J Bioenerg Biomembr (2008) 40:307–314
DOI 10.1007/s10863-008-9155-9

Simvastatin inducing PC3 prostate cancer cell necrosis mediated by calcineurin and mitochondrial dysfunction

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Received: 5 June 2008 / Accepted: 2 July 2008 / Published online: 5 August 2008

Abstract In the present study we analyzed the mechanisms of simvastatin toxicity for the PC3 human prostate cancer cell line. At 10 μM, simvastatin induced principally apoptosis, which was prevented by mevalonic acid but not by cyclosporin A, the inhibitor of calcineurin and mitochondrial permeability transition (MPT). At 60 μM, simvastatin induced the necrosis of PC3 cells insensitive to mevalonic acid. Cell necrosis was preceded by a threefold increase in cytosolic free Ca^{2+} concentration and a significant decrease in both respiration rate and mitochondrial membrane potential. Both mitochondrial dysfunction and necrosis were sensitive to the compounds cyclosporin A and bongkrekic acid, as well as the calcineurin inhibitor FK506. We have concluded that simvastatin-induced PC3 cells apoptosis is dependent on 3-hydroxy-3-methylglutaryl coenzyme-A reductase inhibition and independent of MPT, whereas necrosis is dependent on mitochondrial dysfunction caused, at least in part, by calcineurin.

Keywords Statin · Mitochondrial permeability transition · Intracellular calcium homeostasis · Apoptosis · Cell death

Introduction

Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase, which catalyzes the rate-limiting step in cholesterol synthesis. These compounds are widely used in the treatment of hypercholesterolemia (Shepherd et al. 1995; Collins et al. 2003). The beneficial effects of statin therapy do not seem to be limited to patients with hypercholesterolemia, as increasing evidence suggests that statins may be useful in the prevention and/or treatment of cancer. Indeed a large study of the effects of statins on the risks of prostate cancer have shown a significantly reduced risk of developing advanced prostate cancer (especially metastatic or fatal) in comparison to nondrug users (Platz et al. 2006).

The anticancer effects of the statins are still incompletely characterized, and the mechanisms responsible for these effects can vary, depending on the specific type of cancer (Demierre et al. 2005). Some studies suggest that the ability of statins to reduce cholesterol levels and inhibit reactions in the mevalonic acid pathway may be associated with their antiproliferative, proapoptotic and antimetastatic effects (Hindler et al. 2006). However, some of the effects of the statins are not clearly related to the inhibition of the HMG-CoA reductase and need to be clarified (Demierre et al. 2005).

Recent results from our laboratory have demonstrated that liver mitochondria isolated from hypercholesterolemic LDL receptor knockout mice treated with statins are more susceptible to Ca^{2+}-induced mitochondrial permeability transition (MPT) than are liver mitochondria from control mice (Velho et al. 2006). MPT is a nonselective permeabilization of the inner mitochondrial membrane, typically promoted by an excessive accumulation of Ca^{2+} ions (Gunter and Gunter 1994) and oxidative stress (Kowaltowski et al. 2001). It may be implicated in either necrosis or apoptosis, depending on the pathological situations (Vercesi et al. 2006).

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In the present study, we analyzed the possible involvement of MPT in the death induced by the hydrophobic statin, simvastatin, in the androgen-independent PC3 human prostate cancer cell line. The results indicate that the statin can induce both apoptosis and necrosis in a dose- and time-dependent manner. Apoptosis was dependent on the inhibition of mevalonic acid biosynthesis while necrosis was mediated by MPT and dependent on calcineurin, a calcium-phosphatase reported to lead to cell death in different cell types (Ankarcrona et al. 1996; Wang et al. 1999; Springer et al. 2000; Zecchin et al. 2007; Hara and Snyder 2007).

Material and methods

Chemicals

Simvastatin (99.5% purity) was purchased from Galena Química e Farmacêutica Ltda (Campinas, SP, Brazil). RPMI 1640 medium and fetal bovine serum (FBS) were obtained from Cultilab (Campinas, SP, Brazil), and Annexin V-FITC from the Laboratory of Immunology, Universidade de São Paulo (São Paulo, SP, Brazil). 1,2-bis (2-aminophenoxy) ethane *N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA)-AM, Fluo3-AM and pluronic acid were obtained from Molecular Probes (Eugene, OR, USA), while cyclosporin A, carbonyl cyanide *p*-(trifluo-methoxy) phenylhydrazone (FCCP), adenosine 5-triphosphate (ATP), dimethyl sulfoxide (DMSO), propidium iodide, mevalonic acid, bongkrekic acid and digitonin were obtained from Sigma (St. Louis, MO, USA). All other chemicals were standard commercial products of reagent-grade quality.

PC3 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) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The cell density of the cultures was routinely maintained below 80% confluence. To evaluate the effects of simvastatin, the cells were plated in cell culture dishes with supplemented RPMI 1640 medium for 24 h. The medium was then replaced with a medium supplemented with 1% FBS and 10 mM HEPES, containing either 0.1% DMSO or simvastatin dissolved in DMSO (stock solution of 100 mM) and diluted in the medium before each experiment. Mevalonic acid, cyclosporin A, FK506, bongkrekic acid and BAPTA-AM were also added during some of the experiments to challenge the effectiveness of statin. In the treatments with cyclosporin A, the PC3 cells were pretreated with this compound for 30 min prior to treatment with simvastatin. For BAPTA-AM loading, the PC3 cells were pretreated with this compound for 40 min and washed prior to treatment with simvastatin.

Trypan blue dye exclusion assay

To determine the antiproliferative and cytotoxic effects of simvastatin, cells were stained with 0.1% trypan blue after incubation with different doses of this statin. The total number of cells was counted using a Neubauer chamber, and viability was determined by exclusion of cells marked by trypan blue and those presenting apoptotic morphology (apoptotic bodies and cell shrinkage).

Flow cytometry analysis

The samples were analyzed 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.

Analysis of cell viability by annexin V-FITC and propidium iodide staining

PC3 cells were labeled with annexin V-FITC following the manufacturer's instructions. Briefly, 10^6 cells were harvested at each point in time, washed with PBS and resuspended in a binding buffer (10 mM HEPES (pH7.4), 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$) containing annexin V-FITC (1:500). After 20 min of incubation in the dark at room temperature, cells were also stained with propidium iodide (PI, 1:50). Apoptosis was quantified by FACS analysis as the number of annexin V-FITC positive and PI negative cells divided by the total number of cells, while necrosis was quantified as the number of PI positive cells divided by the total number of cells. Most of the PI positive cells (>96%) did not appear positive for annexin V-FITC.

Measurement of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$)

PC3 cells (10^6 cells) were loaded with 3 μM Fluo3-AM fluorescent probe in the presence of 1 μM pluronic acid and 30 μg/ml BSA at 37°C in a humidified atmosphere of 5% CO_2 for 40 min. Non hydrolyzed Fluo3-AM was removed by washing the cells in their respective medium prior to

acquisition of fluorescence by flow cytometry (Degasperi et al. 2006).

Determination of mitochondrial membrane potential ($\Delta\Psi_m$) in digitonin-permeabilized PC3 cells

$\Delta\Psi_m$ in digitonin-permeabilized cells was estimated as changes in the fluorescence of safranine O (Holden and Sze 1989), recorded using a spectrofluorometer (Hitachi, model F4500, Tokyo, Japan) operating at excitation and emission wavelengths of 495 and 586 nm, respectively, with slits widths 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, and the total potential was determined after the addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP; 1 μM).

Determination of oxygen consumption

Oxygen consumption of PC3 cells (2×10^6 cells/ml) was measured using a Clark-type electrode (Hansatech Instruments Limited, Norfolk, UK) in standard reaction medium (described above) at 37°C, in a 0.5 ml thermostatically-sealed glass cuvette equipped with a magnetic stirrer.

Statistical analysis

The results from at least three independent experiments performed in duplicate are displayed as means ± SEM. Comparisons between groups were performed using a one-way Analysis of Variance (ANOVA) with Tukey's post-hoc analysis. The level of significance was set at $p < 0.05$.

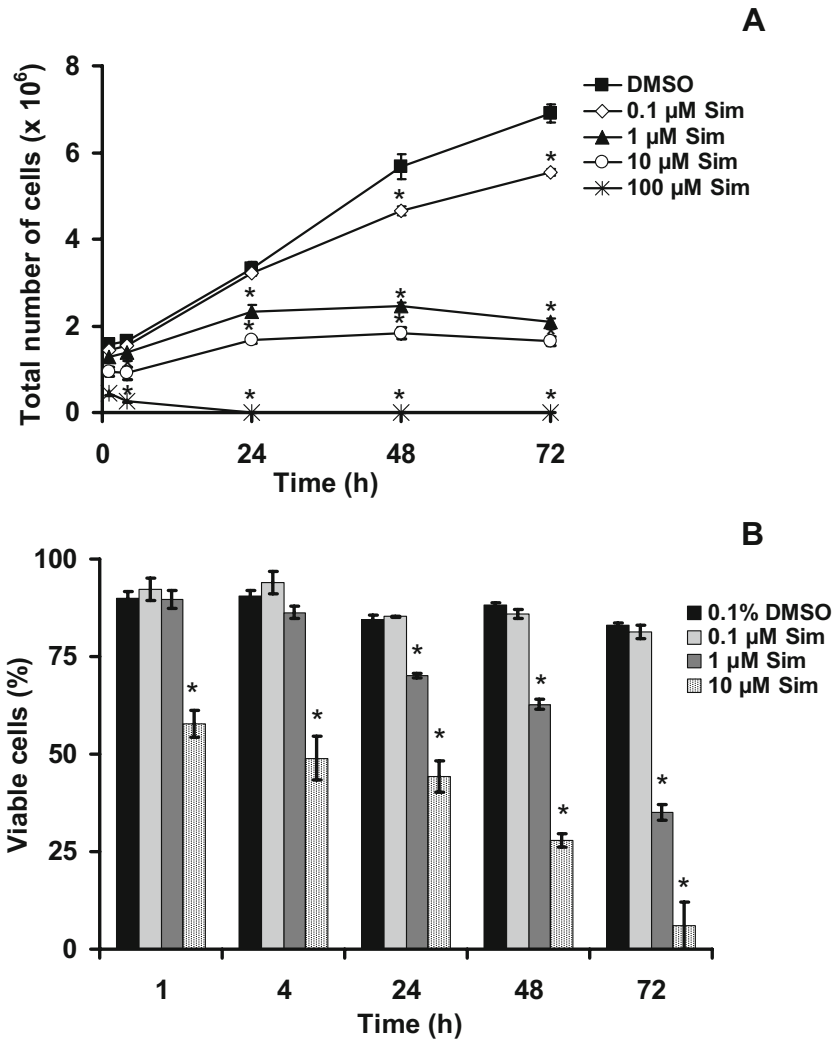

Fig. 1 Inhibition of proliferation and promotion of death of PC3 cells by simvastatin. Total number (**A**) and viability (**B**) of PC3 cells treated with 0.1% DMSO (control) or with simvastatin (Sim) (0.1 to 100 μM). For determination of cell number, cells were counted using a Neubauer chamber. The viability of PC3 cells was determined by exclusion of cells marked by trypan blue and cells presenting apoptotic morphology (apoptotic bodies and cell shrinkage). One hundred micromolars simvastatin caused 100% cell death after 1 h of treatment. The data represent mean±SEM of four independent experiments. *Asterisk* Significant in relation to DMSO; $p<0.05$

Results

Simvastatin induced inhibition of proliferation and promotion of PC3 cells death

PC3 cells were treated with 0.1 to 100 μM simvastatin for up to 72 h, and cell proliferation and viability were analyzed. Cell proliferation was inhibited by statin concentrations equal and above 0.1 μM, and cell death was induced at concentrations from 1 μM to 100 μM (Fig. 1A, B). The effects of simvastatin on cell proliferation and viability were dose- and time-dependent. Next, PC3 cells were treated with simvastatin concentrations in the range of 1 to 100 μM and stained with annexin V and propidium iodide. In the range of 1 to 20 μM and periods of 24 to 72 h, simvastatin induced mainly apoptosis, while at 60 to 100 μM and periods of 2 to 4 h, it induced mainly necrosis (data not shown). Based on these results, we chose

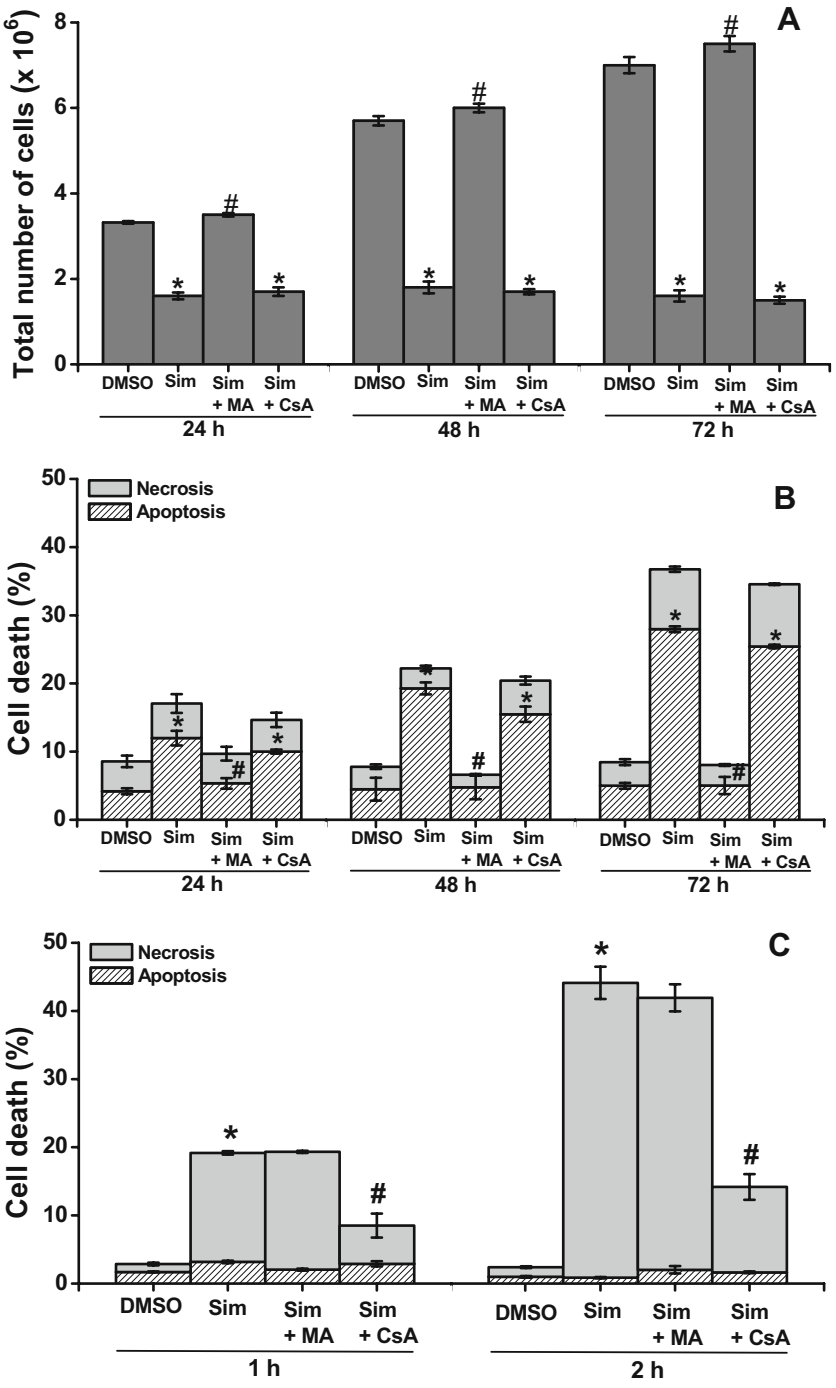

Fig. 2 Simvastatin induced apoptosis dependent on HMG-CoA reductase inhibition and necrosis mediated by calcineurin and/or MPT in PC3 cells. PC3 cells were treated with 0.1% DMSO (control) or with 10 (**A** and **B**) or 60 μM (**C**) simvastatin (Sim) in the absence or presence of 100 μM mevalonic acid (*MA*) or 0.5 μM cyclosporin A (*CsA*), as indicated in the figure. After treatment, total number of cells was estimated using a Neubauer chamber (**A**) and cells were stained with propidium iodide and annexin V to estimate dead cells (**B** and **C**). *Bars* represent mean±SEM of four independent experiments. *Asterisk* Significant in relation to DMSO and *pound sign* significant in relation to Sim; $p<0.05$

simvastatin concentrations of 10 and 60 μM, which caused about 40% cell death, for the following experiments.

Effect of simvastatin on apoptosis dependent on inhibition of HMG-CoA reductase, and necrosis mediated by calcineurin and MPT

In order to ascertain whether the processes of death were dependent on MPT and/or inhibition of mevalonic acid synthesis, PC3 cells were treated with 10 or 60 μM simvastatin in the presence of 100 μM mevalonic acid or 0.5 μM cyclosporin A. Both inhibition of cell proliferation and apoptosis induced by 10 μM simvastatin were totally prevented by mevalonic acid, but not by cyclosporin A (Fig. 2A,B). Necrosis induced by 60 μM simvastatin was not prevented by mevalonic acid, but was significantly (73%) inhibited by cyclosporin A (Fig. 2C).

In order to understand whether the effect of cyclosporin A was dependent on calcineurin and/or MPT inhibition, PC3 cells were treated with either 0.5 μM FK506 (a calcineurin inhibitor), or 0.5 μM bongkrekic acid (an MPT inhibitor). It can be seen in Fig. 3 that both FK506 and bongkrekic acid partially inhibited necrosis; being their effects additive and quantitatively similar to the effect of cyclosporin A. Inhibition by BAPTA, an intracellular Ca^{2+} chelator suggests the participation of this cation in the process.

Effect of simvastatin on cytosolic free calcium concentration ($[Ca^{2+}]_{cyt}$) in PC3 cells

Both MPT (Gunter and Gunter 1994; Kowaltowski et al. 2001) and calcineurin activation (Shibasaki and McKeon 1995) are Ca^{2+} dependent. In order to estimate $[Ca^{2+}]_{cyt}$ in PC3 cells treated with 60 μM simvastatin, the cells were loaded with Fluo3-AM. PC3 cells incubated for 1 h with simvastatin resulted in a threefold increase in $[Ca^{2+}]_{cyt}$ insensitive to the calcineurin and MPT inhibitors tested (Fig. 4), suggesting that the rise in $[Ca^{2+}]_{cyt}$ is a more upstream event than MPT and calcineurin activation. Protection of PC3 cells from necrosis by chelation of cytosolic Ca^{2+} with BAPTA (Fig. 3) supports this hypothesis.

Protection from simvastatin-induced mitochondrial dysfunction by MPT and calcineurin inhibitors

The experiments depicted in Fig. 5 analyzed oxygen consumption and changes in $\Delta\Psi_m$ after 30 min incubation of PC3 cells with 60 μM simvastatin in the presence or absence of MPT (cyclosporin or bongkrekic acid) and calcineurin inhibitors (FK506 or cyclosporin). Simvastatin caused a significant decrease in both $\Delta\Psi_m$ and respiration rate, which were significantly protected by all the inhibitors used. These results indicate that mitochondrial dysfunction precedes simvastatin-induced necrotic cell death. Although simvastatin decreased the initial rate of respiration, the mitochondria were still able to phosphorylate the ADP added.

Bongkrekic acid at the concentration used (0.5 μM) did not completely inhibit the ADP/ATP carrier, since oxidative phosphorylation was still present (trace Sim + BA). In contrast, 1 μM of bongkrekic acid killed all PC3 cells within 2 h probably through a complete inhibition of oxidative phosphorylation (data not shown).

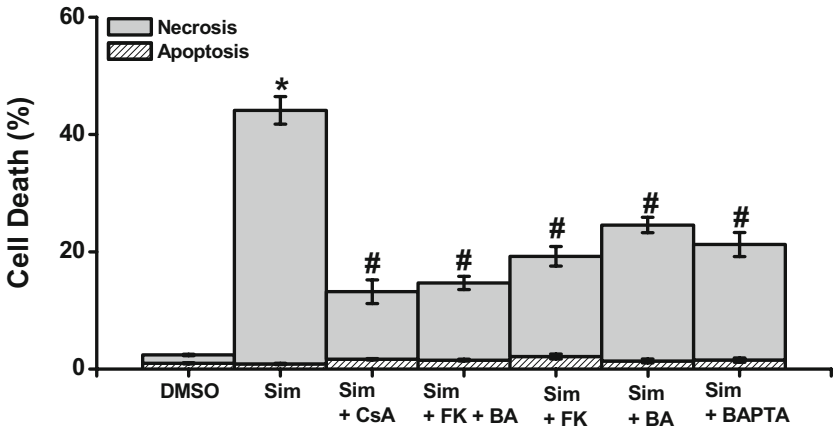

Fig. 3 Simvastatin-induced necrosis of PC3 cells prevented both by calcineurin and MPT-specific inhibitors and by intracellular calcium chelation. PC3 cells were treated with 0.1% DMSO (control) or with 60 μM simvastatin (*Sim*) for 2 h in the absence or presence of 0.5 μM cyclosporin A (*CsA*), 0.5 μM FK506 (*FK*) or 0.5 μM bongkrekic acid (*BA*) as indicated in the figure. For treatment with BAPTA, PC3 cells were pre-incubated with 2.5 μM BAPTA-AM for 40 min and washed. Cells were then stained with propidium iodite and annexin V. *Bars* represent mean±SEM of four independent experiments. *Asterisk* Significant in relation to DMSO control and *pound sign* significant in relation to Sim; $p<0.05$

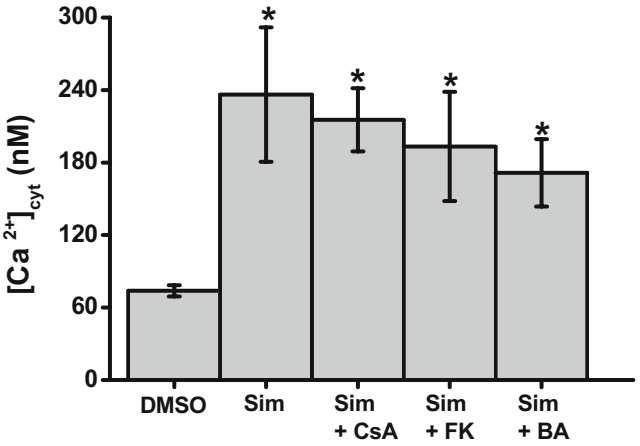

Fig. 4 Simvastatin-induced increase of cytosolic free calcium concentration ($[Ca^{2+}]_{cyt}$) in PC3 cells not prevented by MPT and calcineurin inhibitors. Fluo3-loaded PC3 cells were treated with 0.1% DMSO (control) or with 60 μM simvastatin (Sim) for 1 h in the absence or presence of 0.5 μM cyclosporin A (*CsA*), 0.5 μM bongkrekic acid (*BA*) or 0.5 μM FK506 (*FK*), as indicated in the figure. $[Ca^{2+}]_{cyt}$ in PC3 cells was measured by flow cytometry (10^6 cells/ml). *Bars* represent mean±SEM of four independent experiments. *Asterisk* Significant in relation to DMSO; $p<0.05$

Discussion

Most of the available literature on the reduction of cancer risks or progression by using statins associates these effects with the inhibition of mevalonic acid biosynthesis (for a review see Demierre et al. 2005 and Hindler et al. 2006). This inhibition of biosynthesis leads to modifications in the plasma membrane microdomains (lipid rafts) responsible for signal transduction of key events, such as cell growth and survival; and to decreased formation of downstream lipid isoprenoid intermediates, such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate, thus leading to a deficiency in post-translational prenylation and the impairment of the function of the G-protein (Demierre et al. 2005; Hindler et al. 2006).

In PC3 cells, the inhibition of HMG-CoA biosynthesis leads to the inhibition of proliferation at the G_1-S transition phase (Ukomadu and Dutta 2003; Sivaprasad et al. 2006) and to apoptosis by mechanisms that, in addition to modifications on the lipid rafts (Li et al. 2006), involve the activation of caspase-7 (Marcelli et al. 1998), caspase-3, caspase-8 and caspase-9 (Hoque et al. 2008) as well as inactivation of small GTPase RhoA (Ghosh et al. 1999), down regulation of the E2F-1 transcription factor (Park et al. 2001) and suppression of c-*jun* proto-oncogene expression (Hoque et al. 2008). The present report provides evidence against the participation of MPT and calcineurin in this mechanism of apoptosis (Fig. 2B).

Other reports suggest that statin effects independent of HMG-CoA inhibition also occur such as binding to lymphocyte function-associated antigen 1 (LFA1; Weitz-Schmidt et al. 2001) and inhibition of proteasome in breast cancer cells (Rao et al. 1999). In this regard, our results show that 60 μM simvastatin induces necrosis independent of HMG-CoA inhibition and dependent on MPT and calcineurin in PC3 cells. MPT was evidenced through the sensitivity of PC3 cell necrosis to either cyclosporin A or bongkrekic acid, which interact with cyclofilin D or adenine nucleotide translocase, respectively (Halestrap et al. 1997; Halestrap and Brenner 2003). The protection conferred by FK506 suggests that calcineurin also participates in this process (Fig. 3; Liu et al. 1991).

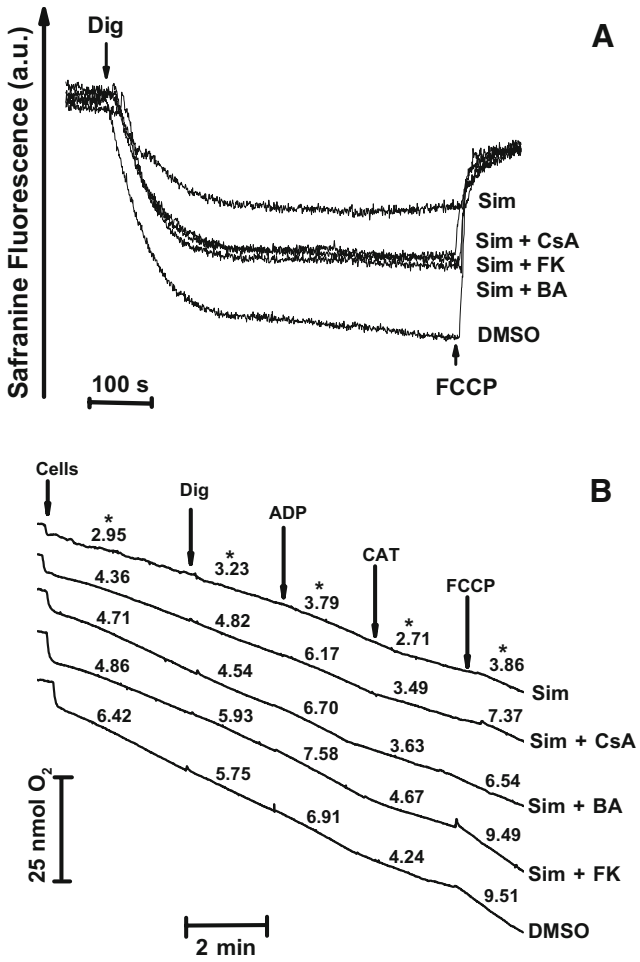

Fig. 5 Statin-induced impairment of mitochondrial function of PC3 cells prevented by cyclosporin A, FK506 and bongkrekic acid. PC3 cells were treated with 0.1% DMSO (control) or with 60 μM simvastatin (*Sim*) for 30 min in the absence or presence of 0.5 μM cyclosporin A (*CsA*), 0.5 μM bongkrekic acid (*BA*) or 0.5 μM FK506 (*FK*) as indicated in the figure. The *arrows* indicate additions of 10^6 PC3 cells, 20 μM digitonin (Dig), 60 μM ADP, 4 μM carboxyatractyloside (CAT) and 1 μM FCCP. Effects on (**A**) mitochondrial membrane potential and (**B**) oxygen consumption of PC3 cells. The *numbers above the lines* indicate mean respiratory rates. Representative figures from four independent experiments. *Asterisk* Significant in relation to DMSO; $p<0.05$

Both MPT and calcineurin activation are Ca^{2+} dependent (Kowaltowski et al. 2001; Shibasaki and McKeon 1995), and their involvement in this process is compatible with the increase in $[Ca^{2+}]_{cyt}$ caused by simvastatin. The significant protection against PC3 cell death offered by intracellular Ca^{2+} chelation with BAPTA supports this interpretation (Fig. 3). In contrast, MPT and calcineurin inhibitors did not prevent the increase in $[Ca^{2+}]_{cyt}$ (Fig. 4), suggesting that this increase precedes both MPT (Kowaltowski et al. 2001) and calcineurin activation (Shibasaki and McKeon 1995).

Mitochondrial participation in simvastatin-induced necrosis was investigated through the measurement of both $\Delta\Psi_m$ and respiration 30 min after the cell incubation with simvastatin in the presence or absence of either cyclosporin A, FK506 or bongkrekic acid (Fig. 5). It is interesting to note that both $\Delta\Psi_m$ and respiration were decreased by simvastatin via a mechanism sensitive to all of these compounds. The sensitivity to cyclosporin A and bongkrekic acid suggests the participation of MPT whereas the sensitivity to FK506 suggests a relationship between calcineurin and mitochondria impairment, although bongkrekic acid and calcineurin inhibition have revealed, at least in part, additive effects in Fig. 3. The oxidative phosphorylation ability of the simvastatin-treated cells suggests that the remaining respiration is supported mainly by the viable cells present. The increase in respiration caused by the MPT and/or calcineurin inhibitors (cyclosporin A, FK506 and bongkrekic acid) supports the results presented in Fig. 3, showing that they decrease the necrosis.

The role of calcineurin in cell death is not completely understood. It can exhibit either anti or pro-apoptotic properties, depending on the stimulus, the cell type, and the phosphorylation status of the p38 mitogen-activated protein kinase (Ankarcrona et al. 1996; Almeida et al. 2004; Campos et al. 2004; Zecchin et al. 2007). Calcineurin can also induce necrosis of cortical (Dawson et al. 1993) and cerebellar granule cell cultures (Manev et al. 1993). Similarly to what we have observed here, Ankarcrona et al. (1996) reported that both cyclosporin A and the calcineurin-inhibitor FK506 protected cells against the development of necrosis and the collapse of $\Delta\Psi_m$ observed during the exposure of neurons to glutamate. The relationship between calcineurin and mitochondrial dysfunction is not clear, but Wang et al (Wang et al. 1999) reported that calcineurin can mediate Bad dephosphorylation followed by heterodimerization with Bcl-X_L, a process that facilitates mitochondrial involvement in Ca^{2+}-induced cell death.

We can conclude that simvastatin can induce not only apoptosis but also necrosis in a dose and time dependent manner. Apoptosis was dependent on the inhibition of HMG-CoA reductase but independent of MPT and calcineurin while necrosis was independent of mevalonic acid biosynthesis and mediated by MPT and calcineurin. Considering that recent studies have shown that susceptibility to necrosis may be partially determined by the cell, and not only by the stimulus (Kroemer et al. 2007), this study indicates that a better understanding of the molecular mechanisms by which MPT and calcineurin mediated cell death may help to identify targets for future prostate cancer therapies.

Acknowledgements The authors would like to thank Edilene S. Santos and Elisangela J. Gomes for their technical assistance, and Natália M. Inada for assistance with FACSCalibur flow cytometer. This work was supported by *Fundação de Amparo à Pesquisa do Estado de São Paulo* (FAPESP), *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq) and *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES).

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