European Journal of Pharmacology 701 (2013) 82–86

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Molecular and cellular pharmacology

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

Rute A.P. Costa [a], Mariana P. Fernandes [a], Nadja C. de Souza-Pinto [b], Aníbal E. Vercesi [a,*]

[a] Departamento de Patologia Clínica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas (UNICAMP), CEP: 13083-887, Campinas, São Paulo, Brazil
[b] Departamento de Bioquímica, Instituo de Química, Universidade de São Paulo (USP), São Paulo, Brazil

ARTICLE INFO

Article history:
Received 29 August 2012
Received in revised form
20 December 2012
Accepted 8 January 2013
Available online 17 January 2013

Keywords:
Cell necrosis
Mitochondrial permeability transition
L-Carnitine
Piracetam
Simvastatin

ABSTRACT

Mitochondrial oxidative stress followed by membrane permeability transition (MPT) has been considered as a possible mechanism for statins cytotoxicity. 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.

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, 1996; 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; Mondul 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 Ca^{2+} 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 Ca^{2+} 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

* Corresponding author. Tel: +55 19 35217370, fax: +55 19 35217330.
E-mail address: anibal@unicamp.br (A.E. Vercesi).

0014-2999/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ejphar.2013.01.001

or piracetam, either alone or in combination, prevents simvastatin induced superoxide generation thus protecting against MPT and cell necrosis.

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]piperazine-*N*,-[2-ethanesulfonic acid]; HEPES). The cells were maintained at 37 °C in air atmosphere with 5% CO_2. 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 also 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 ($\Delta\Psi_m$) in digitonin-permeabilized PC3 Cells

Mitochondrial membrane potential ($\Delta\Psi_m$) 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 (10^6 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 Na_2HPO_4, 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's 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. 1 A 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 piracetan, 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 piracetan 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 $\Delta\Psi_m$ decrease that occur prior to necrosis mediated by MPT. PC3 cells were treated with 60 μM simvastatin (Fig. 3) in the presence or absence

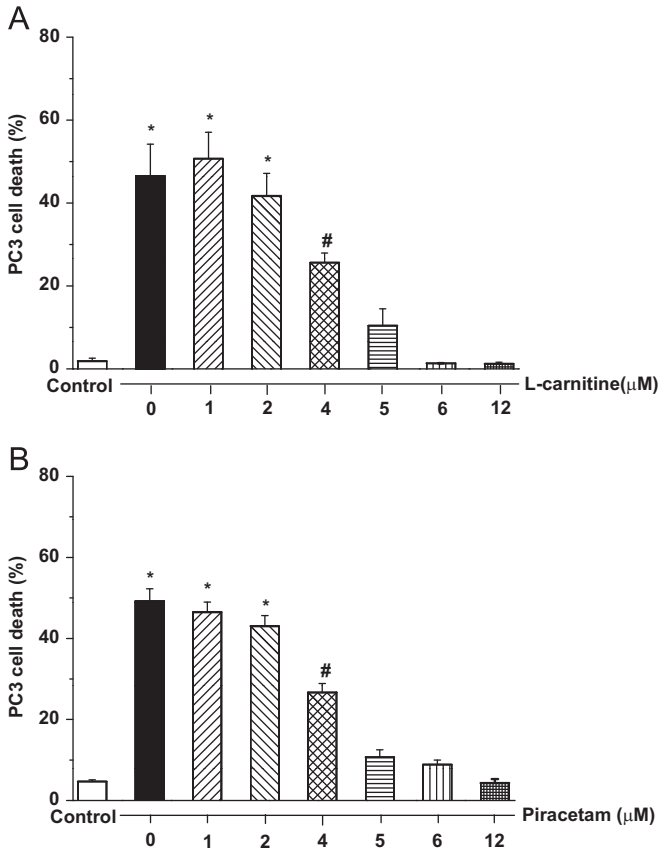

Fig. 1. L-Carnitine or piracetam protect against PC3 cell necrosis induced by simvastatin. PC3 cells were treated with 0.1% DMSO (control) or with 60 μM simvastatin+L-carnitine (A) or 60 μM simvastatin+piracetam (B) for 2 h. After treatment, cells were stained with propidium iodide to estimate dead cells.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

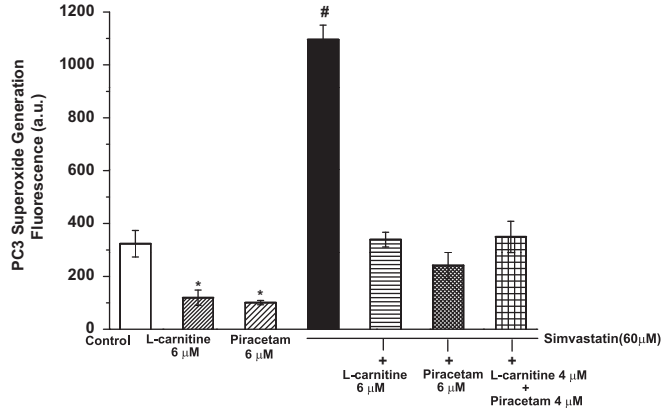

Fig. 2. Protection by L-carnitine or piracetam against superoxide generation induced by simvastatin in PC3 cells. Cells were treated with 6 μM L-carnitine or 6 μM piracetam or 4 μM L-carnitine+4 μM piracetam in the presence (or absence) of 60 μM simvastatin for 2 h. After this treatment they were stained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

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

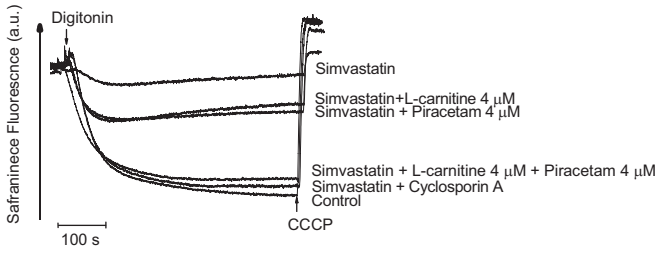

Fig. 3. Protective effects of Cyclosporin A, L-carnitine or piracetam on $\Delta\Psi_m$ decrease of PC3 cells caused by simvastatin. PC3 cells (1×10^6 cells) were treated with 0.1% DMSO (control) or with 60 μM simvastatin in the presence (or absence) of 0.5 μM Cyclosporin A, 4 μM L-carnitine, 4 μM piracetam or 4 μM L-carnitine+4 μM 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 Na_2HPO_4, 0.33 mM EGTA, 5 μM safranine *O* and 5 mM succinate) for $\Delta\Psi_m$ measurements. The *arrows* indicate additions of 20 μM digitnonin and 1 μM CCCP. The figure is representative of four independent experiments.

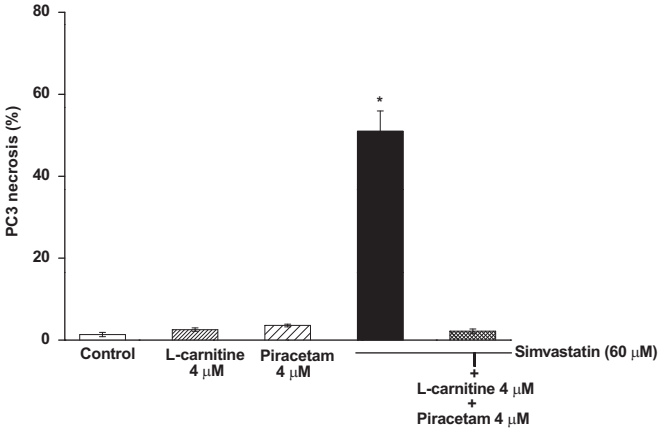

Fig. 4. Protection by the association of L-carnitine plus piracetam against PC3 cell death. PC3 cells were treated with 4 μM L-carnitine, 4 μM piracetam or 4 μM L-carnitine+4 μM piracetam in the presence (or absence) of 60 μM simvastatin. Values represent the mean ± SEM ($n=6$). $^{*}P<0.05$ control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

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 simivastatin+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 μM, 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 4 μM. Fig. 4 shows that the association of these compounds increased their protective effects against cell necrosis from 50% to about 100%.

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; Yapar et al., 2007; Nishimura et al., 2008; Shen et al., 2008; Elinos-Calderon et al., 2009; Ye et al., 2010; Ghavami 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 (Kaufmann et al., 2006) and partial inhibition of respiration (Kwak et al., 2012). Interestingly, all these alterations stimulate reactive oxygen generation by mitochondria supporting 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 (Castilho 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 cardiolipins 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 μmol/L (Kaufmann 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; Rasola 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).

Acknowledgments

The authors would like to thank Edilene S. Santos for technical assistance. This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Biolab Sanus Farmacêutica Ltda (convênio Biolab/UNICAMP, n° 4198, 2009), Instituto Nacional de Ciência e Tecnologia (INCT) em Diabetes e Obesidade (CNPq/FAPESP) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

References

Barazzoni, R., Zanetti, M., Cappellari, G.G., Semolic, A., Boschelle, M., Codarin, E., Pirulli, A., Cattin, L., Guarnieri, G., 2012. Fatty acids acutely enhance insulin-induced oxidative stress and cause insulin resistance by increasing mitochondrial reactive oxygen species (ROS) generation and nuclear factor-kappaB inhibitor (IkappaB)-nuclear factor-kappaB (NFkappaB) activation in rat muscle, in the absence of mitochondrial dysfunction. Diabetologia 55, 773–782.

Bhuiyan, J., Seccombe, D.W., 1996. The effects of 3-hydroxy-3-methylglutaryl-CoA reductase inhibition on tissue levels of carnitine and carnitine acyltransferase activity in the rabbit. Lipids 31, 867–870.

Binienda, Z., Przybyla-Zawislak, B., Virmani, A., Schmued, L., 2005. L-Carnitine and neuroprotection in the animal model of mitochondrial dysfunction. Ann. NY Acad. Sci. 1053, 174–182.

Binienda, Z.K., 2003. Neuroprotective effects of L-carnitine in induced mitochondrial dysfunction. Ann. NY Acad. Sci. 993 (289–295), 289–345 (discussion).

Brill 2nd, L.B., Bennett Jr., J.P., 2003. Dependence on electron transport chain function and intracellular signaling of genomic responses in SH-SY5Y cells to the mitochondrial neurotoxin MPP(+). Exp. Neurol. 181, 25–38.

Campos, C.B., Degasperi, G.R., Pacifico, D.S., Alberici, L.C., Carreira, R.S., Guimaraes, F., Castilho, R.F., Vercesi, A.E., 2004. Ibuprofen-induced Walker 256 tumor cell death: cytochrome c release from functional mitochondria and enhancement by calcineurin inhibition. Biochem. Pharmacol. 68, 2197–2206.

Castilho, R.F., Kowaltowski, A.J., Meinicke, A.R., Vercesi, A.E., 1995. Oxidative damage of mitochondria induced by Fe(II)citrate or t-butyl hydroperoxide in the presence of Ca^{2+}: effect of coenzyme Q redox state. Free Radical Biol. Med. 18, 55–59.

Collins, R., Armitage, J., Parish, S., Sleigh, P., Peto, R., 2003. MRC/BHF Heart Protection Study of cholesterol-lowering with simvastatin in 5963 people with diabetes: a randomised placebo-controlled trial. Lancet 361, 2005–2016.

Cook, G.A., Khan, B., Heimberg, M., 1988. Feeding of lovastatin to rats increases the activity of the hepatic mitochondrial outer carnitine palmitoyltransferase. Biochem. Biophys. Res. Commun. 150, 1077–1082.

Elinos-Calderon, D., Robledo-Arratia, Y., Perez-De La Cruz, V., Pedraza-Chaverri, J., Ali, S.F., Santamaria, A., 2009. Early nerve ending rescue from oxidative damage and energy failure by L: -carnitine as post-treatment in two neurotoxic models in rat: recovery of antioxidant and reductive capacities. Exp. Brain Res. 197, 287–296.

Endo, A., 1992. The discovery and development of HMG-CoA reductase inhibitors. J. Lipid Res. 33, 1569–1582.

Fagian, M.M., Pereira-da-Silva, L., Martins, I.S., Vercesi, A.E., 1990. Membrane protein thiol cross-linking associated with the permeabilization of the inner mitochondrial membrane by Ca^{2+} plus prooxidants. J. Biol. Chem. 265, 19955–19960.

Figueira, T.R., Barros, M.H., Camargo. A.A., Castilho, R.F., Ferreira, J.C., Kowaltowski, A.J., Sluse, F.E., Souza-Pinto, N.C., Vercesi, A.E. Mitochondria as a source of reactiveoxygen and nitrogen species: from molecular mechanisms to human health. Antioxid. Redox. Signal, http://dx.doi.org/10.1089/ars.2012.4729, in press.

Figueira, T.R., Melo, D.R., Vercesi, A.E., Castilho, R.F., 2012. Safranine as a fluorescent probe for the evaluation of mitochondrial membrane potential in isolated organelles and permeabilized cells. Methods Mol. Biol. 810, 103–117.

Ghavami, S., Yeganeh, B., Stelmack, G.L., Kashani, H.H., Sharma, P., Cunnington, R., Rattan, S., Bathe, K., Klonisch, T., Dixon, I.M., Freed, D.H., Halayko, A.J., 2012. Apoptosis, autophagy and ER stress in mevalonate cascade inhibition-induced cell death of human atrial fibroblasts. Cell Death Dis. 3, e330.

Ghosh, S., Kewalramani, G., Yuen, G., Pulinilkunnil, T., An, D., Innis, S.M., Allard, M.F., Wambolt, R.B., Qi, D., Abrahani, A., Rodrigues, B., 2006. Induction of mitochondrial nitrative damage and cardiac dysfunction by chronic provision of dietary omega-6 polyunsaturated fatty acids. Free Radical Biol. Med. 41, 1413–1424.

Grijalba, M.T., Vercesi, A.E., Schreier, S., 1999. Ca^{2+}-induced increased lipid packing and domain formation in submitochondrial particles. A possible early step in the mechanism of Ca^{2+}-stimulated generation of reactive oxygen species by the respiratory chain. Biochemistry 38, 13279–13287.

Gulcin, I., 2006. Antioxidant and antiradical activities of L-carnitine. Life Sci. 78, 803–811.

Guzman, M., Cortes, J.P., Castro, J., 1993. Effects of lovastatin on hepatic fatty acid metabolism. Lipids 28, 1087–1093.

Hsu, M., Muchova, L., Morioka, I., Wong, R.J., Schroder, H., Stevenson, D.K., 2006. Tissue-specific effects of statins on the expression of heme oxygenase-1 in vivo. Biochem. Biophys. Res. Commun. 343, 738–744.

Hunter, D.R., Haworth, R.A., Southard, J.H., 1976. Relationship between configuration, function, and permeability in calcium-treated mitochondria. J. Biol. Chem. 251, 5069–5077.

Jun, D.W., Cho, W.K., Jun, J.H., Kwon, H.J., Jang, K.S., Kim, H.J., Jeon, H.J., Lee, K.N., Lee, H.L., Lee, O.Y., Yoon, B.C., Choi, H.S., Hahm, J.S., Lee, M.H., 2011. Prevention of free fatty acid-induced hepatic lipotoxicity by carnitine via reversal of mitochondrial dysfunction. Liver Int. 31, 1315–1324.

Kaufmann, P., Torok, M., Zahno, A., Waldhauser, K.M., Brecht, K., Krahenbuhl, S., 2006. Toxicity of statins on rat skeletal muscle mitochondria. Cell Mol. Life Sci. 63, 2415–2425.

Keil, U., Scherping, I., Hauptmann, S., Schuessel, K., Eckert, A., Muller, W.E., 2006. Piracetam improves mitochondrial dysfunction following oxidative stress. Br. J. Pharmacol. 147, 199–208.

Kim, J.S., Wang, J.H., Lemasters, J.J., 2012. Mitochondrial permeability transition in rat hepatocytes after anoxia/reoxygenation: role of Ca^{2+}-dependent mitochondrial formation of reactive oxygen species. Am. J. Physiol. Gastrointest. Liver Physiol. 302, G723–G731.

Kowaltowski, A.J., Castilho, R.F., Vercesi, A.E., 2001. Mitochondrial permeability transition and oxidative stress. FEBS Lett. 495, 12–15.

Kwak, H.B., Thalacker-Mercer, A., Anderson, E.J., Lin, C.T., Kane, D.A., Lee, N.S., Cortright, R.N., Bamman, M.M., Neufer, P.D., 2012. Simvastatin impairs ADP-stimulated respiration and increases mitochondrial oxidative stress in primary human skeletal myotubes. Free Radical Biol. Med. 52, 198–207.

Lemasters, J.J., Theruvath, T.P., Zhong, Z., Nieminen, A.L., 2009. Mitochondrial calcium and the permeability transition in cell death. Biochim. Biophys. Acta 1787, 1395–1401.

Li, J., Sun, Y.M., Wang, L.F., Li, Z.Q., Pan, W., Cao, H.Y., 2010. Comparison of effects of simvastatin versus atorvastatin on oxidative stress in patients with coronary heart disease. Clin. Cardiol. 33, 222–227.

Madesh, M., Balasubramanian, K.A., 1997. Activation of liver mitochondrial phospholipase A2 by superoxide. Arch. Biochem. Biophys. 346, 187–192.

Manfredini, V., Biancini, G.B., Vanzin, C.S., Dal Vesco, A.M., Cipriani, F., Biasi, L., Tremea, R., Deon, M., Peralba Mdo, C., Wajner, M., Vargas, C.R., 2010. Simvastatin treatment prevents oxidative damage to DNA in whole blood leukocytes of dyslipidemic type 2 diabetic patients. Cell Biochem. Funct. 28, 360–366.

Marcella, S.W., David, A., Ohman-Strickland, P.A., Carson, J., Rhoads, G.G., 2011. Statin use and fatal prostate cancer: a matched case-control study. Cancer 118, 4046–4052.

Mondul, A.M., Weinstein, S.J., Virtamo, J., Albanes, D., 2011. Serum total and HDL cholesterol and risk of prostate cancer. Cancer Causes Control 22, 1545–1552.

Moretti, S., Famularo, G., Marcellini, S., Boschini, A., Santini, G., Trinchieri, V., Lucci, L., Alesse, E., De Simone, C., 2002. L-Carnitine reduces lymphocyte apoptosis and oxidant stress in HIV-1-infected subjects treated with zidovudine and didanosine. Antioxid. Redox. Signal 4, 391–403.

Nishimura, M., Okimura, Y., Fujita, H., Yano, H., Lee, J., Suzaki, E., Inoue, M., Utsumi, K., Sasaki, J., 2008. Mechanism of 3-nitropropionic acid-induced membrane permeability transition of isolated mitochondria and its suppression by L-carnitine. Cell Biochem. Funct. 26, 881–891.

Oliveira, K.A., Zecchin, K.G., Alberici, L.C., Castilho, R.F., Vercesi, A.E., 2008. Simvastatin inducing PC3 prostate cancer cell necrosis mediated by calcineurin and mitochondrial dysfunction. J. Bioenerg. Biomembr. 40, 307–314.

Panov, A., Dikalov, S., Shalbuyeva, N., Taylor, G., Sherer, T., Greenamyre, J.T., 2005. Rotenone model of Parkinson disease: multiple brain mitochondria dysfunctions after short term systemic rotenone intoxication. J. Biol. Chem. 280, 42026–42035.

Payne, C.M., Weber, C., Crowley-Skillicorn, C., Dvorak, K., Bernstein, H., Bernstein, C., Holubec, H., Dvorakova, B., Garewal, H., 2007. Deoxycholate induces mitochondrial oxidative stress and activates NF-kappaB through multiple mechanisms in HCT-116 colon epithelial cells. Carcinogenesis 28, 215–222.

Rasola, A., Sciacovelli, M., Pantic, B., Bernardi, P., 2010. Signal transduction to the permeability transition pore. FEBS Lett 584, 1989–1996.

Ricchelli, F., Barbato, P., Milani, M., Gobbo, S., Salet, C., Moreno, G., 1999. Photodynamic action of porphyrin on Ca^{2+} influx in endoplasmic reticulum: a comparison with mitochondria. Biochem. J. 338, 221–227, Pt 1.

Ruiz-Ramirez, A., Chavez-Salgado, M., Peneda-Flores, J.A., Zapata, E., Masso, F., El-Hafidi, M., 2011. High-sucrose diet increases ROS generation, FFA accumulation, UCP2 level, and proton leak in liver mitochondria. Am. J. Physiol. Endocrinol. Metab. 301, E1198–E1207.

Sener, G., Paskaloglu, K., Satiroglu, H., Alican, I., Kacmaz, A., Sakarcan, A., 2004. L-Carnitine ameliorates oxidative damage due to chronic renal failure in rats. J. Cardiovasc. Pharmacol. 43, 698–705.

Shen, W., Liu, K., Tian, C., Yang, L., Li, X., Ren, J., Packer, L., Head, E., Sharman, E., Liu, J., 2008. Protective effects of R-alpha-lipoic acid and acetyl-L-carnitine in MIN6 and isolated rat islet cells chronically exposed to oleic acid. J. Cell Biochem. 104, 1232–1243.

Shepherd, J., Cobbe, S.M., Ford, I., Isles, C.G., Lorimer, A.R., MacFarlane, P.W., McKillop, J.H., Packard, C.J., 1995. Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. West of Scotland Coronary Prevention Study Group. N. Engl. J. Med. 333, 1301–1307.

Silva-Adaya, D., Perez-De La Cruz, V., Herrera-Mundo, M.N., Mendoza-Macedo, K., Villeda-Hernandez, J., Binienda, Z., Ali, S.F., Santamaria, A., 2008. Excitotoxic damage, disrupted energy metabolism, and oxidative stress in the rat brain: antioxidant and neuroprotective effects of L-carnitine. J. Neurochem. 105, 677–689.

Sirvent, P., Mercier, J., Vassort, G., Lacampagne, A., 2005. Simvastatin triggers mitochondria-induced Ca^{2+} signaling alteration in skeletal muscle. Biochem. Biophys. Res. Commun. 329, 1067–1075.

Solesio, M.E., Saez-Atienzar, S., Jordan, J., Galindo, M.F., 2012. Nitropropionioc acid induces autophagy by mitochondrial permeability transition pore formation rather than activation of the mitochondrial fission pathway. Br. J. Pharmacol..

Toman, J., Fiskum, G., 2011. Influence of aging on membrane permeability transition in brain mitochondria. J. Bioenerg. Biomembr. 43, 3–10.

Tonin, A.M., Grings, M., Busanello, E.N., Moura, A.P., Ferreira, G.C., Viegas, C.M., Fernandes, C.G., Schuck, P.F., Wajner, M., 2010. Long-chain 3-hydroxy fatty acids accumulating in LCHAD and MTP deficiencies induce oxidative stress in rat brain. Neurochem. Int. 56, 930–936.

Vamos, E., Voros, K., Vecsei, L., Klivenyi, P., 2010. Neuroprotective effects of L-carnitine in a transgenic animal model of Huntington's disease. Biomed. Pharmacother. 64, 282–286.

Vaseva, A.V., Marchenko, N.D., Ji, K., Tsirka, S.E., Holzmann, S., Moll, U.M., 2012. p53 opens the mitochondrial permeability transition pore to trigger Necrosis. Cell 149, 1536–1548.

Vercesi, A.E., 1984. Dissociation of NAD(P)+-stimulated mitochondrial Ca^{2+} efflux from swelling and membrane damage. Arch. Biochem. Biophys. 232, 86–91.

Virmani, A., Gaetani, F., Binienda, Z., 2005. Effects of metabolic modifiers such as carnitines, coenzyme Q10, and PUFAs against different forms of neurotoxic insults: metabolic inhibitors, MPTP, and methamphetamine. Ann. NY Acad. Sci. 1053, 183–191.

Virmani, A., Gaetani, F., Binienda, Z., Xu, A., Duhart, H., Ali, S.F., 2004. Role of mitochondrial dysfunction in neurotoxicity of MPP+: partial protection of PC12 cells by acetyl-L-carnitine. Ann. NY Acad. Sci. 1025, 267–273.

Virmani, A., Gaetani, F., Imam, S., Binienda, Z., Ali, S., 2002. The protective role of L-carnitine against neurotoxicity evoked by drug of abuse, methamphetamine, could be related to mitochondrial dysfunction. Ann. NY Acad. Sci. 965, 225–232.

Virmani, A., Gaetani, F., Imam, S., Binienda, Z., Ali, S., 2003. Possible mechanism for the neuroprotective effects of L-carnitine on methamphetamine-evoked neurotoxicity. Ann. NY Acad. Sci. 993 (197–207), 198–287 (Discussion).

Yao, D., Shi, W., Gou, Y., Zhou, X., Yee, T., Zhou, Y., Liu, Z., 2005. Fatty acid-mediated intracellular iron translocation: a synergistic mechanism of oxidative injury. Free Radical Biol. Med. 39, 1385–1398.

Yapar, K., Kart, A., Karapehlivan, M., Atakisi, O., Tunca, R., Erginsoy, S., Citil, M., 2007. Hepatoprotective effect of L-carnitine against acute acetaminophen toxicity in mice. Exp. Toxicol. Pathol. 59, 121–128.

Ye, J., Li, J., Yu, Y., Wei, Q., Deng, W., Yu, L., 2010. L-Carnitine attenuates oxidant injury in HK-2 cells via ROS-mitochondria pathway. Regul. Pept. 161, 58–66.

Zhang, H., Jia, H., Liu, J., Ao, N., Yan, B., Shen, W., Wang, X., Li, X., Luo, C., 2010. Combined R-alpha-lipoic acid and acetyl-L-carnitine exerts efficient preventative effects in a cellular model of Parkinson's disease. J. Cell Mol. Med. 14, 215–225.

Zhou, H., Liu, X., Liu, L., Yang, Z., Zhang, S., Tang, M., Tang, Y., Dong, Q., Hu, R., 2009. Oxidative stress and apoptosis of human brain microvascular endothelial cells induced by free fatty acids. J. Int. Med. Res. 37, 1897–1903.