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Rhodamine 123-mediated photodamage to mitochondrial interior does not develop hallmarks of apoptosis in MH22 cells

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Cellular response to photodamage targeted to the mitochondrial interior of murine hepatoma MH22 cells was investigated *in vitro*. For induction of photosensitized damage to the inner space of mitochondria, rhodamine 123 was employed. Another photosensitizer, mTHPC, which diffusely localizes to cellular membranes including those of mitochondria, was chosen as a frame of reference. Even mild doses of mTHPC-induced photodamage triggered the pathways of apoptosis: caspase-3 was activated and chromatin condensation was observed. However, no hallmarks of apoptosis were detected in the cells after rhodamine 123-mediated photosensitization. These results highlight the impact of sub-organellar localization of damage on cell death processes.

Key words: photosensitization, apoptosis, cytochrome c, rhodamine 123, mTHPC

Abbreviations: mTHPC, *meso*-tetra(3-hydroxyphenyl)-chlorine; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; PDT, photodynamic treatment; Rh123, rhodamine 123; ROS, reactive oxygen species.

INTRODUCTION

Recent work on cell death mechanisms has placed mitochondria in the focus of apoptosis regulation and implementation. This organelle comprises several well defined compartments: the matrix, surrounded by an inner mitochondrial membrane and the intermembrane space (IMS), which is surrounded by an outer mitochondrial membrane (OMM). The decision / effector phase of the apoptotic process is characterized by permeabilization of the OMM with a consequent release of soluble IMS proapoptotic proteins into the cytosol. Mitochondria produce low levels of reactive oxygen species (ROS) as an inevitable consequence of oxidative metabolism. It is assumed that a deviated enhanced generation of ROS triggers the activation of apoptosis [1]. Cytochrome c was demonstrated to be released from mitochondria in a ROS-dependent fashion [2].

The aim of this study was to investigate whether damage to the mitochondrial *inner space* could trigger the apoptosis pathway of cell death in murine hepatoma MH22 cells *in vitro*. To damage the inner space of mitochondria, we employed photosensitization. It involves treatment of cells with photosensitizing agents that generate free radicals and / or ROS upon exposure to visible light. Photosensitization is a mechanism underlying photodynamic treatment (PDT), a mode of therapy for eradication of tumours and other formations produced by cell overgrowth (for review, see Ref. 3). PDT mediated by a sensitizer accumulated in mitochondria is a potent inducer of apoptosis in many types of cells [4]. The apoptotic pathway occurs mainly at low PDT doses, while necrotic death predominates at high PDT doses [4]. $^1O_2$, which is supposed to be the main damaging agent in PDT, during its estimated lifetime can diffuse as little as 10–20 nm from the site of generation in a cell [5], and this distance is smaller than the dimensions of a cell and its organeles. Thus, the subcellular localization of a photosensitizer could determine the primary site of damage and the outcome of the treatment.

To induce damage to the mitochondrial interior, rhodamine 123 (Rh123) was chosen as a photosensitizer. As a lipophilic cation, Rh123 is an efficient probe of vital mitochondria. It preferentially accumulates within the inner mitochondrial space due to the mitochondrial inner membrane potential (negative charge inside) and a positive charge of the dye [6]. Hence, the primary damage induced by Rh123-mediated PDT would be targeted to the mitochondrial interior.

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Although the exact mechanism of Rh123 photosensitization is unknown, experiments in solutions show that Rh123-induced photodamage is oxygen-independent [7].

For comparison, another photosensitizer, neutral lipophilic chlorine derivative mTHPC (commercial name Foscan®) was used. It has been demonstrated that mTHPC diffusely localizes to cellular membranes, most commonly accumulating in mitochondria [8–10], endoplasmic reticulum and Golgi [11]. Thus, mTHPC-mediated primary photodamage is produced in the membranes including mitochondria. Necrotic and apoptotic features of cell death in response to mTHPC photosensitization have been reported [12, 13].

Our study on MH22 cells has revealed that, despite the key role of mitochondria in apoptosis propagation, damage to the mitochondrial inner space producing a moderate decrease of cell viability does not result in apoptosis.

MATERIALS AND METHODS

Materials

DMEM, FCS and streptomycin were obtained from Biochrom AG (Germany). Culture flasks and Petri dishes were from Falcon. Rhodamine 123 (Sigma) was dissolved in ethanol as a 1 mg/ml stock solution and stored at –20 °C in the dark. The stock solution of mTHPC (a generous gift of R. Bonnett, Queen Mary, University of London, UK) was prepared in the same way. All experiments were performed using dilutions of the stock solutions with cell incubation media. Caspase-3 substrate acetyl-aspartyl-glutamyl-valyl-aspartyl-7-amino-4-trifluoromethyl coumarin (Ac-DEVD-AFC) and the caspase-8 substrate, acetyl-isoleucyl-glutamyl-threonyl-aspartyl-7-amino-4-trifluoromethyl coumarin (Ac-IETD-AFC), were from Biomol. Other chemicals were from Sigma.

Cell culture

MH-22A cells from murine hepatoma were obtained from the Institute of Cytology, Sankt-Petersburg, Russia. The cells were cultured in monolayer in 25 cm^2 flasks in Dulbecco's minimal essential medium (DMEM) supplemented with 10% FCS, 100 IU/ml of penicillin, 100 μg/ml of streptomycin, and 2 mM of glutamine at 37 °C in a 5% CO_2 atmosphere. Cells were subcultured by dispersal with 0.025% trypsin in 0.02% EDTA and replated at 1 : 3 dilution twice a week.

Fluorescence microscopy

Cells were incubated with Rh123 as described in Figure legends or 5 μg/ml Hoechst 33342 for 30 min at 37 °C. The cells were visualized with an Olympus AX70 fluorescence microscope equipped with ×60, NA 1.25 oil immersion lens. A WIBA filter cube (460–490 / 515–550 nm) was used for visualization of Rh123 and WU filter cube (330–385 / 400 nm) for Hoechst 33342. The images were recorded with a CCD camera Orca (Hamamatsu) and analysed with MicroImage version 4.0 (Media Cybernetics) software.

Measurement of rhodamine 123 intracellular concentration

After incubation with Rh123, the cells were washed twice with DPBS and scraped to 0.5 ml of 0.9% NaCl; 17 μl of 1M NaOH was added to a cell suspension. The Rh123 concentration was measured fluorometrically at 485 nm for excitation and 538 nm for emission with a fluoroscan Ascent FL, Labsystems. For quantification, a minimal amount of a standard solution of the known Rh123 concentration increasing the total fluorescence yield by about 50% was added, and the fluorescence was recorded once more [14]. Rh123 concentration in the cell lysates was normalised to protein amount.

Cell treatment

A nearly confluent monolayer of cells in 35 mm Petri dishes was exposed to chemicals in serum-free DMEM. Precautions were taken to avoid irradiating the samples with room light. At the end of incubation, the extracellular photosensitizer was removed by rinsing the cell monolayer 3 times with room-temperature DPBS, and DMEM with 10% FCS was added. The light exposure was carried out as described below.

Rh123-photosensitization. The cells were incubated with 0.5–50 μg/ml (1.3–130 μM) Rh123 for 5–120 min as indicated in Figures. Cells loaded with Rh123 were exposed to light for 15–75 min as indicated in Figures. The light source was LED array UNIMELA-2 ($\lambda = 509 \pm 5$ nm), VU Laser Research Centre, Lithuania; the fluence rate at the level of the cells was 29 W/m^2.

mTHPC-photosensitization. The cells were incubated with 0.15 μg/ml mTHPC for 18 h. Cells loaded with mTHPC were exposed to light for 15–75 sec as indicated in Figures. The light source was LED array UNIMELA-1 ($\lambda = 660 \pm 5$ nm), VU Laser Research Centre, Lithuania; the fluence rate at the level of the cells was 16 W/m^2.

MTT assay

The incubation medium in 35 mm Petri dishes was replaced with a volume of 1 ml of tetrazolium dye 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution at 0.2 mg/ml in DPBS, and the cells were incubated for 2 h at 37 °C. After incubation, the MTT solution was discarded, and 2 ml of 2-propanol (Avsista, Lithuania) was added. The extraction process was performed during 20 min at room temperature. The optical density (OD) was then recorded at 570 nm. The mean OD_{570} of the control cells exposed to a test-compound-free culture medium was set to represent 100% of viability, and the results were expressed as a percentage of these controls [15].

DEVD-specific caspase activity assay

The activity of DEVD-specific caspases was measured using the Caspase Fluorescent Substrate / Inhibitor QuantiPak (Biomol) based on the cleavage of 7-amino-4-trifluoromethyl coumarin dye from a corresponding peptide derivative. The cleavage was followed by fluorescence measurements

using an AscentFL microplate reader (Labsystems) at exitation wavelength 390 nm and emission wavelength 510 nm; 10^5 cells were used for each measurement. The activity of the caspases was calculated as pmol / min from a slope of the plot 'fluorescence vs time' using a conversion factor obtained from the appropriate calibration curve.

Measurement of protein concentration

Protein concentration was determined using bicinchoninic acid sodium salt (4,4'-dicarboxy-2,2'-biquinoline disodium salt) (BCA) [16].

Data analysis

The data are presented as means ± standard error (S. E.) from three independent experiments. SigmaPlot 2001 for Windows version 7.101 software was used for the statistical analysis.

RESULTS AND DISCUSSION

Uptake of Rh123 within MH22 cells

Rh123 stains intracellularly energized mitochondria with virtually absolute specificity, i. e. with no detectable staining of any other subcellular compartment taking place simultaneously [6, 17]. Rh123, as a lipophilic cationic dye, localizes to the mitochondria interior electrophoretically without passage through endocytic vesicles and lysosomes according to the well-established premise that the diffusion of this dye is directly proportional to the value of the membrane potential, in response to the negative internal charge [18, 19]. On the basis of these findings, we chose Rh123 for inducing damage targeted to the mitochondrial interior.

MH22 cells were saturated with Rh123 by incubation for 60 min (Fig. 1A). Therefore, further experiments were carried out after cell incubation with Rh123 for 60 min. Since the mitochondrial membrane potential $\Delta\psi_m$ conforms with the maintenance of cellular homeostasis, its oscillations are insignificant. As a consequence, cellular uptake of Rh123, the steady probe of $\Delta\psi_m$, does not depend on Rh123 concentration in incubation medium within rather wide limits up to 10 μg/ml (Fig. 1B). At higher concentrations, accumulation of Rh123 in the cells significantly increases, and the increase parallels with the loss of cell viability in the dark (Fig. 1C). Rh123 was found to inhibit the bioenergetic function in mitochondria [20] namely at concentrations above 10 μg/ml [21]. Therefore, it could be suggested that overloading mitochondria with Rh123 undermines homeostasis in the cell. On the other hand, it is well known that the dying cell uncontrollably absorbs significant amounts of materials present in the incubation medium. It should be noted that these effects were observed in the absence of light exposure. Regarding mitochondria, the Rh123 overload affects the distribution pattern

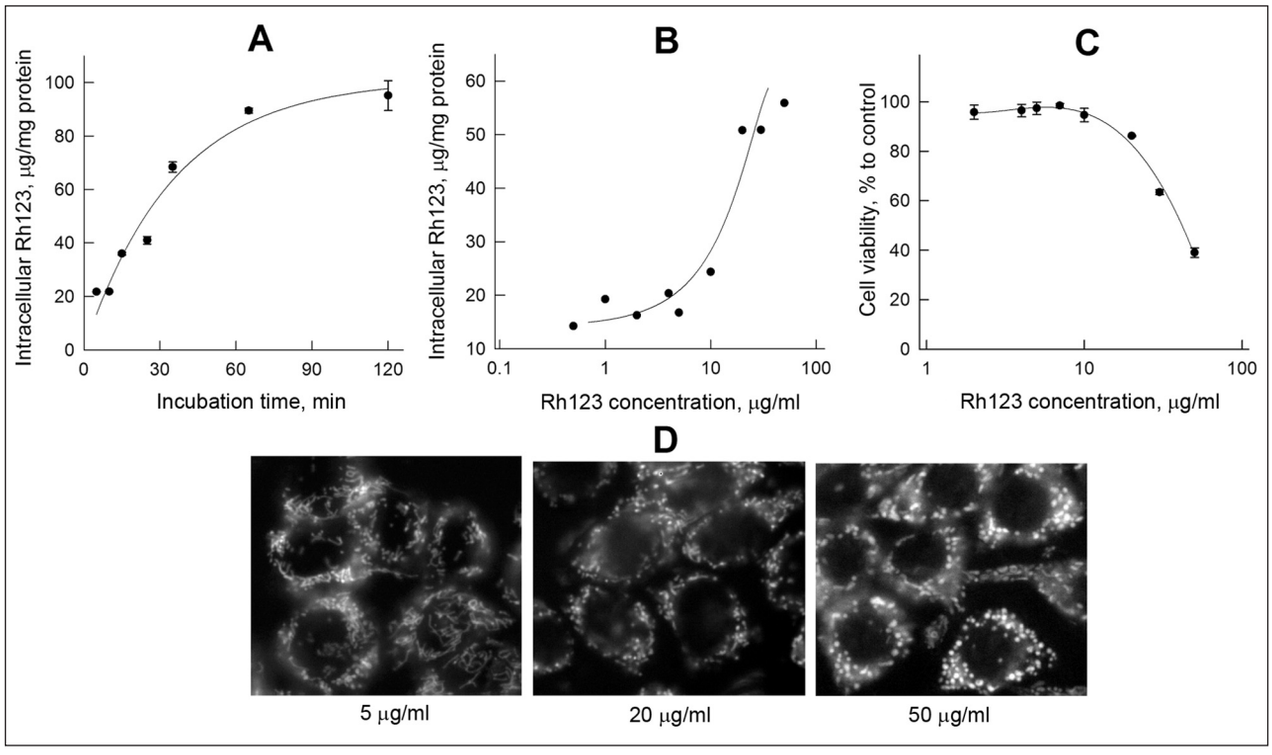

Fig. 1. Uptake of Rh123 into MH-22A cells and dark toxicity. (A) Rh123 accumulation on Rho *vs* duration of incubation; the cells were incubated in the medium containing 5 μg/ml Rh123. (B) Rh123 accumulation *vs* Rh123 amount in the incubation medium; the cells were incubated for 1 h; (C) cell viability as assessed by MTT reduction at 24 h of incubation in medium supplemented with Rh123; (D) cellular localization of Rh123 fluorescence. Cells were incubated with 5 μg/ml, 20 μg/ml or 50 μg/ml of Rh123 for 60 min and examined with a fluorescence microscope as described in Materials and methods

of mitochondria in the cell: mitochondria look rounded up (Fig. 1D). Therefore, when studying cell response to mitochondrial damage, we did not investigate the consequences of Rh123 dark toxicity at high concentrations and prefered the induction of photodamage in the presence of 5 μg/ml of Rh123: at this dose, no major changes in the pattern of mitochondrial distribution and cell viability were observed.

Impact of Rh123-mediated photodamage on cell viability

Rh123 is a popular fluorescent probe of vital mitochondria due to its high fluorescence quantum yield, $\Phi_F = 0.9$ [22]. It means that the quantum yield of the triplet state, which is important for the generation of $^1O_2$, one of the major factors in PDT, is low. Indeed, laser flash photolysis studies found the quantum yield of the triplet state $\Phi_T = 0.1$ [22]. However, phototoxicity mediated by Rh123 was shown to depend on oxygen concentration, implicating ROS as an inducer or mediator of photodamage [23]. A variability of the effectiveness of cell photosensitization by Rh123 has been noted, and this effectiveness was described as low in general [7]. In our study, the impact of Rh123-mediated photodamage on the viability of MH22 cells was compared with that of mTHPC, a potent photosensitizer with the quantum yield of the triplet state $\Phi_T = 0.9$ [24]. The uptake of mTHPC into MH22 cells and the optimal conditions for mTHPC-PDT had been determined earlier [25]. The greatest loss of cell viability achieved by Rh123-PDT was approx. 40% and took 60 min of light exposure (104.4 kJ/m^2) (Fig. 2A). Meanwhile, the same loss by mTHPC-PDT was achieved at 0.72 kJ/m^2 (45 s of light exposure). Interestingly, light exposure of Rh123 treated cells induced a fission of mitochondria, and the mitochondrial network did not recover up to 24 h after light exposure (Fig. 2C). However, an important result was Rh123-mediated inhibition of citrate synthase, an enzyme of the Krebs cycle, enclosed in the mitochondrial matrix, indicating the damage to the inner space of mitochondria (Fig. 2D). A certain loss of citrate synthase activity (approx. 10%) was detected following cell incubation with Rh123 in the dark. However,

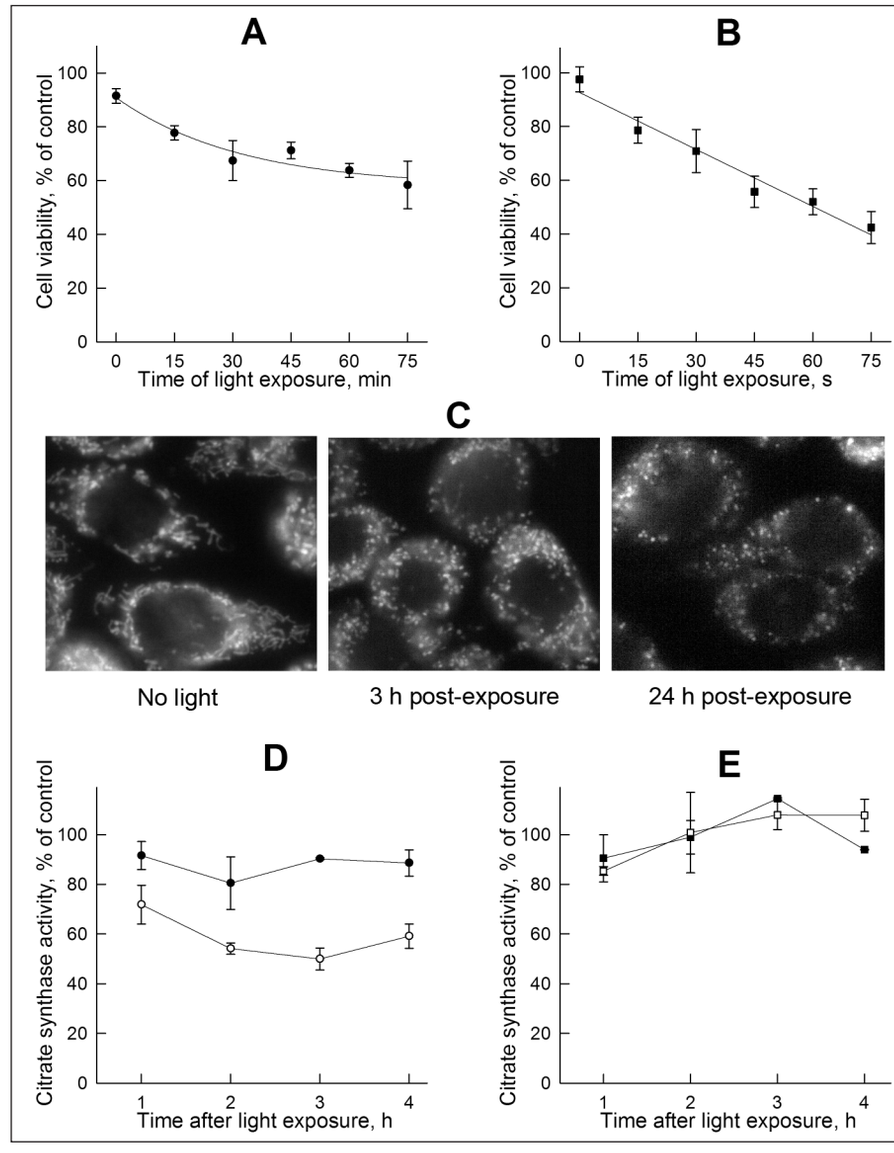

Fig. 2. Effect of photosensitization on MH22 cells. (A) Cell viability at 24 h after light exposure as assessed by MTT reduction. Cells were incubated with 5 μg/ml of Rh123 for 1 h before light exposure, 509 ± 5 nm, 29 W/m^2; (B) cell viability at 24 h after light exposure as assessed by MTT reduction. Cells were incubated with 0.15 μg/ml of mTHPC for 18 h before light exposure, 660 ± 5 nm, 16 W/m^2; (C) Rh123 fluorescence was examined with a fluorescence microscope after incubation with 5 μg/ml Rh123 for 1 h (no light) and at 3 h (3 h post-exposure) or 24 hours (24 h post-exposure) after light exposure at 509 ± 5 nm, 29 W/m^2, for 60 min; (D) citrate synthase activity following light exposure after pre-incubation with 5 μg/ml of Rh123 for 1 h. Open symbols: 509 ± 5 nm, 29 W/m^2, 60 min. Closed symbols: without light exposure; (E) citrate synthase activity following light exposure after pre-incubation with 0.15 μg/ml of mTHPC for 18 h. Open symbols: 660 ± 5 nm; 16 W/m^2, 45 s. Closed symbols: without light exposure

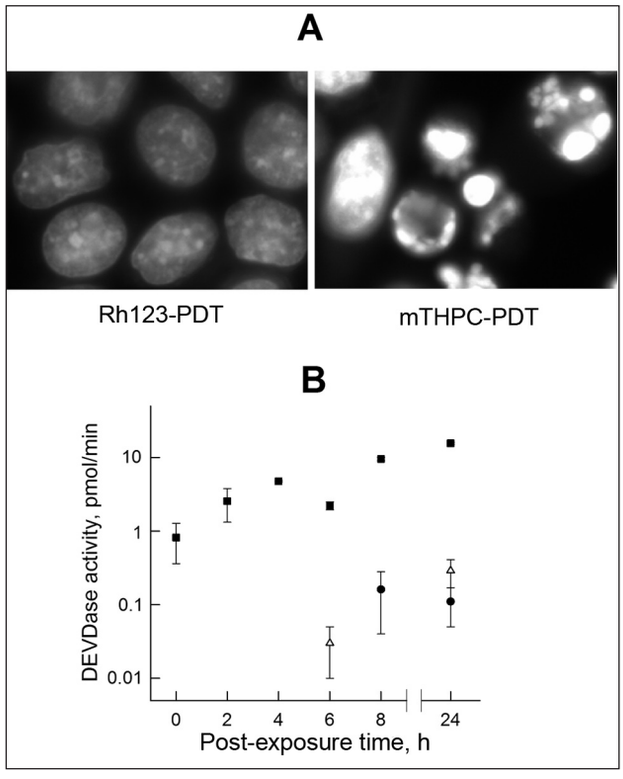

Fig. 3. Hallmarks of PDT-induced apoptosis. Irradiation parameters see in the legend of Figs. 2D and 2E. (A) fluorescence of Hoechst 33342 in cells after PDT. Duration of post-exposure incubation: Rh123-PDT, 4 h; mTHPC-PDT, 1.5 h; (B) DEVDase activity following PDT. Triangles, untreated control cells. Circles: 5 μg/ml of Rh123 for 1 h before light exposure, 509 ± 5 nm, 29 W/m^2, 60 min. Squares: 0.15 μg/ml of mTHPC for 18 h before light exposure, 660 ± 5 nm; 16 W/m^2, 45 s

the activity decrease in Rh123-photosensitized cells was significantly more prominent, reaching 50%. After photosensitization with mTHPC, which localizes to cellular membranes including mitochondrial ones, a temporary inhibition by 10–15% was registered in cells with the same residual viability as after Rh123-photosensitization, but it was absent at 2 h post-exposure (Fig. 2E).

On the basis of these data, we assumed that photosensitization mediated by Rh123 did induce damage to the mitochondrial inner space, while mTHPC-PDT intervened in other cellular compartments or / and mitochondrial subcompartments.

Hallmarks of apoptosis in photosensitized cells

ROS induction by PDT mediated by photosensitizers that localize to mitochondria is known to lead to extensive apoptosis [4]. It is generally accepted that OMM permeabilization is an obligatory event in inducing apoptosis, which is triggered by both external and internal signaling pathways [26]. It releases cytochrome c and other apoptogenic factors from IMS into the cytosol. If the impact of ROS generated in the mitochondrial interior or their products can reach the OMM, it facilitates the realease of apoptogenic factors into the cytosol. To know how the cell would respond to a damage targeted not to the OMM but to the mitochondrial interior, we focused our study on the changes in cell morphology and some biochemical parameters induced by Rh123-photosensitization.

After mTHPC-PDT, the following apoptotic features were observed: chromatin condensation (Fig. 3A), caspase-3 activation (DEVDase activity) (Fig. 3B) and cytochrome c release to the cytosol [27]. After photosensitization mediated by Rh123, only a couple of dead cells exhibiting an apoptotic or necrotic morphology were observed, at the same extent as in control cells; no release of cytochrome c was detected in the cytosolic fraction of the treated cells, and no increase of caspase-3 activity in comparison with control cells was recorded (Fig. 3B). It should be noted that photodamage mediated by either sensitizer induced a small share of necrosis as detected by staining with propidium iodide (not shown).

This study demonstrates that cell response to damage in the mitochondrial interior is different from the response to damage produced in cellular membranes. After PDT mediated by mTHPC, which is localized in cellular membranes, the treated cells undergo cell death through apoptosis associated with caspase activation. Meanwhile, the same cytotoxic dose of Rh123 photosensitization does not display hallmarks of apoptosis, although a direct damage is produced inside the mitochondria, the key point in apoptosis propagation. The loss of cell viability indicates that growth arrest, senescence or other pathways of cell death such as autophagy should be involved.

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RODAMINU 123 SUKELTOS FOTOPAŽAIDOS MITOCHONDRIJŲ VIDINĖJE ERTMĖJE NEINDUKUOJA APOPTOZĖS POŽYMIŲ

Santrauka

Šiame darbe buvo tiriamas pelių hepatomos MH22 ląstelių atsakas į mitochondrijų vidinės ertmės fotopažaidas *in vitro*. Fotosensibilizuotos pažaidos mitochondrijų vidinėje ertmėje buvo sukeltos rodaminu 123. Palyginimui naudotas kitas fotosensibilizatorius – mTHPC, difuziškai pasiskirstantis ląstelės membranose, tarp jų ir mitochondrijų membranose. Net ir nedidelės mTHPC fotodinaminio poveikio dozės inicijavo apoptozę: chromatino kondensaciją bei kaspazės 3 aktyvaciją. Tuo tarpu ląstelių fotosensibilizacija rodaminu 123 nesukėlė apoptozės. Šie rezultatai pabrėžia fotopažaidų lokalizacijos organelių skyriuose svarbą ląstelių žūčiai.