tris–HCl (pH 7.2), 2.5 mM Na₂HPO₄, 0.33 mM EGTA, 5 mM succinate, and 5 μM safranine O) under constant stirring at 37 °C, in a 0.5 ml thermostatically sealed glass cuvette equipped with a magnetic stirrer.

2.7. Statistical analysis

The results from six independent experiments, except for mitochondrial membrane potential (n = 4) and reactive oxygen species (n = 3) measurements, are presented as means ± SEM. Comparisons between groups were performed using a one-way Analysis of Variance (ANOVA) with Kruskal–Wallis post-hoc analysis. The level of significance was set at P < 0.05.

3. Results

3.1. Either l-carnitine or piracetam protect against PC3 cell death induced by simvastatin

The effects of l-carnitine or piracetam on simvastatin-induced cell death were investigated in PC3 cells treated during 2 h with 60 μM simvastatin (Oliveira et al., 2008). As analyzed by flow cytometry (Fig. 1A and B), this treatment induced significant necrotic cell death. Either l-carnitine (A) or piracetam (B) caused a dose-dependent protection; being 50% inhibition obtained at concentrations in the range of 4 μM of either compound. An almost complete protection was obtained at the concentration of 6 μM of either l-carnitine (A) or piracetam (B).

3.2. l-Carnitine or piracetam decreased the rate of mitochondrial superoxide generation induced by simvastatin in PC3 cells

The effects of l-carnitine or piracetam on the rates of reactive oxygen production by mitochondria were estimated by the MitoSox method in PC3 cells treated or not with simvastatin for 2 h (Fig. 2). Simvastatin increased the rate of superoxide generation by 73% (black bar). Either l-carnitine or piracetam, at 6 μM, significantly decreased the rates of superoxide generation both in the presence or absence of simvastatin. In the presence of simvastatin the inhibition by 6 μM of either l-carnitine or piracetam shifted the rates of superoxide production to the control levels. In addition, Fig. 2 shows that the combination of l-carnitine and piracetam, at the concentrations of 4 μM, also shifted the rates of superoxide production to the levels induced by the individual compounds, at the concentration of 6 μM.

3.3. Cyclosporin A, l-carnitine or piracetam protects PC3 cells against MPT caused by simvastatin

Simvastatin-induced cell death had been associated with MPT opening in PC3 cells (Oliveira et al., 2008). Thus, we investigated whether l-carnitine or piracetam protect against ΔΨₘ decrease that occur prior to necrosis mediated by MPT. PC3 cells were treated with 60 μM simvastatin (Fig. 3) in the presence or absence
of Cyclosporin A, L-carnitine or piracetam. After these treatments cells were washed and placed in the standard reaction medium in the presence of the fluorescent dye safranine O. The permeabilization of the plasma membrane by digitonin allows the entry of safranine O in the cells, which binds to the inner mitochondrial membrane in response to the proton gradient. This decreases the intensity of the fluorescence emitted (Figueira et al., 2012). The addition of CCCP leads to \( \Delta \Psi_m \) elimination and release of safranine O from the membrane, thus allowing for \( \Delta \Psi_m \) estimation. Simvastatin caused a large decrease in \( \Delta \Psi_m \) sensitive to the MPT inhibitor Cyclosporin A (Fig. 3, compare lines simvastatin and simvastatin + Cyclosporin A). Similar to the effects observed with Cyclosporin A, the \( \Delta \Psi_m \) decrease induced by simvastatin was inhibited either by L-carnitine or piracetam alone, or additively by the combination of these compounds (Fig. 3).

### 3.4. The combination of L-carnitine and piracetam has additive effects on the protection against necrosis induced by simvastatin

The dose–response experiments shown in Fig. 1 indicated that, at 4 \( \mu \)M, either L-carnitine or piracetam protected the PC3 cells against necrosis by 50%. In addition the experiments of Fig. 3 indicated that the combination of these compounds have additive effects on the protection against simvastatin induced \( \Delta \Psi_m \) decrease. In order to ascertain the different molecular mechanisms of these compounds on the protection against cell necrosis we used a combination of both, at the concentrations of 20 \( \mu \)M digitonin and 1 \( \mu \)M CCCP. The figure is representative of four independent experiments.

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**Fig. 1.** L-Carnitine or piracetam protect against PC3 cell necrosis induced by simvastatin. PC3 cells were treated with 0.1% DMSO (control) or with 60 \( \mu \)M simvastatin + l-carnitine (A) or 60 \( \mu \)M simvastatin + piracetam (B) for 2 h. After treatment, cells were stained with propidium iodide to estimate dead cells. Values represent the mean ± SEM (\( n = 6 \)). *\( P < 0.05 \) control versus l-carnitine 0–2 \( \mu \)M, *\( P < 0.05 \) control versus l-carnitine 4 \( \mu \)M.

**Fig. 2.** Protection by l-carnitine or piracetam against superoxide generation induced by simvastatin in PC3 cells. Cells were treated with 6 \( \mu \)M l-carnitine or 6 \( \mu \)M piracetam or 4 \( \mu \)M l-carnitine + 4 \( \mu \)M piracetam in the presence (or absence) of 60 \( \mu \)M simvastatin for 2 h. After this treatment they were stained with MitoSox as described in Material and methods. Values represent the mean ± SEM (\( n = 3 \)). *\( P < 0.05 \) control versus l-carnitine 6 \( \mu \)M and Piracetam 6 \( \mu \)M, * \( P < 0.05 \) control versus simvastatin 60 \( \mu \)M.

**Fig. 3.** Protective effects of Cyclosporin A, l-carnitine or piracetam on \( \Delta \Psi_m \) decrease of PC3 cells caused by simvastatin. PC3 cells (1 \( \times 10^6 \) cells) were treated with 0.1% DMSO (control) or with 60 \( \mu \)M simvastatin in the presence (or absence) of 0.5 \( \mu \)M Cyclosporin A, 4 \( \mu \)M l-carnitine, 4 \( \mu \)M piracetam or 4 \( \mu \)M l-carnitine + 4 \( \mu \)M piracetam, as indicated in the figure. After these treatments the cells were washed and resuspended in 0.5 ml of standard reaction medium (125 mM sucrose, 65 mM KCl (pH 7.2), 2.5 mM Na2HPO4, 0.33 mM EGTA, 5 \( \mu \)M safranine O and 5 mM succinate) for \( \Delta \Psi_m \) measurements. The arrows indicate additions of 20 \( \mu \)M digitonin and 1 \( \mu \)M CCCP. The figure is representative of four independent experiments.

**Fig. 4.** Protection by the association of L-carnitine plus piracetam against PC3 cell death. PC3 cells were treated with 4 \( \mu \)M l-carnitine, 4 \( \mu \)M piracetam or 4 \( \mu \)M l-carnitine + 4 \( \mu \)M piracetam in the presence (or absence) of 60 \( \mu \)M simvastatin. Values represent the mean ± SEM (\( n = 6 \)). *\( P < 0.05 \) control versus simvastatin 60 \( \mu \)M.
4. Discussion

The results described above demonstrate that either L-carnitine or piracetam protect against simvastatin-induced PC3 cell death mediated by increased cytosolic Ca\(^{2+}\) concentrations. Considering that calcium is a known signaling agent for mitochondrial reactive oxygen generation, in the present study we investigated whether preventing and/or ameliorating oxidative mitochondrial dysfunction with L-carnitine (Binienda, 2003; Sener et al., 2004; Yaprak et al., 2007; Nishimura et al., 2008; Shen et al., 2008; Elinos-Calderon et al., 2009; Ye et al., 2010; Gavami et al., 2012) or protecting against changes in mitochondrial membrane fluidity by piracetam (Keil et al., 2006) would prevent necrotic cell death induced by toxic concentrations of simvastatin (Oliveira et al., 2008). The results show that the necrotic death of PC3 cells was inhibited in a dose-dependent manner by either L-carnitine or piracetam (Fig. 1). The additive effects of these compounds confirmed their concerted actions through different mechanisms leading to the same final effect, that is, a decreased mitochondrial overproduction of the superoxide anion induced by simvastatin (Fig. 2). Similar results were also obtained with the primary fibroblasts cell line GN16 obtained from normal gengival biopsy and the non-tumorigenic immortalized cell line HaCaT, derived from human keratinocytes (Costa, R.A.P., Fernandes, M.P. and Vercesi, A.E., unpublished results).

Literature data indicate that, at the concentrations used here, statins promote cell death mediated by mitochondrial dysfunction associated with alterations in calcium homeostasis (Sirvent et al., 2005), inhibition of beta-oxidation (Kauffmann et al., 2006) and partial inhibition of respiration (Kwak et al., 2012). Interestingly, all these alterations stimulate reactive oxygen generation by mitochondria suggesting the notion that oxidative stress is a common denominator in statins toxicity. Therefore, we next discuss the possible mechanisms underlying the protective effects of L-carnitine and piracetam on the simvastatin effects described above such as cytosolic Ca\(^{2+}\) increase, MPT and reactive oxygen generation.

As it might occur in some degree in our experiments, L-carnitine has been shown to stimulate beta-oxidation by increasing the mRNA expression of carnitine palmitoyltransferase 1A. This prevented free fatty acid induced oxidative stress and reduced hepatotoxicity either in vitro or in vivo by enhancing mitochondrial function (Jun et al., 2011). Indeed, the property of free fatty acids as mitochondrial oxidative damaging agents are very well documented either in vivo (Madesh and Balasubramanian, 1997; Ghosh et al., 2006; Tonin et al., 2010; Ruiz-Ramirez et al., 2011) or in vitro (Yao et al., 2005; Zhou et al., 2009; Barazzoni et al., 2012).

With respect to the effect of statins on the mitochondrial electron transport chain, current literature data indicate that like simvastatin (Sirvent et al., 2005), the metabolic inhibitors 3-nitropropionic acid (3-NTA) (Solesio et al., 2012), rotenone (Panov et al., 2005) and 1-methyl-4-phenylpyridinium (MPP\(^{+}\)) (Brill and Bennett, 2003) inhibit electrons transport, at the levels of Complexes I or II (Virmani et al., 2004, 2005; Binienda et al., 2005; Panov et al., 2005; Vamos et al., 2010). This causes damage to Complex I and II, presumably at the level of 4Fe–4S clusters (Panov et al., 2005). These alterations diminish resistance to Ca\(^{2+}\) thus increasing probability of MPT and necrotic cell death (Panov et al., 2005; Vaseva et al., 2012). Since L-carnitine plays significant neuroprotective effects against all these conditions (Virmani et al., 2002, 2003) we propose that L-carnitine, in addition to its metabolic role, may directly interact with 4Fe–4S clusters decreasing the superoxide radical generation. In this respect, it is worthy to mention that L-carnitine has also the ability to bind Fe\(^{2+}\) (Gulcin, 2006) a transition metal, supposed to participate in the mitochondrial oxidative stress that leads to MPT (Castillo et al., 1995).

Concerning the molecular mechanisms of piracetam protection against simvastatin toxicity (Fig. 1B), we may consider that piracetam protects against changes in membrane fluidity via unspecific interactions with the polar head groups of the cellular membranes (Keil et al., 2006). In this regard, we have previously demonstrated that Ca\(^{2+}\) binding to inner membrane cardioliopins causes alterations in membrane lipid organization that result in a burst of mitochondrial superoxide generation that triggers MPT (Grijalba et al., 1999). Therefore, it can be proposed that inhibition of simvastatin-induced MPT and necrosis by piracetam is mediated by membrane stabilization that minimizes Ca\(^{2+}\) stimulated superoxide production (Fig. 2).

Having investigated PC3 toxicity of statins in vitro and the protection by L-carnitine and piracetam, the question arises to what extent our findings are relevant for the in vivo situation. The plasma concentrations of statins after oral doses of 20–40 mg are around 0.1 \(\mu\)mol/L (Kauffmann et al., 2006) much lower than the toxic concentrations used in our assays. However, the lipophilic nature of simvastatin, presumably, favors its accumulation into the cells, as a function of time, rendering it difficult to estimate its effective concentrations in the mitochondrial environment.

Taken together, our investigations do not offer an explanation for in vivo toxicity of simvastatin. Rather, the findings are more relevant with respect to the understanding of MPT mechanisms and the effects of L-carnitine, piracetam and their combination on the protection against necrosis, either in tumor or non-tumorigenic cells. According mounting evidences accumulated during recent years (Lemasters et al., 2009; Rosala et al., 2010; Toman and Fiskum, 2011; Figueira et al., in press; Kim et al., 2012) MPT-induced necrosis underlies the pathogenesis of leading causes of human death such as cerebral stroke and other vascular diseases (Vaseva et al., 2012).

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