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Effect of oxidative stress on dynamics of mitochondrial reticulum

O.Yu. Pletjushkina*, K.G. Lyamzaev, E.N. Popova, O.K. Nepryakhina, O.Yu. Ivanova, L.V. Domnina, B.V. Chernyak, V.P. Skulachev

A. N. Belozersky Institute, Moscow State University, 119992 Moscow, Russia

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Abstract

Fission of the mitochondrial reticulum (the thread–grain transition) and following gathering of mitochondria in the perinuclear area are induced by oxidative stress. It is shown that inhibitors of the respiratory chain (piericidin and myxothiazol) cause fission of mitochondria in HeLa cells and fibroblasts, whereas a mitochondria-targeted antioxidant (MitoQ) inhibits this effect. Hydrogen peroxide also induced the fission, which was stimulated by the inhibitors of respiration and suppressed by MitoQ. In untreated cells, the mitochondrial reticulum consisted of numerous electrically-independent fragments. Prolonged treatment with MitoQ resulted in drastic increase in size and decrease in number of these fragments. Local photodamage of mitochondria caused immediate depolarization of a large fraction of the mitochondrial network in MitoQ-treated cells. Our data indicate that the thread–grain transition of mitochondria depends on production of reactive oxygen species (ROS) in initial segments of the respiratory chain and is a necessary step in the process of elimination of mitochondria (mitoptosis).

Keywords: Metochondrial reticulum; Fission; Fusion; Metochondrial inhibitors; Reactive oxygen species (ROS); Antioxidants

1. Introduction

Mitochondria in various cell types form a dynamic reticulum [1], which has been suggested to play a role of a cable-like power transporting system of the cell [2]. Respectively, fission of the mitochondrial reticulum (the thread–grain transition) observed in response of local damage of the reticulum [3] was assumed to prevent a short-circuit collapse of membrane potential in the whole network [4]. Moreover, fission and fusion of mitochondria are important for the proper distribution of mitochondria during cell division. It was recently found that fission of mitochondria accompanied early steps of apoptosis, and inhibition of fission delayed cytochrome *c* release into the cytosol and the following apoptotic events [5]. On the other hand, even prolonged fission did not cause the release of cytochrome *c* ([6] and see below) and inhibition of fission was found to suppress a cytochrome *c*-independent apoptosis in *Caenorhabditis elegans* [7]. The role of mitochondrial dynamics in apoptosis remains obscure.

In our studies, fission of mitochondria induced by inhibitors of bioenergetic functions has been described [6]. Effects of uncouplers [8] and respiratory inhibitors [9] were found to be mediated by the dynamin-related GTPase DRP-1 in the same way as described for apoptosis-related fission [5]. We suggested that reactive oxygen species (ROS) produced by the respiratory chain inside mitochondria could also induce mitochondrial fission [6]. It was hypothesized that fission could be involved in elimination of ROS-producing mitochondria (this process was named “mitoptosis”) participating in multi-faceted defense of the cell against oxidative damage [10].

In the experiments described below, we have investigated the effect of mitochondrial ROS production on mitochondrial dynamics and location of mitochondria in the cell.

Abbreviations: DRP-1, dynamin-related protein-1; ROS, reactive oxygen species; EYFP, enhanced yellow fluorescent protein; MtPTP, mitochondrial permeability transition pore; MitoQ, mitochondria-targeted antioxidant, 10-(6′-ubiquinolyl)decyltriphenilphosphonium; CsA, cyclosporine A; FCCP, trifluoromethoxycarbonylcyanidephenylhydrazone; DNP, dinitrophenol; TMRM, tetramethylrhodamine methyl ester; CytC, cytochrome C; MAPK, mitogene activated protein kinase; ZVADfmk, carbobenzoxy’Val–Ala–Asp–fluoromethyl ketone; Myx, myxothiazol

* Corresponding author.
E-mail address: pletjush@genebee.msu.ru (O.Y. Pletjushkina).

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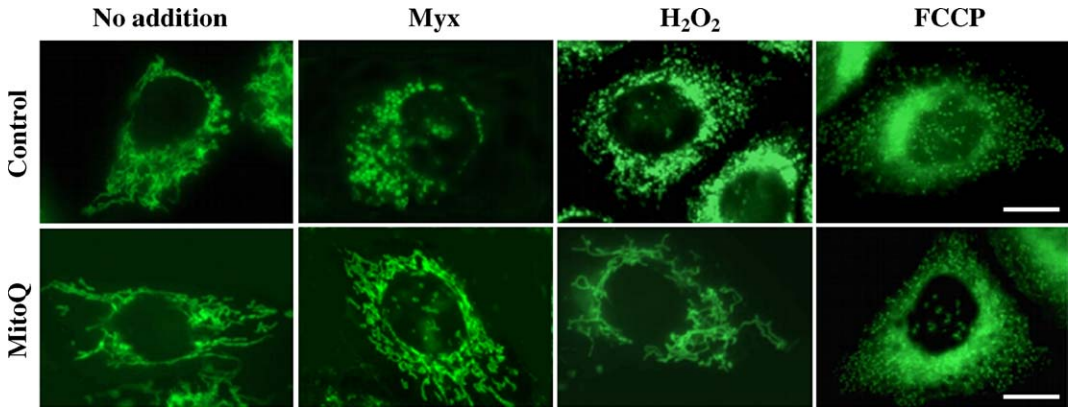

Fig. 1. Fission of mitochondrial reticulum in HeLa cells induced by a respiratory chain inhibitor, uncoupler, or hydrogen peroxide. HeLa cells transfected with Mito-EYFP were treated with myxothiazol (Myx, 2 μM), H_2O_2 (50 μM), or FCCP (10 μM) for 15 h where indicated; the cells were treated with 20 nM MitoQ for 7 days (see Materials and methods). Scale bar 15 μm.

2. Materials and methods

2.1. Cell culture and transfections

HeLa cells and CV1 cells were grown in a Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 U/ml streptomycin, and 100 U/ml penicillin (all from Gibco, CA). Cells were transfected with Mito-EYFP construct (Clontech, USA) containing enhanced yellow fluorescent protein (EYFP) fused with the mitochondrial localization signal of subunit VIII of cytochrome *c* oxidase. Transfections were performed using Lipofectamine 2000 reagents according to the manufacturer's protocol (Invitrogen). The transfected cells were transferred to DMEM containing 500 μg/ml geneticin (Invitrogen). HeLa cells were grown with 20 nm MitoQ in complete medium for 5–7 days (once reseeded) and plated on coverslip glasses 2 days before experiments without MitoQ.

2.2. Chemicals

Unless stated otherwise, all reagents were from Sigma-Aldrich, St. Louis, MO. To affect mitochondrial function, we applied a panel of well characterized inhibitors: (1) mitochondrial electron transport was inhibited at levels of Complex I or Complex III with piericidin (4 μM) and antimycin (2 μM) or myxothiazol (2 μM); ATP synthase was blocked with oligomycin (5 μg/ml); aurovertin B (10 μM), ouabain (0.1 μM), mtPTP was inhibited with CsA (5 μM, Calbiochem); and electron transport was uncoupled from oxidative phosphorylation with FCCP (10 μM) or 2,4 DNP (0.4 mM). MitoQ was synthesized by a modification of the method described in [11].

2.3. Visualization of mitochondria

Mitochondria were visualized with the mitochondria-specific dyes, TMRM and MitoTracker Green (all from Molecular Probes, Eugene, OR) or antibodies against cytochrome *c* (6H2.B4 BD Pharmingene). HeLa and CV1 cells with stable expression of Mito-EYFP were used in some experiments. Images were analyzed with a Zeiss Axiovert microscope and with a Zeiss LSM 510 confocal microscope.

2.4. Immunofluorescence

Cells grown on glass cover slips were fixed with 3.7% formaldehyde in phosphate-buffered saline solution (PBS) for 15 min at room temperature, treated as above, and stained with anti-cytC monoclonal antibodies, with anti-BAX polyclonal antibodies (13666E BD Pharmingene), falloidin-TRITC (actin filaments), and Hoechst 33342 (nuclei) [12].

2.5. Local photodamage of mitochondria

Cells grown on glass cover slips were loaded with TMRM (200 nM) for 15 min at 37 °C, then washed with PBS, transferred to complete medium, and placed onto a heated microscope stage. Cells were imaged using the Zeiss LSM 510 confocal microscope. All imaging was done with a 100× oil immersion objective lens. Mitochondria were photodamaged with an argon laser, the treated region being 6 × 60 pixels.

3. Results and discussion

3.1. Inhibition of respiration caused fission of the mitochondrial reticulum

In HeLa cells most mitochondria had an elongated, tubular shape. Often, mitochondria were organized into networks, particularly in the perinuclear region. We have shown that the inhibitors of respiratory chain, piericidin and myxothiazol

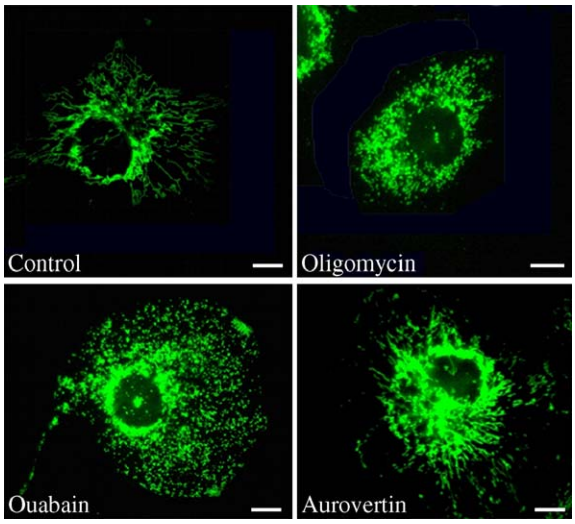

Fig. 2. Fission of mitochondrial reticulum in epithelial CV-1 cells induced by inhibitors of mitochondrial ATP synthase and Na,K-ATPase. The CV-1 cells transfected with Mito-EYFP were treated with oligomycin (5 μg/ml), aurovertin B (10 μM), or oubain (0.5 mM) for 24 h. Scale bar 20 μm.

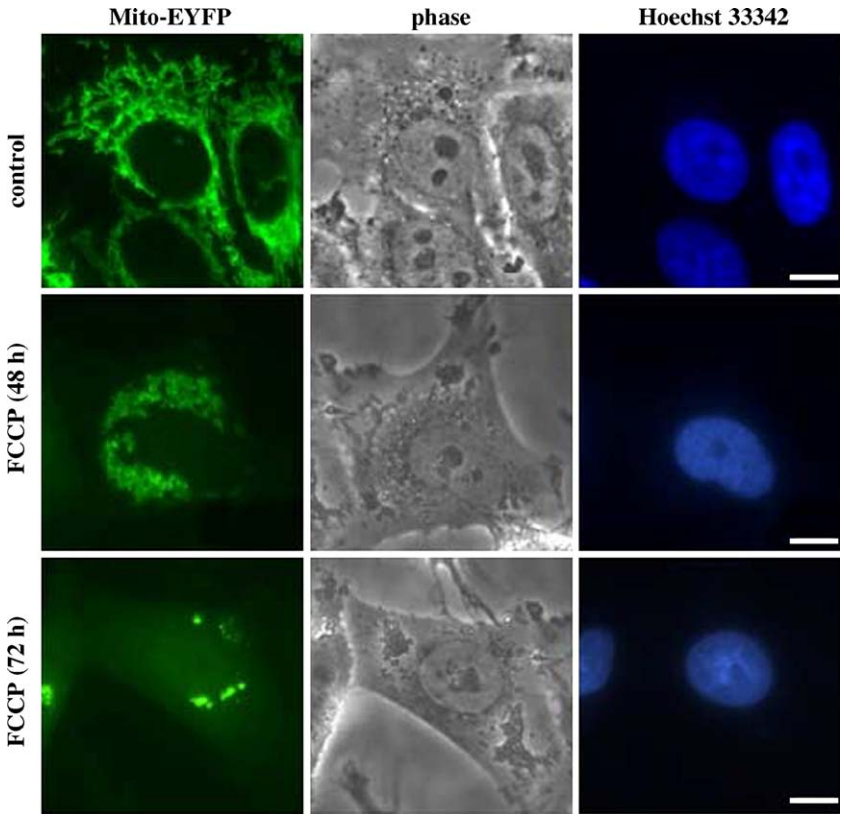

Fig. 3. Dynamics of mitochondria during prolonged treatment FCCP. HeLa cells transfected with Mito-EYFP cells were treated with FCCP (10 μM) for 48 h and 72 h. Nuclei were stained with Hoechst 33342. Scale bar 15 μm.

(inhibitors of complexes I and III, respectively) induce fission of the mitochondrial reticulum (the thread–grain transition) in HeLa cells within 15 h (Fig. 1). Oligomycin (an inhibitor of F_0F_1-ATP synthase) was ineffective, indicating that cessation of ATP synthesis was not critical for fission of mitochondria (not shown in the figures).

In epithelial CV-1 cells, the respiratory inhibitors rotenone and myxothiazol also induced fission of the mitochondrial reticulum. However, in contrast to HeLa, oligomycin caused rapid fission of mitochondria in these cells. The same phenomenon was described recently by K. De Vos et al. [9] and was interpreted as an effect mediated by inhibition of

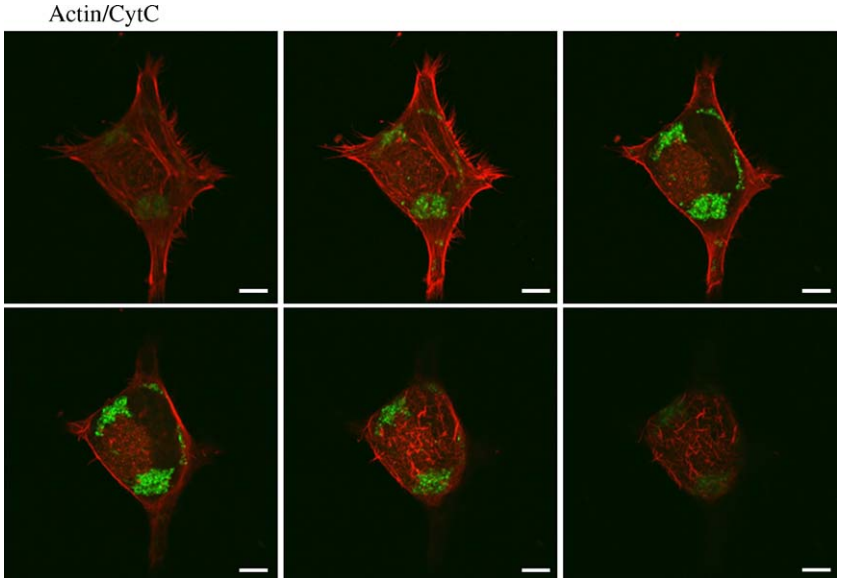

Fig. 4. Structure of actin filaments and localization of cytochrome *c* in cells during mitoptosis. HeLa cells were treated with FCCP (10 μM) for 72 h, washed, and cultured for 48 h without inhibitors. Actin (red) and cytochrome *c* (green) distribution was analyzed using a confocal microscope. Optical slices with the steps of 10 μm are presented. Scale bar 10 μm.

mitochondrial ATP synthase. We found that this is not the case. When we used aurovertin B, another effective inhibitor of ATP synthase, no effect on mitochondrial morphology was observed. Unlike aurovertin, oligomycin is known to have, besides ATP synthase, one more non-mitochondrial target in the cell, namely Na/K-ATPase of the plasma membrane. The specific inhibitor of Na/K-ATPase oubain caused fission of mitochondria in CV-1 (Fig. 2) but not in HeLa (not shown) indicating that ion homeostasis controlled by Na/K-ATPase is critical to support tubular mitochondria and inhibition of this enzyme with oligomycin underlie fission of mitochondria in some cell lines like CV-1.

We have suggested that fission of mitochondria induced by the inhibitors of respiration was mediated by increased production of reactive oxygen species (ROS) in the initial and middle segments of the respiratory chain. Fluorescent ROS-sensitive dyes did not reveal significant increase in ROS production immediately after treatment of the cells with piericidin or myxothiazol (not shown). However, we found that these inhibitors strongly enhance the level of ROS under conditions of exogenous oxidative stresses (Chernyak et al., this issue). It could be suggested that during prolonged incubation with the respiratory inhibitors low amounts of ROS produced in mitochondria initiating endogenous oxidative stress resulted in additional ROS production due to a self-amplification mechanism (ROS-induced ROS release [13]).

3.2. Changes of location of mitochondria following their fission

Large-scale fission of mitochondria has usually been observed in models of apoptosis. We have shown earlier that respiratory inhibitors and uncouplers did not reduce viability of HeLa cultivated in the presence of glucose for 48 h. In the following experiment we have took advantage of this model for investigation of the long-term consequences of mitochondrial fission. The most rapid fission of mitochondria was induced by uncouplers. In the final steps of the treatment, the fragmented mitochondria gathered near the nucleus and formed several aggregates (Fig. 3). When HeLa cells were treated for 72 h with FCCP or DNP, a fraction of the cells (50–60%) died by apoptosis, but the rest of the population was viable, without any signs of apoptosis (normal nucleus, no annexin V staining). Neither actin filaments structure (Fig. 4) nor microtubular cytoskeleton (not shown) were significantly modified. Even the initial events of apoptosis were not initiated: Bax remained in cytosol, cytochrome *c* remained inside mitochondria for more than 48 h after complete fission of the mitochondria (Fig. 5). Staining of mitochondria with anti-cytochrome oxidase and Mitotracker Green (not shown) revealed the same distribution as anti-cytochrome *c* staining. Gathering of mitochondria in the perinuclear area was observed earlier during apoptosis [6]. This phenomenon was attributed to inactivation of kinesin (a motor molecule for movement of organelles to the cellular periphery) while dynein (a motor for movement in the opposite direction) remained active [14]. Kinesin could be inactivated by the kinase of its light chain, activated by p38 kinase (MAPK) [14]. Uncoupler-induced gathering of mitochondria was not related to apoptotic events and was not prevented by the inhibitor of p38 kinase or by pan-caspase inhibitor zVADfmk (not shown).

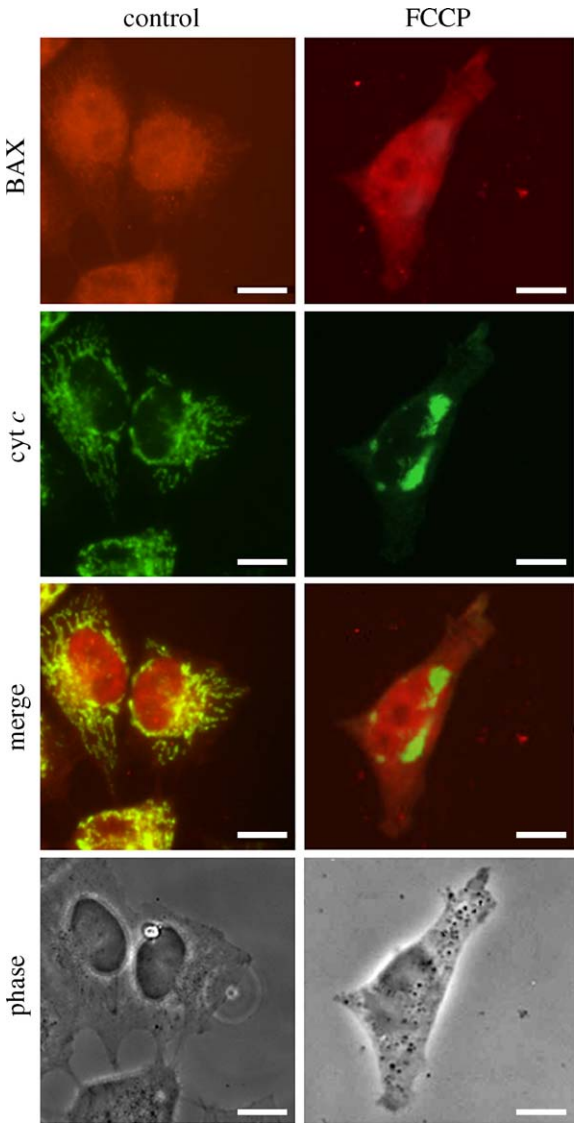

Fig. 5. Localization of cytochrome *c* and Bax in cells during mitoptosis. HeLa cells were treated with FCCP (10 μM) for 72 h, washed, and cultured for 48 h without inhibitors. Scale bar 15 μm.

We found earlier that uncoupler-induced fission of mitochondria resulted in decrease in the total amount of mitochondrial material [15]. This phenomenon (postulated earlier and named “mitoptosis” [10]) was suggested to protect cells against excessive endogenous ROS production [16].

3.3. Protective effect of mitochondria-targeted antioxidant MitoQ on structure of mitochondrial reticulum

The hypothesis on ROS-induced fission of mitochondria was tested using the mitochondria-targeted antioxidant 10-(6′-ubiquinolyl)decyltriphenylphosphonium (MitoQ) introduced by M. P. Murphy, R.A. Smith, and coworkers [11]. This compound is accumulated in the mitochondrial matrix due to the positive charge and its quinone residue is reduced by

the respiratory chain after ROS-dependent oxidation making this antioxidant renewable and very effective. It was shown that MitoQ protects cells from various oxidative stresses at very low (nanomolar) concentrations [17,18]. MitoQ also inhibits accumulation of mitochondrial ROS induced by oxidative stresses (Chernyak et al., this issue).

Prolonged incubation of HeLa cells and normal fibroblasts (data not shown) with 20 nM MitoQ caused significant development of mitochondrial reticulum, initiating the grain–thread transition. As to fission of mitochondria induced by inhibitors of the respiratory chain, it was strongly inhibited by MitoQ (Figs. 1 and 5). Unfortunately, we were not able to prove the dependence of the protective effect on targeting of MitoQ to mitochondria using protonophorous uncouplers for prevention MitoQ accumulation. Uncouplers (FCCP, DNP) induced rapid (in 1–2 h) fission of mitochondria. This effect was accompanied by swelling of mitochondria and probably was mediated by a ROS-independent mechanism. Fission of mitochondria by uncouplers was described earlier and it was suggested that it was mostly due to an inhibition of fusion [8]. In our cell models, the protective effects of MitoQ against apoptotic cell death and endogenous ROS production were always prevented by uncouplers (Chernyak et al., this issue) and have the same time and concentration-dependences as fission of mitochondria. Thus, we have suggested that MitoQ protect mitochondria against fission by scavenging of ROS in the mitochondrial interior (Fig. 6).

3.4. Hydrogen peroxide causes fission of mitochondria due to induction of mitochondrial ROS production

Further support for the key role of intramitochondrial ROS in fission of the mitochondrial reticulum was obtained in experiments on exogenous hydrogen peroxide insult. In this case, the fission was observed 4 h after a single addition of 50 μM H_2O_2 (Fig. 1). We have shown elsewhere (Chernyak et al., this issue) that this treatment initiated some endogenous ROS production that was stimulated by piericidin and myxothiazol and was inhibited by MitoQ. In line with our hypothesis, H_2O_2-induced fission of mitochondria was inhibited by MitoQ (Figs. 1 and 5). Piericidin and myxothiazol accelerated H_2O_2-induced fission, MitoQ being protecting also in this case (Fig. 5). Unfortunately, the last observation could not be unequivocally interpreted since the combined treatment with H_2O_2 and inhibitors caused significant depolarization of mitochondria (not shown). MitoQ prevented depolarization, indicating the key role of mitochondrial ROS, but an indirect effect of ROS on fission could not be excluded. This was not the case in experiments where fission of mitochondria was induced by a respiratory inhibitor or H_2O_2 alone since none of them caused significant depolarization.

The possible mechanism of ROS-dependent fission of mitochondria consists in opening of the permeability transition pore (PTP), which entails swelling of the matrix, cristae remodeling [16] and disruption of the outer mitochondrial membrane. We applied cyclosporin A (CsA) as an inhibitor of the pore and did not observe any protection of mitochondria in HeLa cells treated with respiratory inhibitors or H_2O_2 (not shown). Surprisingly, in epithelial CV-1 cells, we have found that CsA induced rapid fission of mitochondria (De Vos et al. [9] observed the same phenomenon). CsA interacts with cyclophilins and in particular the mitochondrial member of this family (CypD), which is involved in PTP opening [19]. The other cyclophilins outside mitochondria also tightly bind CsA, resulting in inhibition of calcineurin, a protein phosphatase involved in regulation of immune response and some other processes unrelated to mitochondria. To clarify the situation, we have applied the analogs of CsA, 4-MeVal-CsA and Sanglifehrin, which are effective inhibitors of CypD and

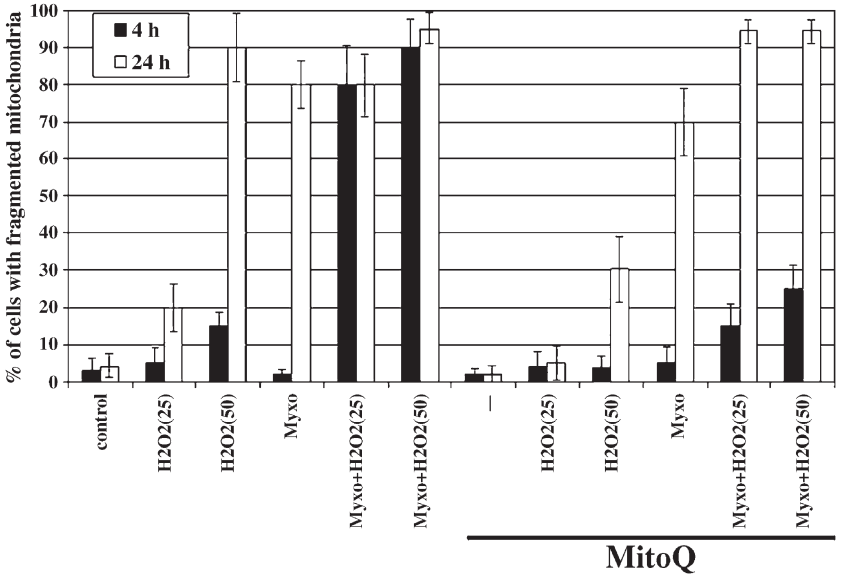

Fig. 6. Combined action of hydrogen peroxide and inhibitor of respiration on fission of mitochondria. Effect of 20 nM MitoQ. HeLa cells transfected with Mito-EYFP were treated with myxothiazol (Myx, 2 μM) or H_2O_2 (25 μM or 50 μM) for 4 h (dark columns) and 24 h (empty columns). The fraction of cells where the mitochondrial population was mainly in a fragmented state was calculated by analyzing 100–200 cells in three independent experiments.

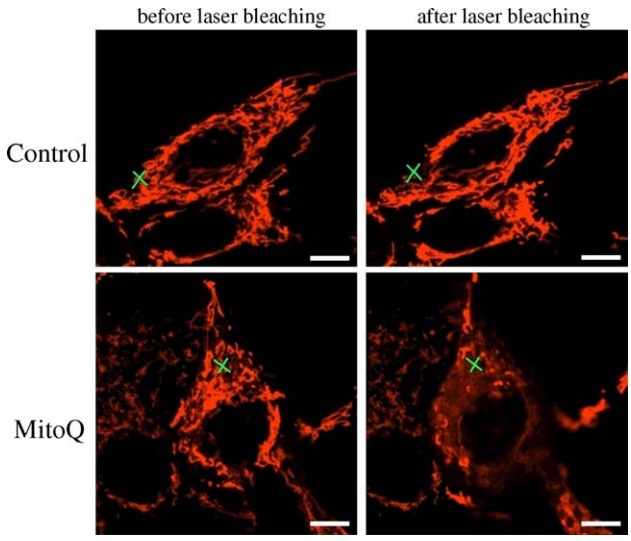

Fig. 7. Depolarization of mitochondrial reticulum with local photodamage. HeLa cells loaded with TMRM (100 nM, 15 min) were illuminated as described in Materials and methods. The center of the region of illumination is marked with cross. Images were obtained 15 min after illumination. Cells were pretreated with MitoQ as in Fig. 1. Scale bar 15 μm.

the PTP opening being ineffective in inhibition of calcineurin [20]. No fission was observed (not shown). It was suggested that calcineurin-dependent processes are somehow involved in regulation of mitochondrial fission in CV-1 cells. Probably these pathways are related to Na/K-ATPase-supported ion flows, which affect mitochondrial dynamics in these cells but not in HeLa cells (see above).

3.5. ROS-dependent functional changes of mitochondrial reticulum

Earlier it was suggested that the structural changes in the mitochondrial reticulum could be correlated with the size of electrically-connected areas (electrical syncytium) of the mitochondrial network [2]. It was found that fission of such a syncytium resulted in formation of numerous small electrically independent organelles. We have used the same experimental approach based on local photodamage of mitochondria to study ROS-dependent functional changes in the mitochondrial reticulum.

It was shown that local photodynamic damage of rhodamine-loaded tubular mitochondria in HeLa cells, using a laser focused by the confocal microscope, resulted in depolarization (detected by release of rhodamine from mitochondria) of a limited number of mitochondria closest to the region of damage (Fig. 7). This indicated that the size of electrically-connected mitochondrial clusters in the HeLa cells is rather small. When the cells were pretreated with MitoQ, the area of depolarization around local photodamage drastically increased and reached 30–50% of total mitochondrial population. Clusters of electrically connected mitochondria were described earlier in fibroblasts [3]. We have repeated this experiment and found that the clusters are larger in these cells than in HeLa cells, but the effect of MitoQ is still clearly visible (not shown). These data indicate that mitochondrial ROS production can modulate mitochondrial dynamics (structural and functional) both under stress and normal conditions in the cell culture. It is necessary to note that during cultivation cells are subjected to 3–4 times higher oxygen tension than in vivo. An attractive possibility is that mitochondria-targeted antioxidants could improve functioning of the mitochondrial reticulum not only in cell cultures but also in normal tissues. This effect could be used for therapy of the pathologies where excessive fission of mitochondria plays a key role.

Acknowledgements

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