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

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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 Ca2+ 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 Ca2+-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 Ca2+ 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).
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) phenyl-hydrazone (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 \( ([\text{Ca}^{2+}]_{\text{cyt}}) \)

PC3 cells (10\(^6\) cells) were loaded with 3 \(\mu\)M Fluo3-AM fluorescent probe in the presence of 1 \(\mu\)M pluronic acid and 30 \(\mu\)g/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