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Production of reactive oxygen species in mitochondria of HeLa cells under oxidative stress

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Abstract

Mitochondria can be a source of reactive oxygen species (ROS) and a target of oxidative damage during oxidative stress. In this connection, the effect of photodynamic treatment (PDT) with Mitotracker Red (MR) as a mitochondria-targeted photosensitizer has been studied in HeLa cells. It is shown that MR produces both singlet oxygen and superoxide anion upon photoactivation and causes photoinactivation of gramicidin channels in a model system (planar lipid bilayer). Mitochondria-targeted antioxidant (MitoQ) inhibits this effect. In living cells, MR-mediated PDT initiates a delayed ("dark") accumulation of ROS, which is accelerated by inhibitors of the respiratory chain (piericidin, rotenone and myxothiazol) and inhibited by MitoQ and diphenyleneiodonium (an inhibitor of flavin enzymes), indicating that flavin of Complex I is involved in the ROS production. PDT causes necrosis that is prevented by MitoQ. Treatment of the cell with hydrogen peroxide causes accumulation of ROS, and the effects of inhibitors and MitoQ are similar to that described for the PDT model. Apoptosis caused by H_2O_2 is augmented by the inhibitors of respiration and suppressed by MitoQ. It is concluded that the initial segments of the respiratory chain can be an important source of ROS, which are targeted to mitochondria, determining the fate of the cell subjected to oxidative stress.

Keywords: Mitochondria; Reactive oxygen species; Oxidative stress; Photo dynamic treatment

1. Introduction

It is widely accepted that mitochondria play a key role in development of oxidative stress. The major endogenous sources of reactive oxygen species (ROS) are localized to mitochondria and can be related to the respiratory chain, substrate dehydrogenases in the matrix, monoamine oxidase and cytochrome P450. On the other hand, significant antioxidant capacity is inherent in mitochondria (ubiquinol, MnSOD, glutathione- and thioredoxin-dependent systems). As a result, mitochondria could either amplify or suppress the general oxidative stress provoked by exogenous stimuli. In turn, stress is known to initiate programmed cell death (apoptosis) or necrosis, which could be controlled by mitochondria as well. The details of this complicated picture are not clear [1,2].

We have addressed the problem using oxidative insult caused by photodynamic treatment and directed to mitochondria due to mitochondria-targeted photosensitizer. Photodynamic therapy is an important field of biomedical research and a powerful approach in clinical oncology. The new generations of photosensitizers are very effective and can be applied to treat tumors of various locations using excitation with far red light. However, high therapeutic efficiency is usually limited by side effects mediated by inflammation and photodynamic damage to tissues after illumination. Mitochondria are considered as

Abbreviations: ROS, reactive oxygen species; PDT, photodynamic treatment; MR, Mitotracker Red; CM-DCF-DA, 5-(and-6)-chloromethyl-2′, 7′-dichlorodihydrofluorescein diacetate; BLM, bilayer lipid membrane; DPhPC, diphytanoyl-phosphatidyl-choline; DPhPG, diphytanoyl-phosphatidyl glycerol; $AlPcS_4$, aluminum phthalocyanine; MitoQ, 10-(6′-ubiquinolyl)decyltriphenyl-phosphonium; DPI, diphenyleneiodonium; zVADfmk, carbobenzoxy Val-Ala-Asp-fluoromethyl ketone; FCCP, trifluoromethoxycarbonylcyanide phenylhydrazone; TMRM, tetramethylrhodamine methyl ester; PTP, permeability transition pore; CsA, cyclosporin A

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attractive targets for PDT since the pioneering works of L. B. Chen and coworkers [3]. It was found that cationic photosensitizers could be selectively accumulated by mitochondria in tumor cells and be entrapped there even after depolarization of the mitochondrial membrane. The nature of this selectivity remains unknown, and photosensitizers of this type have not yet reached the clinic. Some modern photosensitizers were announced as mitochondria-targeted but appeared to be located in different compartments including lysosomes [4]. Cationic mitochondrial photosensitizers seem promising since (i) they should be accumulated in the negatively charged mitochondrial interior only and (ii) a delicate damage to mitochondria could preferentially induce apoptosis, which is not accompanied, in contrast to necrosis, by inflammation.

Studies on mitochondria-mediated oxidative stress were strongly stimulated by development of mitochondria-targeted antioxidants by M. P. Murphy and his colleagues [5]. MitoQ proved to be especially effective. It is accumulated in the mitochondrial matrix due to its positive charge, and its antioxidant capacity is rechargeable due to reduction of the quinol by the respiratory chain. As a result, this antioxidant appeared effective at very low (nM) concentrations [5,6].

The data presented below indicate that mitochondria are involved in generation of ROS after oxidative insult caused by PDT or exogenous hydrogen peroxide. Mitochondrial ROS are important in determining the fate of cells subjected to oxidative stress.

2. Materials and methods

Mitotracker Red (MR), C11-BODIPY, and CM-DCF-DA were from Molecular Probes. 10-(6′-ubiquinolyl) decyltriphenylphosphonium (MitoQ) was synthesized essentially as described by Kelso et al. [5]. All the other reagents and HeLa and HeLa-Bcl2 cells were from the same sources as described earlier [7,8].

The experiments on photoinactivation of gramicidin were performed as described previously [9]. In brief, bilayer lipid membrane (BLM) was formed from a solution of 2% DPhPC or DPhPC and DPhPG (Avanti Polar Lipids, Alabaster, AL) in *n*-decane on a 0.55-mm diameter hole in a Teflon partition separating two aqueous compartments. The bathing aqueous solutions contained 100 mM KCl, 10 mM MES, and 10 mM Tris, pH 7.0. At both sides of the BLM, 10 nM gramicidin A was added. The addition resulted in an increase in the membrane conductivity, which reached a steady state within 15 min. The electric current was recorded under voltage-clamp conditions at 30 mV. A halogen lamp (Novaflex, World Precision Instruments, USA) providing a fluence rate of 30 mW/cm^2 was used for illumination. Mitotracker Red (MR) or aluminum phthalocyanine bearing four sulfonate groups ($AlPcS_4$, Porphyrin Products, Logan, UT) was added to the bathing solution at the *trans*-side of the BLM (the *cis*-side corresponded to the side of illumination). MitoQ and the other reagents were added to both sides. The experiments were carried out at room temperature (T=24–26 °C).

HeLa cells were cultivated as described earlier [8]. For PDT, the cells were loaded with 200 nM MR for 15 min at 37 °C. The cells on plastic Petri dishes were illuminated by the tungsten lamp of an Axiovert 200 M microscope (Carl Zeiss) using a green filter (bandpass 545/25 nm) and Neofluor 20× objective for 1–1.5 min to reach the fluence of 3.65 J/cm^2. For induction of cell death, illumination was increased to 23.4 J/cm^2 (“mild illumination”) or to 34.8 J/cm^2 (“strong illumination”).

For induction of oxidative stress, the cells were treated with 200 μM H_2O_2 for 45 min in complete medium. ROS production was measured after loading the cells with 5 μM CM-DCF-DA for 15 min at 37 °C immediately after washing out of MR (before illumination) or after washing out of H_2O_2. Fluorescence was analyzed using the Axiovert 200 M microscope or a Nikon Eclipse TE2000-U microscope equipped with confocal C1 unit and with a program written in Matlab language described in [10]. The same device was used for local photoactivation of MR with 543 nm laser light. Loading and analysis of BODIPY-C11 fluorescence were described elsewhere [10]. Flow cytometric analyses were performed using a Partec PAS-III particle analyzing system (Partec GmbH), which was equipped with a 488-nm argon-ion laser. For immunostaining, the cells were grown on coverslips. The cells were treated as above and stained with anti-cytochrome *c* monoclonal antibodies (6H2.B4, BD Pharmingene), Hoechst 33342 (to reveal apoptotic nuclei), and propidium iodide (to reveal necrosis) as described earlier [8].

3. Results

3.1. Photodynamic effect of Mitotracker Red

Mitotracker Red (MR) is a cationic fluorescent dye introduced for selective labeling of mitochondria. It is not used for PDT in clinics due to low efficiency and inconvenient position of the excitation maximum, which is in the green spectral region. MR was applied as a mitochondria-targeted photosensitizer by Nagley and coworkers [11,12]. They found that PDT with MR resulted in depolarization of the mitochondrial membrane, release of cytochrome *c* into cytosol, and apoptosis. The details of photodynamic action of MR were not studied. In particular, the origin of ROS produced after illumination of MR was not analyzed.

It has been recently shown that the sensitivity of gramicidin channels to ROS can be used for evaluation of photodynamic efficacy of different photosensitizers [9]. The photosensitized suppression of the gramicidin-mediated current across a BLM has proven to be highly specific, as it is caused by selective damage to tryptophan residues located near the channel gate [13]. The photoinactivation of gramicidin channels can be considered as a model of a widespread phenomenon of the photomodification of natural ion channels.

We found that MR mediates rapid photoinactivation of gramicidin channels in a BLM (Fig. 1A). The process ceased immediately after switching off the light. This suggests that the effect is due to primary ROS produced by photosensitizer and secondary lipid radicals are not involved. MR appeared less effective compared to $AlPcS_4$, which is known as one of the most powerful photosensitizers with high quantum yield of singlet oxygen production [14]. When negatively charged membrane was used, the efficacy of MR strongly increased and exceeded that of $AlPcS_4$, which is negatively charged (Fig. 1B).

We tested the mitochondria-targeted antioxidant MitoQ in this model and for the first time demonstrated pronounced protective effect against photodynamic damage of a peptide molecule. To compare the effect of MitoQ in different membranes, we used different concentrations of MR that showed similar photodynamic effects. The efficacy of MitoQ appeared practically the same in neutral versus negatively charged membrane (Fig. 1C), indicating that hydrophobicity plays the major role in the binding of MitoQ to the membrane.

The effect of MR was suppressed by azide (a known scavenger of singlet oxygen [15]), suggesting the participation of singlet oxygen in the gramicidin photodamage (Fig. 2).

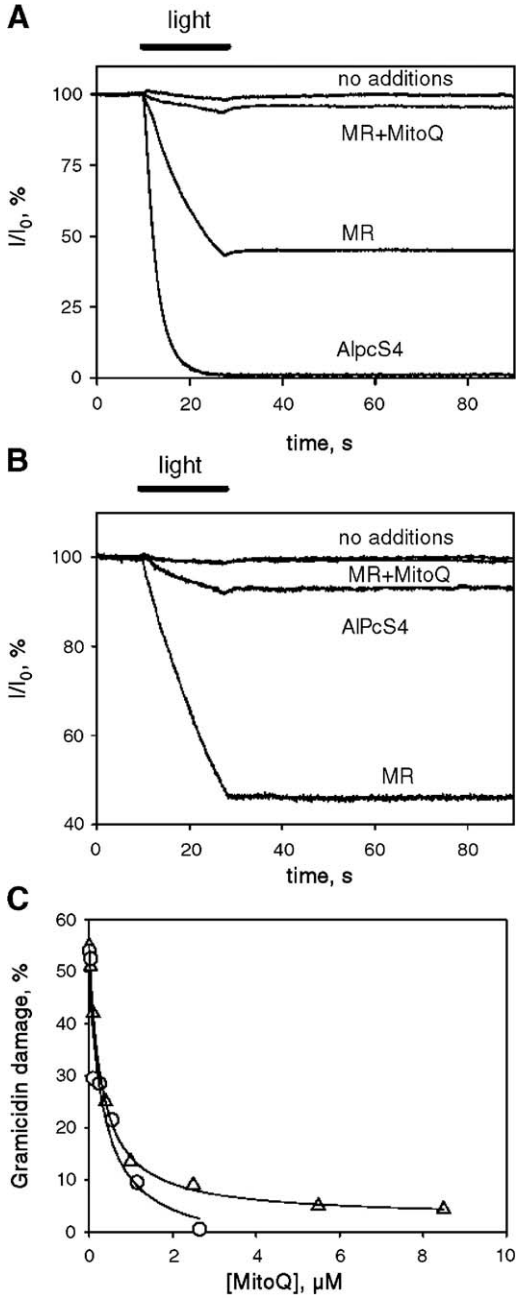

Fig. 1. Photodynamic inactivation of gramicidin and protective effect of MitoQ. The ratio between currents across BLM measured before (I_0) and after (I) illumination is presented. The average I_0 was 0.5 μA. Typical recordings from 3 to 5 experiments are presented. (A) Neutral BLM (DPhPC). Photosensitizers $AlPcS_4$ (0.7 μM) or MR (0.7 μM) were added 2 min before illumination. MitoQ (8 μM) was added together with MR. Control — illumination without photosensitizers. (B) Negatively charged BLM (DPhPC/DPhG 70/30 mol%). $AlPcS_4$ (0.1 μM) and MR (0.1 μM) and MitoQ (8 μM) were added where indicated. (C) Protection by MitoQ against gramicidin damage (decrease in the current) induced by MR-mediated PDT in neutral BLM (triangles, 0.7 μM MR) and negatively charged BLM (circles, 0.1 μM MR). Different concentrations of MR (0.7 and 0.1 μM) were selected in order to have similar gramicidin damage without MitoQ.

The effect of azide was not complete (around 60%) even at a saturating concentration, indicating the participation of other ROS. Superoxide anion may well be such a ROS since photoinactivation of gramicidin was inhibited also by SOD (Fig. 2). Inasmuch as MitoQ almost completely prevented the MR-mediated effect, one can conclude that MitoQ is able to scavenge both singlet oxygen and superoxide.

3.2. PDT-dependent ROS production in HeLa cells

The effect of PDT with MR as a photosensitizer was studied in HeLa cells loaded with CM-DCF (a probe for ROS production). It was found that immediately after illumination fluorescence of CM-DCF was preferentially located to mitochondria as indicated by co-localization with Mitotracker Red (Fig. 3). This localization was not due to a specific accumulation of CM-DCF in mitochondria since direct photoactivation of this dye did not reveal selective staining of mitochondria or any other organelles (not shown). During 5–10 min after illumination, CM-DCF fluorescence appeared in the whole cell and became homogenously spread in cytosol and nucleus (Fig. 3A). The same redistribution of CM-DCF was observed after local illumination of a part of the cell (Fig. 3B). In this case, immediately after illumination CM-DCF fluorescence was observed only in illuminated mitochondria and then slowly spread all over the cell.

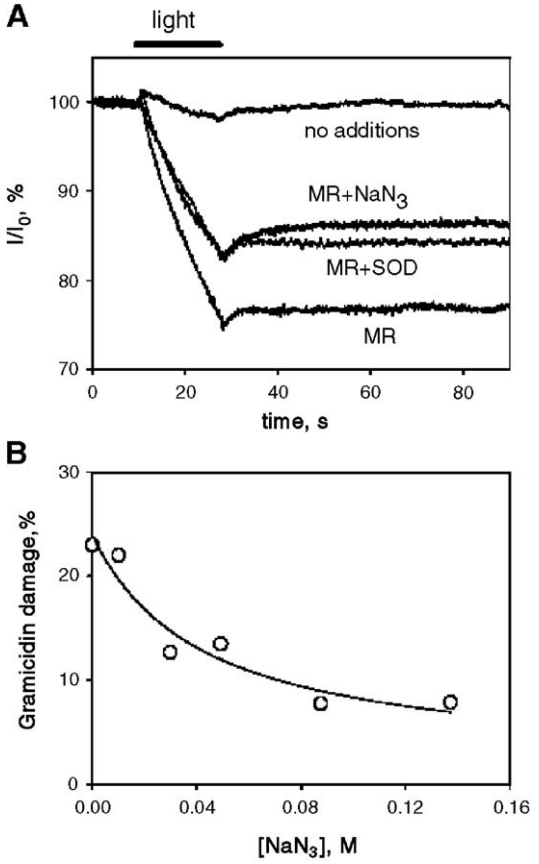

Fig. 2. Photodynamic effect of MR depends on singlet oxygen and superoxide production. (A) Gramicidin damage (decrease in the current) induced by MR-mediated PDT in neutral BLM (for conditions see Fig. 1). Concentrations: 0.3 μM MR, 50 mM NaN_3 and 450 EU/ml SOD. (B) Dependence of the effect on the concentration of NaN_3. The data of the typical experiment of three are presented.

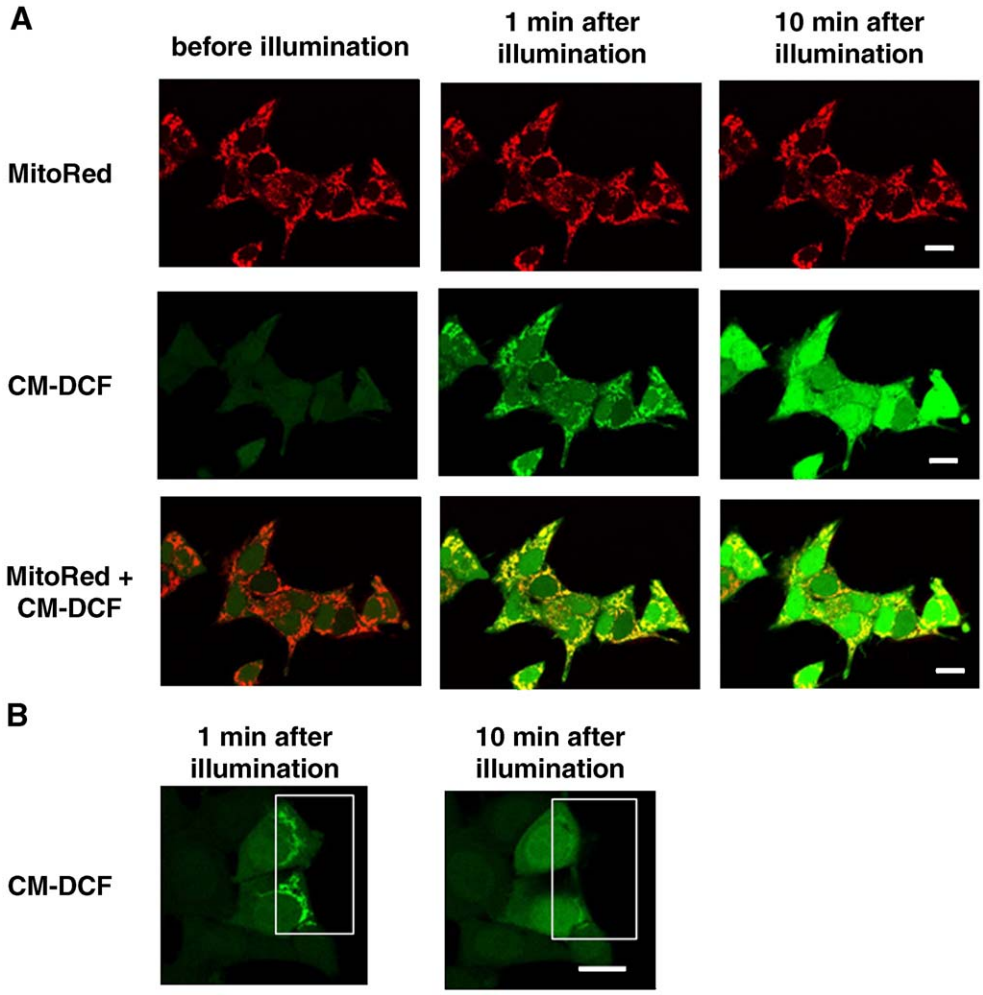

Fig. 3. Accumulation of ROS in HeLa cells after photoactivation of MR. (A) Cells were loaded with MR (200 nM, 15 min) and CM-DCFH-DA (5 μM, 15 min) and illuminated and analyzed with a confocal microscope as described in Materials and methods. Typical images obtained before illumination and 1 min and 10 min after illumination are presented. (B) CM-DCF fluorescence 1 min and 10 min after local illumination with laser light (543 nm) of part of the MR-loaded cell (shown with solid line).

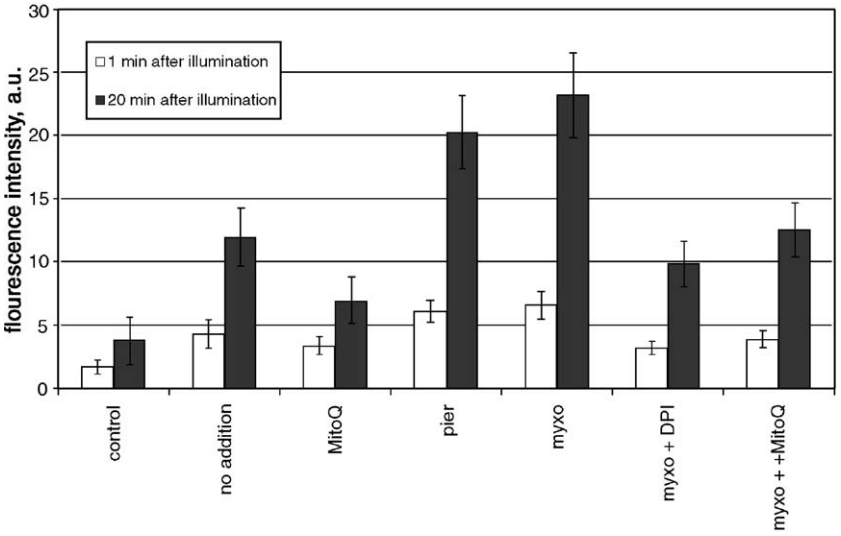

Fig. 4. Inhibitors of respiration stimulate accumulation of ROS after MR-mediated PDT. Piericidin A (Pier, 10 μM) and myxothiazol (Myx, 2 μM) were added simultaneously with CM-DCFH-DA before illumination. Where indicated, the cells were preincubated with DPI (10 μM) for 1 h, or with MitoQ (20 nM) for 8 days. The averages of three experiments are presented.

Quantitative measurements of increase in CM-DCF fluorescence after illumination are shown in Fig. 4. After control illumination without MR, an increase in CM-DCF fluorescence was very small. The rate and the amplitude of fluorescent response were significantly enhanced by inhibitors of the respiratory chain, i.e., rotenone (not shown), piericidin, and myxothiazol (Fig. 4). The first two inhibitors prevent reduction of coenzyme Q catalyzed by Complex I, and myxothiazol is an inhibitor of Complex III. Our data indicate that the major ROS production after PDT originates from the initial segment of the respiratory chain upstream from the rotenone (piericidin) block. Increase in CM-DCF fluorescence was completely arrested by diphenyleneiodonium (DPI), an inhibitor of flavin-containing enzymes (Fig. 4), indicating that flavin of Complex I was required for ROS production. The same relationships were revealed by Kunz and coworkers in a study of ROS production by isolated mitochondria [16].

The very fact that CM-DCF fluorescence response measured 1 min after illumination and located to mitochondria showed similar inhibitor pattern as after 10 min (when fluorescence was delocalized in the whole cell) (Fig. 4) indicated that ROS production in the mitochondrial interior was catalyzed mainly by Complex I. Preincubation of the cells with MitoQ suppressed CM-DCF fluorescence immediately after illumination and during a prolonged post-illumination period (Fig. 4). These data demonstrate for the first time the antioxidant efficacy of MitoQ in a PDT model and support a suggestion that the mitochondrial interior is a major source of ROS during light-induced oxidative stress.

3.3. The PDT-induced cell death

HeLa cells loaded with MR remained viable in the dark. Mild illumination with green light did not cause immediate cell death, but after 24 h in the dark cell death with the typical

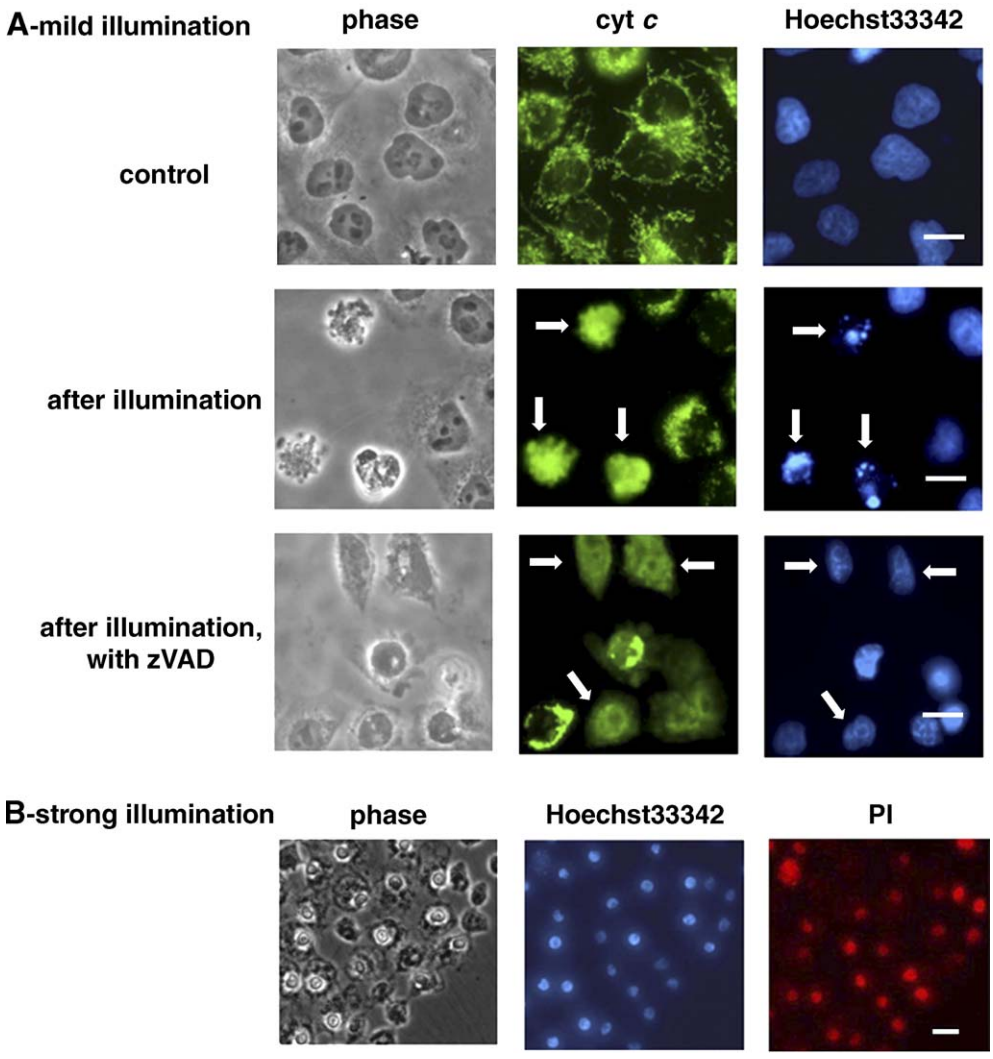

Fig. 5. MR-mediated PDT causes apoptosis or necrosis under different illumination. (A) Mild illumination induced the release of cytochrome *c* from mitochondria into the cytosol and apoptosis of HeLa cells. Cells were stained with Hoechst 33342 and antibodies to cytochrome *c* 18 h after illumination. Inhibition of caspases with zVADfmk (25 μM, 1 h before illumination) did not prevent cytochrome *c* release but blocked apoptotic fragmentation of chromatin. Arrows show cells with released cytochrome *c* and their nuclei. Scale bar, 30 μm. (B) Necrosis of HeLa cells was observed 5 h after strong illumination. Staining with Hoechst 33342 and PI. Scale bar, 30 μm.

features of apoptosis was observed (Fig. 5A). Cell death was accompanied by blebbing of the plasma membrane, externalization of phosphatidyl serine (detected by staining with annexin V, not shown), and condensation and fragmentation of chromatin. The plasma membrane remained impermeable for propidium iodide (PI) indicating the low level of necrosis (not shown). Apoptosis was prevented by pan-caspase inhibitor zVADfmk (Fig. 5A) and by overexpression of Bcl-2 (not shown). Complete release of cytochrome *c* from mitochondria into the cytosol was observed and appeared to be insensitive to zVADfmk (Fig. 5A), in contrast to various apoptotic models where massive release of cytochrome *c* was stimulated by executive caspases-3 and-7 [17].

Apoptosis induced by mild PDT with MR was not augmented by piericidin or myxothiazol. Some stimulation of apoptosis was observed with rotenone (not shown), but this effect was probably not related to inhibition of respiration but rather to suppression of cell division mediated by disturbance of the cytoskeleton [18]. Preincubation with MitoQ did not protect against cell killing either in the presence or in the absence of the respiratory inhibitors (Fig. 6). Thus induction of apoptosis did not correlate with oxidative stress detected with CM-DCF under similar conditions (see above).

Increase in the fluence of illumination from 23.4 J/cm^2 to 34.8 J/cm^2 dramatically changed the features of the cell death. In this case, massive necrosis was detected 3–5 h after illumination without any signs of apoptosis (Fig. 5B). zVADfmk did not prevent the cell death, indicating that "secondary necrosis" was not responsible for the cell killing. Overexpression of Bcl-2 also was not protective. Preincubation with MitoQ protected against necrosis and revealed some apoptosis (Fig. 6). These data represent the first example of protection against primary necrotic cell death by MitoQ. They suggest that necrosis induced by PDT depends on massive production of intramitochondrial ROS. From a practical point of view our data indicate that MitoQ and similar antioxidants could be promising co-treatment in photodynamic therapy. They do not interfere with apoptotic killing of the target cells but prevent necrosis, which is followed by inflammation of the surrounding regions of the tissue.

3.4. Oxidative stress and cell death caused by exogenous H_2O_2

It is well known that addition of H_2O_2 can cause oxidative stress and cell death since it easily permeates through the membranes. For the same reason, H_2O_2 added to a cell culture is quickly decomposed due to activity of catalase and other cellular H_2O_2-consuming systems. For example, we found that 20 μM H_2O_2 added to a culture of HeLa cells was completely decomposed in 15–20 min [19]. On the other hand, significant accumulation of ROS in the H_2O_2-treated cells was detected 45 min after addition of peroxide (Fig. 7), indicating activation of endogenous ROS production by added H_2O_2. Production of ROS was strongly stimulated by piericidin and myxothiazol (Fig. 7) and inhibited by DPI (not shown), resembling the ROS production after PDT (Fig. 4). Preincubation with MitoQ also protected against ROS production (Figs. 7 and 8).

The ROS production induced by exogenous H_2O_2 and stimulated by the inhibitors of respiration was detected not only with water-soluble dye CM-DCF, but also with the lipophilic dye C11-BODIPY (not shown). This compound is a ratiometric probe that reports on oxidative processes in membranes [20].

Thus, the data described above clearly indicated that production of ROS in mitochondria is critical for development of oxidative stress caused by a single addition of hydrogen peroxide. When cell death was analyzed under the same conditions, it was found that both apoptosis and necrosis, initiated by H_2O_2, were stimulated by the inhibitors of the respiratory chain (Fig. 9). zVADfmk prevented both modes of

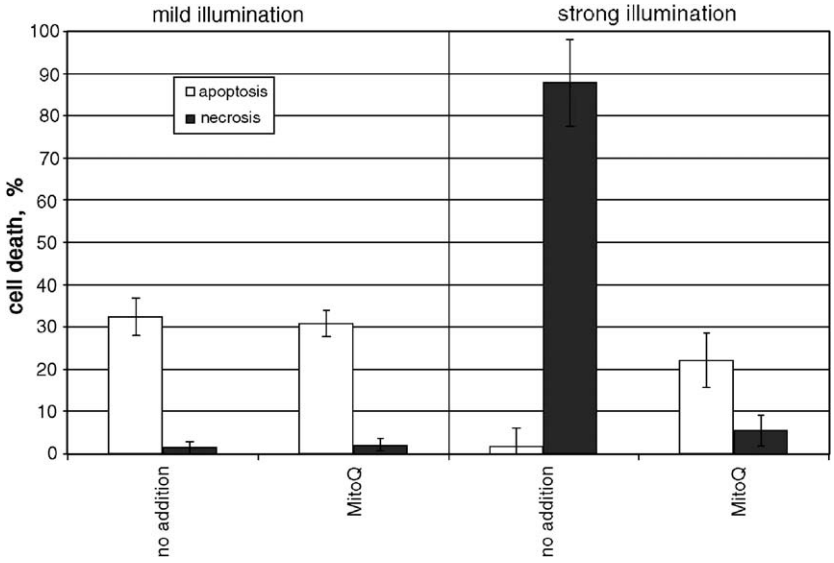

Fig. 6. MitoQ protected against necrosis and did not protect against apoptosis induced by MR-mediated PDT. HeLa cells were preincubated with MitoQ (1 μM, 1 h) loaded with MR and illuminated as in Fig. 5. Apoptosis was measured by chromatin condensation after staining with Hoechst 33342 (open columns) and necrosis was measured after staining with PI (filled columns). The averages of three experiments are presented.

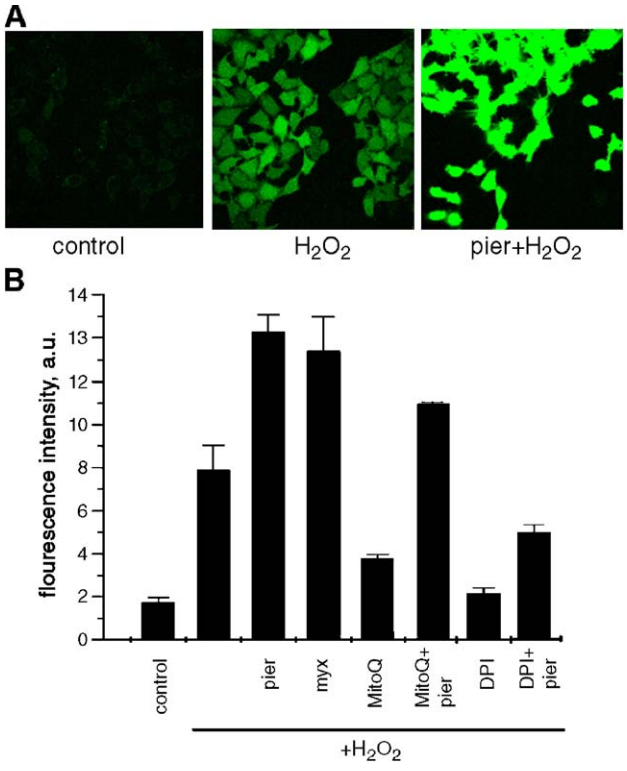

Fig. 7. Accumulation of ROS in HeLa cells after treatment with hydrogen peroxide. Cells were treated with 200 μM H_2O_2 , incubated for 45 min, washed, loaded with CM-DCFH-DA (5 μM, 15 min), and analyzed. Piericidin (Pier, 2 μM), myxothiazol (Myx, 2 μM), and DPI (10 μM) were added 1 h before peroxide. MitoQ (20 nM) was added for 8 days (2 passages). A day before addition of H_2O_2 cells were washed and cultivated for 24 h without MitoQ. (A) Images made with confocal microscope. (B) Measurements of CM-DCF fluorescence. The averages of three experiments are presented.

cell death, indicating that in this case necrosis was caspase-dependent and, probably, a secondary event, being preceded by apoptosis. Preincubation with MitoQ protected against apoptosis caused by H_2O_2 (Fig. 9). The uncoupler FCCP added during preincubation with MitoQ completely abolished the protective effect, in line with the suggestion that electrophoretic accumulation of MitoQ in mitochondria is critical for its action. MitoQ was less protective when ROS production (Figs. 7 and 8) and apoptosis (Fig. 9) was induced by combination of H_2O_2 and the respiratory inhibitors. The effect could be partly explained by strong depolarization of mitochondria caused by this combined treatment, as it was confirmed by decreased staining with TMRM (not shown). Moreover, the inhibitors could prevent regeneration of reduced MitoQ from its oxidized form.

The release of cytochrome *c* from mitochondria into the cytosol was observed during apoptosis induced by H_2O_2 (Fig. 10). It was preceded by translocation of Bax from the cytosol into mitochondria suggesting a possible role of Bax-dependent permeabilization of the outer mitochondrial membrane in release of cytochrome *c* and apoptosis (not shown). On the other hand, translocation of the both cytochrome *c* (Fig. 10) and Bax (not shown) was prevented by zVADfmk. One can suggest that "early" caspases (caspase 2, for example) are involved in the process of upstream activation of Bax and release of cytochrome *c*. Alternatively, cytochrome *c* release is activated by executive caspases 3 and 7, as it was suggested in some other models of apoptosis [17]. Anyway, the protective effect of MitoQ accumulated in mitochondria strongly supports the conclusion of a critical role of intramitochondrial ROS in apoptosis caused by exogenous hydrogen peroxide.

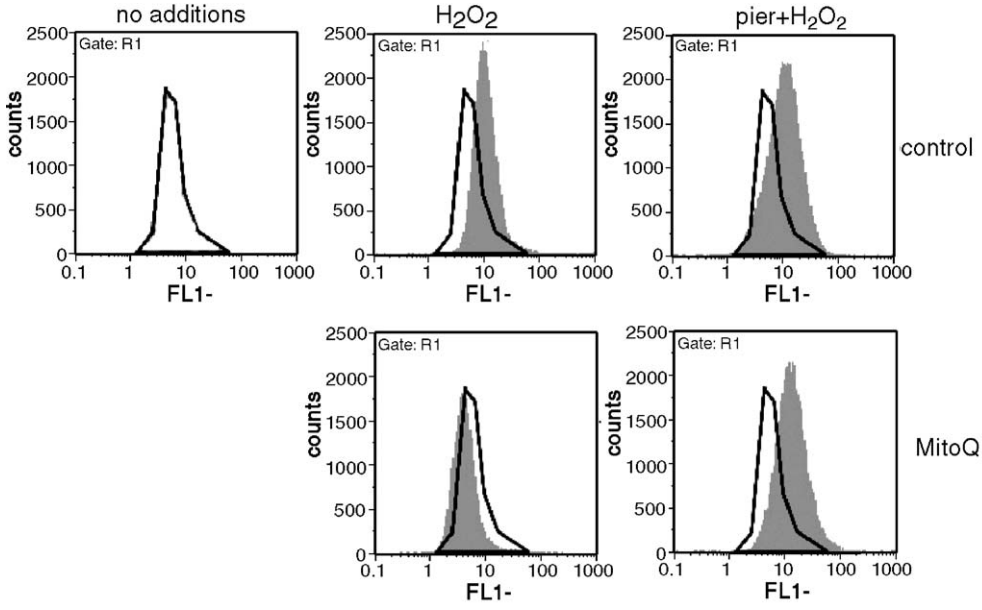

Fig. 8. MitoQ suppresses accumulation of ROS induced by H_2O_2. HeLa cells were treated and loaded with CM-DCF-DA as described in Fig. 7 and analyzed by flow cytometry. Distribution of control (non-treated) cells is shown with solid contour, peroxide-treated cells with filled gray area. A typical experiment of three experiments is presented.

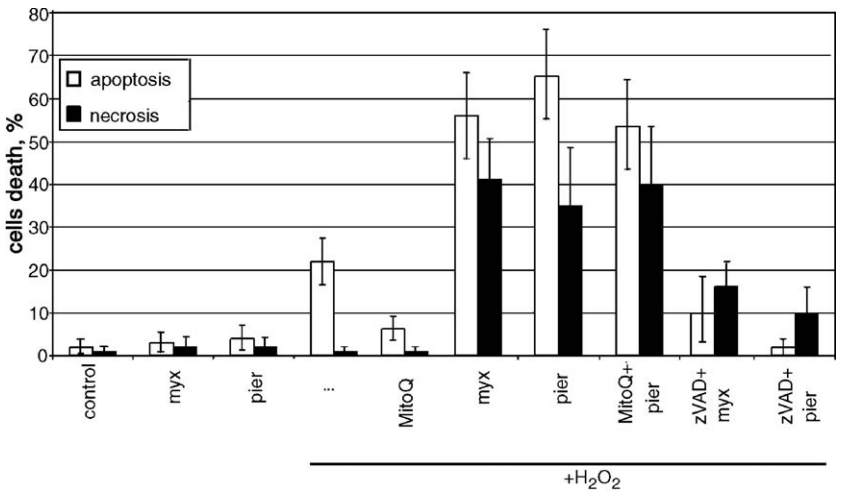

Fig. 9. Cell death caused by H_2O_2. Effects of respiratory chain inhibitors and of MitoQ. HeLa cells were treated with 200 μM H_2O_2. Apoptotic or necrotic cell death was measured after 17 h. The respiratory inhibitors and MitoQ were added as in Fig. 7. zVADfmk (100 μM) was added 1 h before H_2O_2. The averages of three experiments are presented.

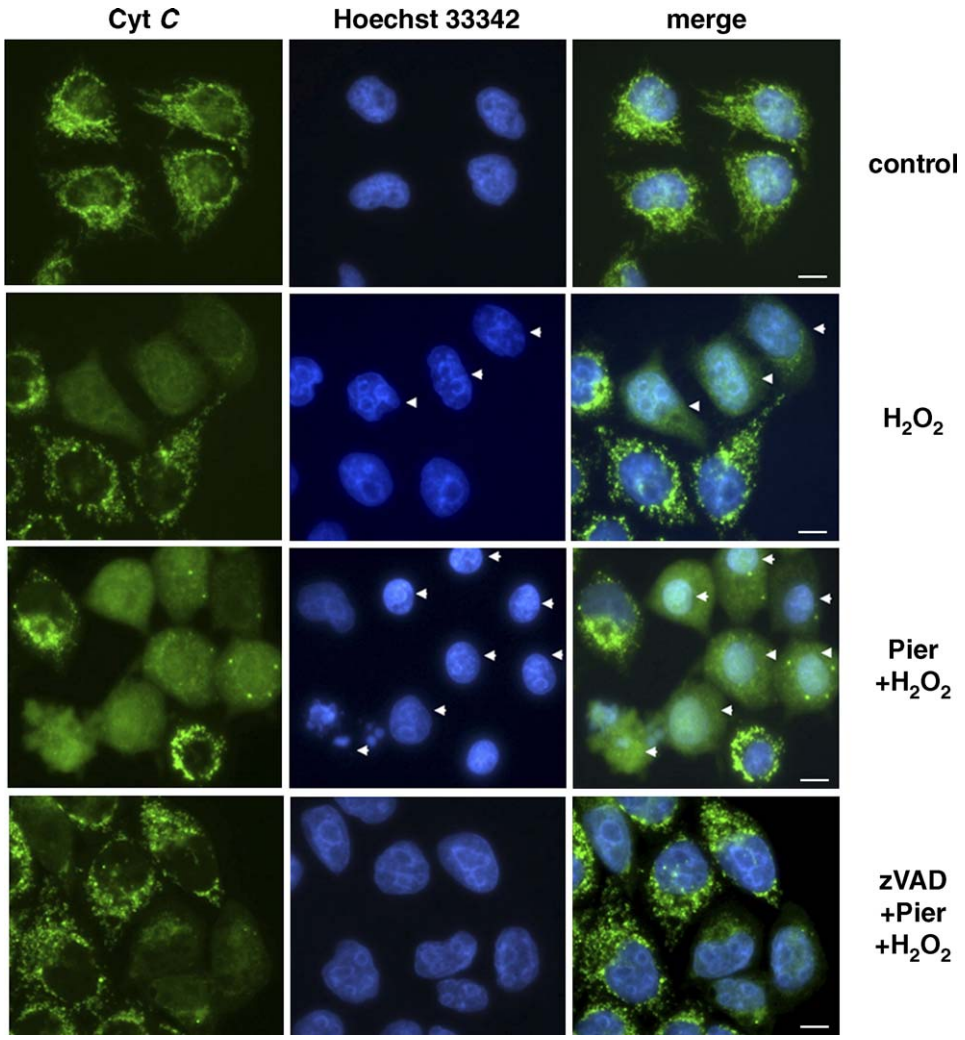

Fig. 10. Release of cytochrome *c* from mitochondria into the cytosol induced by H_2O_2. HeLa cells were treated with 200 μM H_2O_2 and analyzed after 17 h as in Fig. 5. Piericidin and zVADfmk were added as in Fig. 9. Arrowheads show apoptotic cells. Scale bar, 30 μm.

4. Discussion

The data described above indicated that active production of ROS by mitochondria can be induced by various oxidative insults and become critical for cell death. A similar phenomenon was described by Zorov et al. [21] in cardiomyocytes loaded with positively charged rhodamines and illuminated with a laser beam. The effect was coined "ROS-induced ROS release" [21]. It was suggested that ROS accumulation is mediated by the opening of the permeability transition pore (PTP) in the inner mitochondrial membrane. Rotenone blocks ROS production in Zorov's model [21]. In the two models described in this paper we did not find any indications for a role of the PTP opening in either ROS production or cell death. Cyclosporin A (CsA, an inhibitor of the PTP) alone or in combination with trifluoroperasine (a phospholipase inhibitor that promotes arrest of TNF-induced apoptosis in HeLa cells by CsA [8]) did not affect ROS production or cell death. Rotenone enhanced ROS production in both photodynamic or H_2O_2 treated cells indicating that the mechanism of "ROS-induced ROS release" catalyzed by mitochondria could be different in cardiomyocytes and in HeLa cells.

Overproduction of ROS in Complex I observed above is suggested to play a key role in some pathologies such as ischemia–reperfusion [22] or neurodegenerative diseases [23], as well as in apoptosis induced by various stimuli [24]. It was shown that rotenone-induced ROS production could initiate apoptosis [25] and suggested that endogenous rotenone-like inhibitors of Complex I could be responsible for overproduction of ROS in a Parkinson's disease model [26]. In the study of Complex I in submitochondrial membrane vesicles Vinogradov et al. [27] have shown, that it did not produce ROS due to NAD (H) dependent inhibition of this reaction. It could be suggested that ROS production in living cell is a result of specific regulation or, alternatively, of damage of Complex I. Recently it was found that caspase-dependent cleavage of the p75 subunit of this complex could be responsible for excessive ROS production during apoptosis [23]. Moreover, oxidative damage of protein components or cardiolipin [28] also could be involved in stimulation of ROS production by Complex I in ishemic/reperfused hearts. Our data indicate that photodynamic treatment and exogenous peroxide insult could be added to this list.

Acknowledgements

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References

[1] V.P. Skulachev, Mitochondrial physiology and pathology; concepts of programmed death of organelles, cells and organisms, Mol. Aspects Med. 20 (1999) 139–184.
[2] V.P. Skulachev, How to clean the direct place in the cell: cationic antioxidants as intramitochondrial ROS scavengers, IUBMB Life 57 (2005) 305–310.
[3] L.B. Chen, Fluorescent labeling of mitochondria, Methods Cell Biol. 29 (1989) 103–123.
[4] R.D. Almeida, B.J. Manadas, A.P. Carvalho, C.B. Duarte, Intracellular signaling mechanisms in photodynamic therapy, Biochim. Biophys. Acta 1704 (2004) 59–86.
[5] G.F. Kelso, C.M. Porteous, C.V. Coulter, G. Hughes, W.K. Porteous, E.C. Ledgerwood, R.A. Smith, M.P. Murphy, Selective targeting of a redox-active ubiquinone to mitochondria within cells: antioxidant and anti-apoptotic properties, J. Biol. Chem. 276 (2001) 4588–4896.
[6] M.L. Jauslin, T. Meier, R.A. Smith, M.P. Murphy, Mitochondria-targeted antioxidants protect Friedreich Ataxia fibroblasts from endogenous oxidative stress more effectively than untargeted antioxidants, FASEB J. 13 (2003) 1972–1974.
[7] O.Y. Pletjushkina, E.K. Fetisova, K.G. Lyamzaev, O.Y. Ivanova, L.V. Domnina, M.Y. Vyssokikh, A.V. Pustovidko, J.M. Vasiliev, M.P. Murphy, B.V. Chernyak, V.P. Skulachev, Long-distance apoptotic killing of cells is mediated by hydrogen peroxide in a mitochondrial ROS-dependent fashion, Cell Death Differ. 11 (2005) 1442–1444.
[8] L.A. Shchepina, O.Y. Pletjushkina, A.V. Avetisyan, L.E. Bakeeva, E.K. Fetisova, D.S. Izyumov, V.B. Saprunova, M.Y. Vyssokikh, B.V. Chernyak, V.P. Skulachev, Oligomycin, inhibitor of H+ATP-synthase, suppress the TNF-induced apoptosis, Oncogene 21 (2002) 8149–8157.
[9] T.I. Rokitskaya, M. Block, Y.N. Antonenko, E.A. Kotova, P. Pohl, Photosensitizer binding to lipid bilayers as a precondition for the photoinactivation of membrane channels, Biophys. J. 78 (2000) 2572–2580.
[10] D.V. Sakharov, E.D. Elstak, B.V. Chernyak, K.W. Wirtz, Prolonged lipid oxidation after photodynamic treatment. Study with oxidation-sensitive probe C11-BODIPY581/591, FEBS Lett. 579 (2005) 1255–1260.
[11] T. Minamikawa, A. Sriratana, D.A. Williams, D.N. Bowser, J.S. Hill, P. Nagley, Chloromethyl-X-rosamine (Mitotracker Red) photosensitizes mitochondria and induces apoptosis in intact human cells, J. Cell Sci. 112 (1999) 2419–2430.
[12] M.G. Lum, P. Nagley, Two phases of signaling between mitochondria during apoptosis leading to early depolarization and delayed cytochrome c release, J. Cell Sci. 116 (2003) 1437–1447.
[13] M. Straessler, G. Stark, Photodynamic inactivation of ion channel: gramicidin A, Photochem. Photobiol. 55 (1992) 461–463.
[14] I. Rosenthal, Phthalocyanines as photodynamic sensitizers, Photochem. Photobiol. 53 (1991) 859–870.
[15] C.S. Foote, Photosensitized oxidation and singlet oxygen: consequences in biological systems, in: W.A. Pryor (Ed.), Free Radicals in Biology, vol. II, Academic Press, New York, 1976, pp. 85–133.
[16] A.P. Kudin, N.Y. Bimpong-Buta, S. Vielhaber, C.E. Elger, W.S. Kunz, Characterization of superoxide-producing sites in isolated brain mitochondria, J. Biol. Chem. 279 (2004) 4127–4135.
[17] S.J. Riedl, Y. Shi, Molecular mechanisms of caspase regulation during apoptosis, Nat. Rev., Mol. Cell Biol. 5 (2004) 897–907.
[18] B.R. Brinkley, S.S. Barham, S.C. Barnanco, G.M. Fuller, Rotenone inhibition of spindle microtubule assembly in mammalian cells, Exp. Cell Res. 85 (1974) 41–46.
[19] O.Y. Pletjushkina, E.K. Fetisova, K.G. Lyamzaev, O.Y. Ivanova, L.V. Domnina, M.Y. Vyssokikh, A.V. Pustovidko, A.V. Alexeevsky, D.A. Alexeevsky, J.M. Vasiliev, M.P. Murphy, B.V. Chernyak, V.P. Skulachev, Biochemistry (Moscow) 71 (2006) 60–67.
[20] E.H. Pap, G.P. Drummen, J.A. Post, P.J. Rijken, K.W. Wirtz, Fluorescent fatty acid to monitor reactive oxygen in single cells, Methods Enzymol. 319 (2000) 603–612.
[21] D.B. Zorov, C.R. Filburn, L.O. Klotz, J.L. Zweier, S.J. Solott, Reactive oxygen species (ROS)-induced ROS release: a new phenomenon

accompanying induction of the mitochondrial permeability transition in cardiac myocytes, J. Exp. Med. 192 (2000) 1001–1014.
[22] A.J. Tompkins, L.S. Burwell, S.B. Digerness, C. Zaragoza, W.L. Holman, P.S. Brookes, Mitochondrial dysfunction in cardiac ischemia–reperfusion injury: ROS from complex I, without inhibition, Biochim. Biophys. Acta 1762 (2006) 223–231.
[23] J. Emerit, M. Edeas, F. Bricaire, Neurodegenerative diseases and oxidative stress, Biomed. Pharmacother. 58 (2004) 39–46.
[24] J.E. Ricci, C. Munoz-Pinedo, P. Fitzgerald, B. Bailly-Maitre, G.A. Perkins, N. Yadava, I.E. Scheffler, M.H. Ellisman, D.R. Green, Disruption of mitochondrial function during apoptosis ia mediated by caspase cleavage of the p75 subunit of complex I of the electron transport chain, Cell 117 (2004) 773–786.
[25] N. Li, K. Ragheb, G. Lawler, J. Sturgis, B. Raiwa, J.A. Melendez, J.P. Robinson, Mitochondria complex I inhibitor rotenone induces apoptosis through enhancing mitochondrial reactive oxygen species production, J. Biol. Chem. 278 (2003) 8516–8525.
[26] J.T. Greenamyre, T.B. Sherer, R. Betarbet, A.V. Panov, Complex I and Parkinson's disease, IUBMB Life 52 (2001) 135–141.
[27] A.D. Vinogradov, V.G. Grivennikova, Generation of superoxide-radical by the NADH:ubiquinone oxidoreductase of heart mitochondria, Biochemistry (Moscow) 70 (2005) 120–127.
[28] G. Paradies, G. Petrosillo, M. Pistolese, N. Di Venosa, A. Federici, F.M. Ruggiero, Decrease in mitochondrial complex I activity in ischemic/reperfused rat heart: involvement of reactive oxygen species and cardiolipin, Circ. Res. 94 (2003) 53–59.