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Interrelations of mitochondrial fragmentation and cell death under ischemia/reoxygenation and UV-irradiation: Protective effects of SkQ1, lithium ions and insulin

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Abstract Mitochondria-targeted antioxidant 10-(6-plastoquinonyl)decyltriphenyl-phosphonium (SkQ1) as well as insulin and the inhibitor of glycogen-synthase kinase, Li^+ are shown to (i) protect renal tubular cells from an apoptotic death and (ii) diminish mitochondrial fission (the thread-grain transition) induced by ischemia/reoxygenation. However, SkQ1 and LiCl protected the mitochondrial reticulum of skin fibroblasts from ultraviolet-induced fission but were ineffective in preventing a further cell death. This means that mitochondrial fission is not essential for apoptotic cascade progression.

Keywords: Mitochondria; Fission; Fusion; Apoptosis; Ischemia/reoxygenation

1. Introduction

Mitochondria are known to be critical intracellular elements integrating a complex system of apoptotic and surviving signaling pathways (i.e., see [1]). Mitochondrial bioenergetics and involvement in the cell death regulation are tightly linked with mitochondrial structure through the balance of mitochondrial fragmentation (fission) [2] and fusion (the thread-grain transition [2]).

These intracellular organelles are very dynamic [2–4]. Their fusion and fission are apparently required for normal mitochondrial functioning including stability of the mitochondrial genome [5], lower heterogeneity of transmembrane potential in the mitochondrial population within the cell, optimal oxygen consumption as well as adequate energy supply and transmission [6].

Mitochondrial fission is speculated to prevent the collapse of the entire mitochondrial network through the isolation of damaged compartments and occurs under various stressful conditions including oxidative stress [7]. Frequently, mitochondrial fission is associated with induction of apoptosis [2,8,9], moreover, it occurs in parallel with successive changes in mitochondrial ultrastructure [10]. The basic steps of this pathway are: disintegration of the space-orientated mitochondrial reticulum into small fragments, swelling of mitochondrial matrix and the release of pro-apoptotic factors (cytochrome *C*, AIF, etc.) [11,12].

While mitochondrial fission plays a pro-apoptotic role, mitochondrial fusion may protect the cell from the death. Particularly, overexpression of mitofusins (Mfn) may diminish the probability of apoptosis [13], while depletion of OPA1 or Mfn enhances the sensitivity to apoptotic stimuli [14,15].

However, there is some evidence that mitochondrial fission and apoptotic cell death may be regulated by separate signaling mechanisms. It has been demonstrated that replacement of Bcl-2 by a homologous protein, CED-9 in mammalian cells protects mitochondrial reticulum from fragmentation but does not protect against the cytochrome *C* release from mitochondria induced by different stimuli [16].

In this study, we explored the possibility of a divergence of signaling pathways of mitochondrial fragmentation and cell death under various pro-apoptotic stimuli. As tentative protective agents we used (i) lithium ions and insulin, involved in the glycogen-synthase kinase-3β (GSK-3β) protecting cascade and having as end-effector the mitochondrial permeability transition pore complex [17], and (ii) mitochondria-targeted antioxidant 10-(6-plastoquinonyl)decyltriphenyl-phosphonium (SkQ1) [18].

2. Materials and methods

2.1. Primary renal tubular epithelium and fibroblasts cell cultures

Kidneys were excised aseptically from 3 to 7-day-old rats, then homogenized and placed in the balanced Hanks solution, pH 7.4. After several washes the dispensed tissue was placed in 0.1% collagenase and incubated for 20–30 min at 37 °C. Large pieces were removed, and cells were sedimented at $1000 \times g$ for 3 min. The pellet was resuspended in DMEM/F-12 1:1 containing 10% fetal calf serum (FCS) and seeded in cultural plates and glass-bottom dishes. Cells were cultivated in CO_2 (5%) incubator for 1–2 days before the experiments.

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Abbreviations: SkQ1, 10-(6-plastoquinonyl)decyltriphenyl-phosphonium; ROS, reactive oxygen species; GSK, glycogen-synthase kinase; TMRE, tetramethylrhodamine ethyl ester; UV, ultraviolet

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Human fetal dermal fibroblasts (obtained from elective termination of pregnancies following local ethical approval; gestation <12 weeks) were established by explanting tissue. Cells were grown in DMEM medium supplemented with 10% FCS, 2 mM glutamine and 100 U/ml penicillin.

Cell viability was evaluated by a standard MTT-test. Micromodification of the method was used for 96-well plates for microcultivation [19].

2.2. Ischemia modeling

Renal cell culture was washed from the cultivated medium using Hanks solution followed by bubbling with pure nitrogen for 15 min. The cells were incubated in anoxic solution for 24 h at 37 °C.

2.3. Ultraviolet (UV) irradiation

The UV source was a TL20W/12 bulb (Philips, The Netherlands) with emission at 280–370 nm. UV exposure was performed in culture plates or dishes containing prewarmed PBS. No increase in temperature was observed during irradiation. The irradiation doses were chosen to achieve appropriate cell death. This resulted in an experimental model using 60 and 30-s irradiation.

2.4. Evaluation of the mitochondrial state in the kidney cells

Mitochondrial transmembrane potential $\Delta\psi$ was evaluated with tetramethylrhodamine ethyl ester (TMRE), a probe having a lypophilic monocationic structure with delocalized charge which makes it permeable to the mitochondrial membrane for its translocation into mitochondrial matrix [20] according to $\Delta\psi$ on the inner mitochondrial membrane.

The cell culture was stained with 200 nM TMRE immediately after reoxygenation started in a medium DMEM/F-12 supplemented with 20 mM HEPES. Kidney cells were examined using the laser scanning confocal microscope LSM510 (Carl Zeiss, Germany) with the laser excitation at 543 nm and emission in the 560–590 nm range. Image analysis was performed using ImageJ (W. Rasband, Bethesda, USA, http://rsb.info.nih.gov/ij). For the quantitative analysis of the mitochondrial shapes we used the function Analyze Particles in a software package ImageJ. Confocal images were analyzed and determined a weighted size (square) and a number of mitochondrial fragments in the cells (average number of fragments per cell). From 12 to 14 confocal images per each group were analyzed with 8–12 cells in average per each image.

2.5. Flow cytometry

Ischemia-induced cell death was analyzed by staining with Annexin V-FITC. After 24 h ischemia following by a 24 h reoxygenation, the cultural supernatant was collected and attached cells were dissociated with 0.25% trypsin and combined with supernatant. The cell suspension was centrifuged at 2000 × g for 5 min. The pellet was resuspended with Annexin V-FITC (with the dilution of the stock solution according to the manufacturer recommendation) on DMEM with 0.1% NaN_3. After 20-min incubation, the cells were washed out of the dye and analyzed in a flow cytometer CyFlow (Partec GmbH, Germany).

2.6. Fluorescence evaluation

Total fluorescence of the mitochondrial dye, TMRE was measured by the universal microplate fluorescence reader Zenyth (Anthos Labtec, Austria). The cells were cultivated in a 96-well plate, exposed to 24 h ischemia and further stained with 200 nM TMRE for 30 min. Thereafter, the cells were washed out of the dye with fresh medium and analyzed in a plate reader under "bottom fluorescence" protocol at excitation filter 535 nm and emission filter 595 nm; integration time 0.4 s. Twenty wells of 96-well plate were taken for each experiment; all experiment were repeated three times.

2.7. Chemicals

TMRE was from Molecular Probes (Oregon, USA) LiCl and Annexin V-FITC were from Sigma–Aldrich, insulin was from Novo Nordisk (Denmark), SkQ1 (chemical formula is shown in Fig. 1) was synthesized in the Center of Mitoengineering (Moscow, Russia) according to Skulachev [21]. Cultural media and serum were from Gibco (USA).

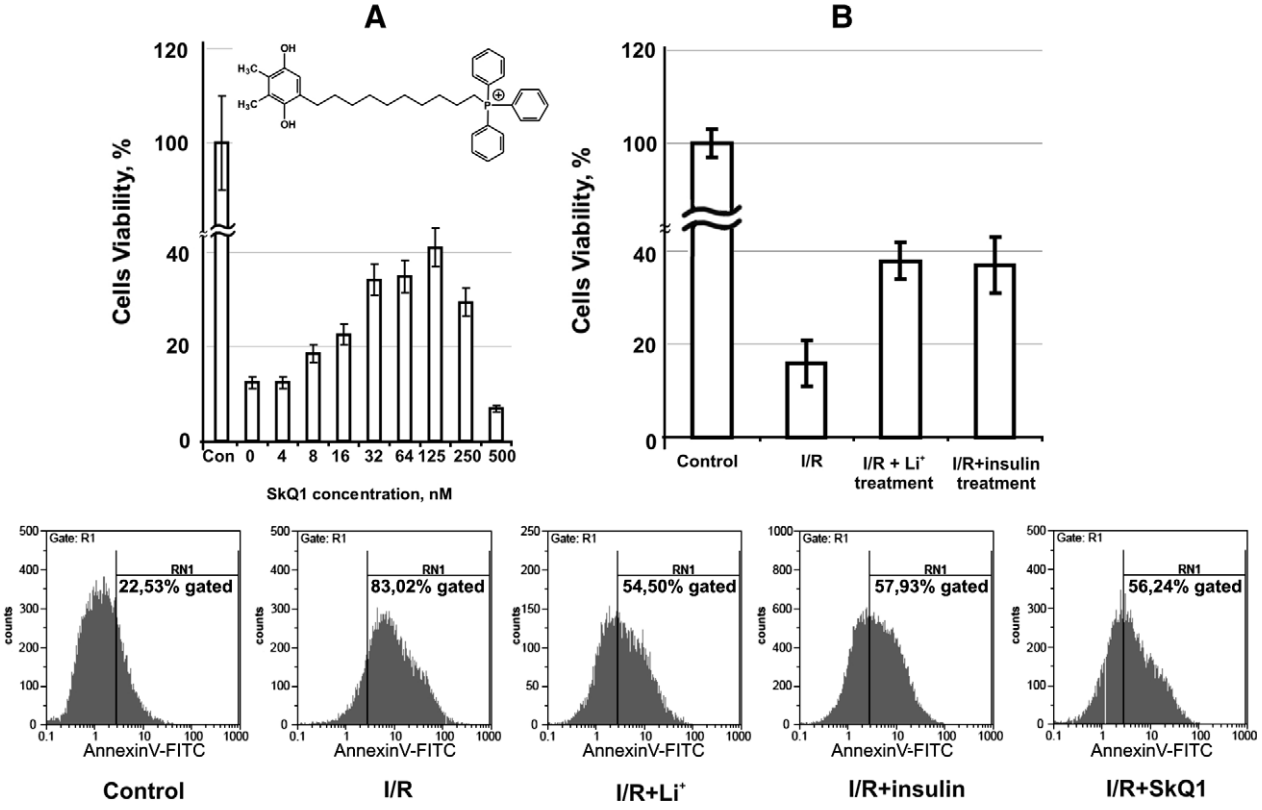

Fig. 1. The effect of SkQ1 (120 nM, 5 days cultivation, A), insulin (120 nM just prior ischemia) and LiCl (3 mM just prior ischemia, B) on the renal tubular epithelium cells survival after 24-h ischemia/24-h reoxygenation (I/R). Bottom: increasing of Annexin V-positive cells population under I/R and prevention of such events by LiCl, insulin and SkQ1.

3. Results

3.1. The effects of SkQ1, insulin and LiCl on cell death due to ischemia/reoxygenation

After exposure of cultivated cells to 24-h ischemia followed by 24-h reoxygenation, cell death exceeded 80% (Fig. 1A). To study the effect of SkQ1, the cells were supplemented with different concentrations of SkQ1 for 5 days prior to 24-h ischemia followed by 24-h reoxygenation. The protective effect of SkQ1 (Fig. 1A) has been demonstrated at concentrations between 8 and 250 nM; the effect at >250 nM SkQ1 is lower than at 125 nM apparently due to some toxic (possibly prooxidant) effect of SkQ1 at high concentrations.

Using the same model we analyzed the ability of Li^+ and insulin to protect the cells from death. Fig. 1B demonstrates that the number of live cells doubled after preincubation with

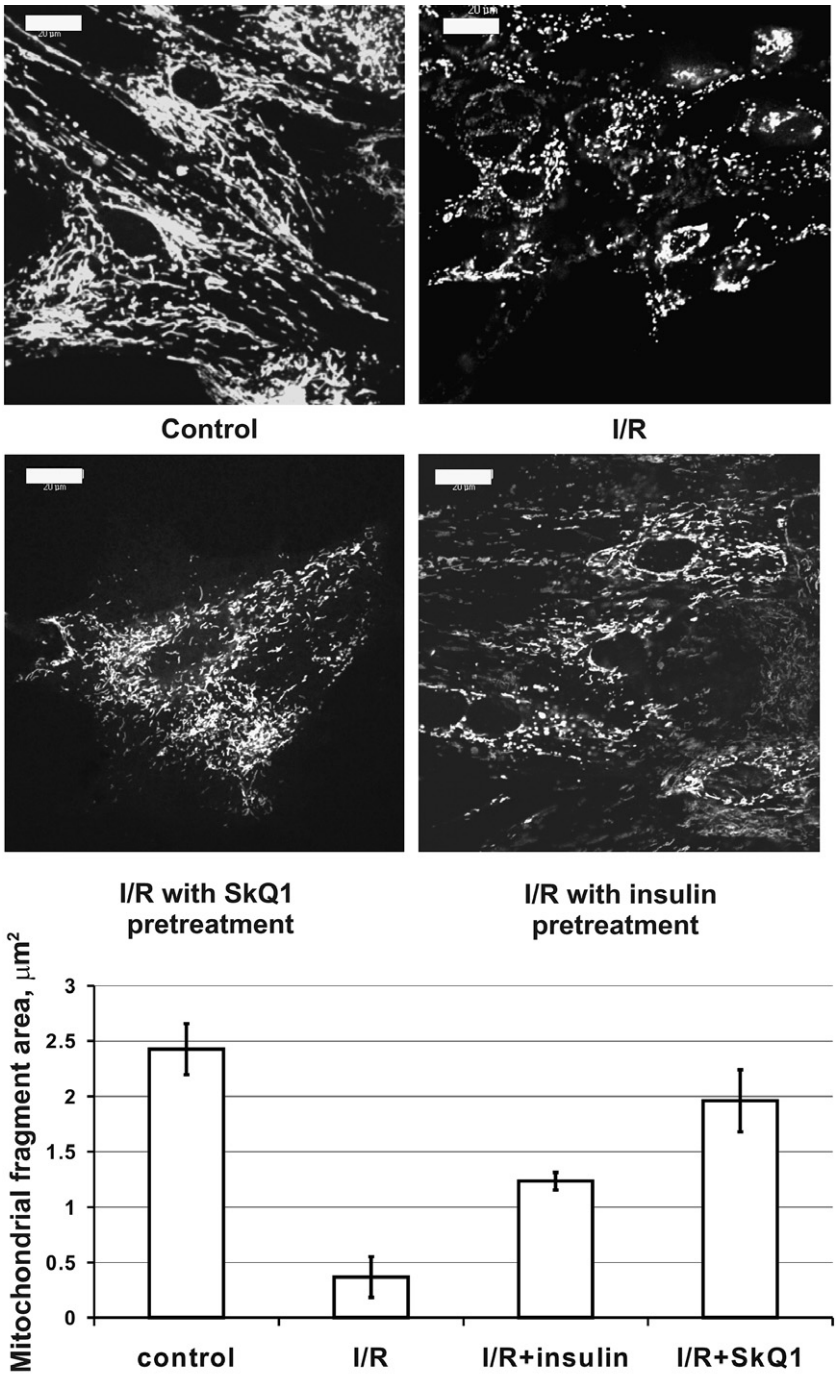

Fig. 2. Mitochondrial fragmentation in renal cells after ischemia/reoxygenation. TMRE staining. In control cells, mitochondria keep filamentous shape while after ischemia/reoxygenation (I/R) the mitochondrial fragmentation takes place. Cells cultivated with 120 nM SkQ1 (5 days) are protected from I/R-induced fragmentation. In the cells incubated with 120 nM insulin during ischemia the mitochondrial fragmentation is also diminished. Bar, 20 μm. Diagram illustrates alterations of average mitochondria fragments size (area occupied by a single mitochondrial fragment) under these particular conditions.

3 mM LiCl or 120 nM insulin when compared to that after ischemia/reoxygenation alone. We conclude that mitochondria-targeted antioxidant as well as Li^+ and insulin, are protectors against ischemia/reoxygenation-induced cell death.

Annexin V-FITC-staining of the cultivated cells 30 min after ischemia demonstrate only a few positive cells (not shown) pointing that significant apoptotic cell degradation did not occur yet within this short period of time. However, 24 h after ischemia revealed a significant number of Annexin-positive cells appear. Since under these conditions we observed an essential detachment of cells from the substrate we used flow cytometry to analyze cultural medium combined with trypsin-dissociated cells. Ischemia/reoxygenation was found to cause a significant rise of Annexin-positive cells when referred to the control (Fig. 1, bottom). However, after Li or insulin or SkQ1 pretreatment the number of these cells drops to about two-fold.

3.2. The alteration of a spatial organization of mitochondrial reticulum after ischemia/reoxygenation

We revealed a dramatic change of the mitochondrial morphological state in cell cultures exposed to ischemia/reoxygenation. We found that after 15 min of reoxygenation following the 24-h ischemia, mitochondrial filaments have been significantly fragmented forming round-shape bodies instead of conventional filament-like structures with frequent branches (Fig. 2). These grain-like mitochondria retain their membrane potential since accumulating TMRE to approximately the same level as filamentous precursors.

To explore the possible prevention of mitochondrial fission by SkQ1, we supplemented the cell culture with this agent 5 days prior to ischemia. We observed that mitochondria in the kidney cells exposed to 120 nM SkQ1 displayed significantly lower fission abilities after 15-min reoxygenation following the 24-h ischemia (Fig. 2). In spite of the fact that some cells exposed to ischemia/reoxygenation died, in the surviving cells the mitochondrial structure was almost normal. The analysis of the mitochondrial energization using a fluorescent plate reader demonstrated that after ischemia rather small fall of the mitochondrial membrane potential takes place (TMRE fluorescence changes from 154960 ± 5230 to 134785 ± 4980 arb. units). After pretreatment of the cells with LiCl or insulin the total TMRE fluorescence intensity after ischemia rose to 142969 ± 3251 and 147311 ± 4523, correspondingly. Thus, we consider that the visible difference in fluorescence intensity (seen in Fig. 2) seems mostly to be caused by out-of-a-focal-plane location of mitochondria when we catch only the portion of the TMRE fluorescence from mitochondrion occupying the border of the focal-plane. It was proved by the z-sectioning analysis of the TMRE fluorescence intensity (not shown). However, we cannot exclude that mitochondria in some cells are intrinsically different in their level of energization, i.e., even in control cells there are mitochondrial populations with different energization status and this heterogeneity is not changing much after ischemia. We must admit that the same conclusion on the mitochondrial membrane potential heterogeneity has been made after the treatment of the cells with 1 μM nigericin converting ΔpH into $\Delta\psi$ (not shown).

We observed a similar effect on the spatial mitochondrial organization in cells treated with insulin (Fig. 2) or LiCl (not shown). In these cases, mitochondrial fragmentation caused by 24-h ischemia followed by 15-min reoxygenation was significantly diminished.

Counting cells with partially and totally fragmented mitochondria showed that while control cells mostly contain non-fragmented mitochondrial filaments (around 90% of the whole cell population), only about 10–20% cells exposed to ischemia/reoxygenation retain filamentous mitochondrial structure while in the rest 80–90% mitochondria were partially or totally fragmented. These data are presented in Fig. 2 where calculated average mitochondrial dimensions (and/or their fragments) are given for control cells and after different exposure. LiCl treatment before ischemia partially retained normal mitochondrial structure. The same protective effect was observed when the cells were pretreated with 60 nM SkQ1 for 5 days before ischemia.

Since mitochondrial fission was observed in the early phase after ischemia/reoxygenation, we suggest that the prevention of the fission provides better cell survival under such conditions. In this respect, the mitochondrial fission and further cell survival look tightly linked when different agents like mitochondria-targeted antioxidant (SkQ1), an inhibitor of GSK-3 kinase (LiCl), and a signaling molecule (insulin) are equally protective in both prevention of mitochondrial fission and induced cell death.

3.3. The effect of SkQ1 and LiCl on the death of UV-exposed fibroblasts

In another series of experiments, skin fibroblasts were irradiated with UV known to induce oxidative stress and apoptosis [22]. UV exposure caused the death of more than 80% of fibroblasts. However, the agents used to prevent ischemia/reoxygenation-induced kidney cell death proved to be rather ineffective in preventing a UV-induced fibroblasts death. Namely, 3 mM LiCl supplemented to fibroblasts 3 h prior the UV exposure resulted in insignificant improvement of the cell survival (Fig. 3). A similar effect was observed with 120 nM SkQ1 (which was most effective in the experiments with ischemia/reoxygenation-induced mitochondrial fragmentation and kidney cell death) supplemented 5 days prior to exposure of fibroblasts to UV. In the latter case, the cell survival after UV exposure of cells treated with 120 nM SkQ1 was 25% versus 15% in untreated irradiated cells.

Moreover, cells dying within 1 day after UV exposure were Annexin V-positive and propidium iodide negative apparently demonstrating the apoptotic nature of cell death. Similarly, the cells treated with LiCl displayed the same properties (not shown). Thus, these agents were incapable of preventing cell death caused by a 60-s UV exposure.

Thereafter, we tested lower doses of UV exposure (30 s instead of 60 s) in their ability to induce fibroblast cell death. In these experiments, the cell death was analyzed 24 h after UV exposure. In this case, about 80% of cells survived (Fig. 4) and such a cell death was completely prevented by LiCl or insulin treatment (Fig. 4). A similar protective effect in case of a moderate UV exposure was observed in the cells pretreated for 5 days with 60 nM SkQ1 (Fig. 4).

3.4. The alteration of the mitochondrial structure in UV-exposed fibroblasts

Further analysis of the effect of UV-irradiation on the mitochondrial dynamics in fibroblasts revealed a lack of coinci-

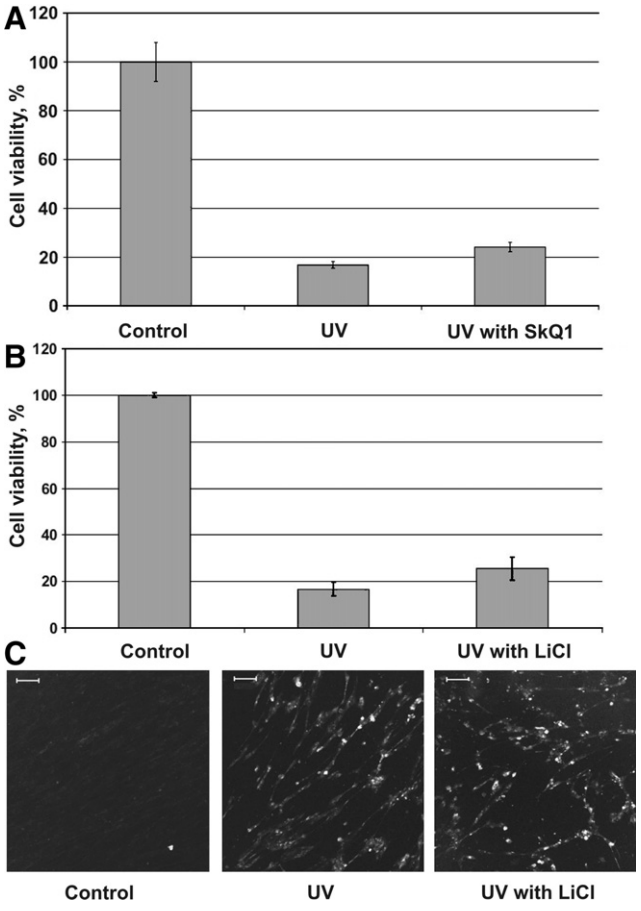

Fig. 3. The effects of SkQ1 (5 days cultivation, A) and LiCl (3-h preincubation, B) on fibroblasts survival after strong (60-s) UV exposure. After irradiation, the cells were incubated for 24 h. (C) Annexin V-FITC-staining. Bar, 100 μm.

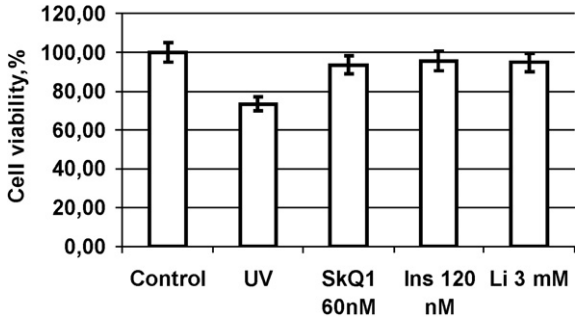

Fig. 4. The effect of SkQ1, insulin and LiCl on fibroblast survival after moderate UV exposure. LiCl and insulin were supplemented 3 h prior to UV exposure. After irradiation, the cells were incubated for 24 h.

dence of LiCl and SkQ1 effects on cell death and mitochondrial fission.

As in the case of ischemia/reoxygenation-induced cell death, essential alterations of the spatial mitochondrial organization have been revealed. We found that just 60 s after UV exposure, mitochondria in fibroblasts are significantly fragmented (Fig. 5) apparently demonstrating an extremely fast reaction to the applied stress. Within such short time, no signs of the death progression were observed in spite of the fact that mitochondrial fragmentation occurs in about 90% cells. Mitochondria in the cells still retain the membrane potential since accumulating TMRE but they do not form a reticulum as observed in the untreated cells. Cells pretreated with SkQ1 (120 nM, 5 days) did not show mitochondrial fragmentation after 60-s UV exposure (Fig. 5). Thus, SkQ1 prevented mitochondrial fission induced by strong UV exposure and incubation with 3 mM LiCl prevented alterations of the cell chondriome as well (Fig. 5).

We conclude that at long UV exposure SkQ1 and Li^+ prevent mitochondrial fragmentation being, however almost ineffective in their ability to prevent further cell death. Only in case of moderate UV exposure, SkQ1, insulin and Li^+ were capable of preventing cell death. However, we admit that in this case when dead cells comprise 20–25% only (Fig. 4) mitochondria practically showed no signs of global fragmentation (not shown).

4. Discussion

Global mitochondrial fragmentation (mitochondrial fission) was discovered 25 years ago in the cells treated with the mitochondrial benzodiazepine receptor ligand [23] and later has been induced by a number of mitochondrial drugs (rotenone, cyanide, antimycin A, uncouplers, oligomycin, etc.) [24,25]. Now it became clear that there is a correlation between the induction of apoptotic or mitoptotic cascade and mitochondrial fission (“the thread-grain transition”) [2].

A similar effect was observed under photo-dynamic generation of reactive oxygen species (ROS) in mitochondria [26,27]. It is apparent that mitochondrial fission occurs in parallel with ROS generation and is directly linked to the distortion of mitochondrial function [2]. Fission can be irreversible and represents one of the steps in the apoptotic cell death cascade [28].

In this study, we have demonstrated that under oxidative stress, induced by ischemia/reoxygenation, the use of mitochondria-targeted antioxidant, SkQ1, as well as Li^+ and insulin, enhance cell survival. Similarly, this kind of treatment essentially diminished mitochondrial fragmentation (fission) observed after ischemia/reoxygenation. Thus, by prevention of early events in the progression of the apoptotic cascade one can expect the prevention of the terminal phase of ischemia/reoxygenation, namely cell death. It is tentative to speculate that ischemia/reoxygenation-induced apoptotic signaling severely depends on mitochondrial function and what is most important, from the activity of proteins regulating mitochondrial fission/fusion.

It is apparent that mitochondria-targeted antioxidant may have an influence on the expression of some signaling molecules associated with dynamin/mitofusin system, since time is needed for the progression of this effect (5 days under our conditions). Another explanation may consist in a time-consuming redistribution of SkQ1 between inner mitochondrial membrane and other cell membranes and lipid intracellular droplets [29].

At the same time, Li^+ and insulin have an effect on both mitochondrial fragmentation and cell survival during ischemia/reoxygenation, i.e., their action is mediated by some fast reactions. These fast reactions may include protein phosphorylation with the involvement of some kinases, i.e., GSK-3β [30].

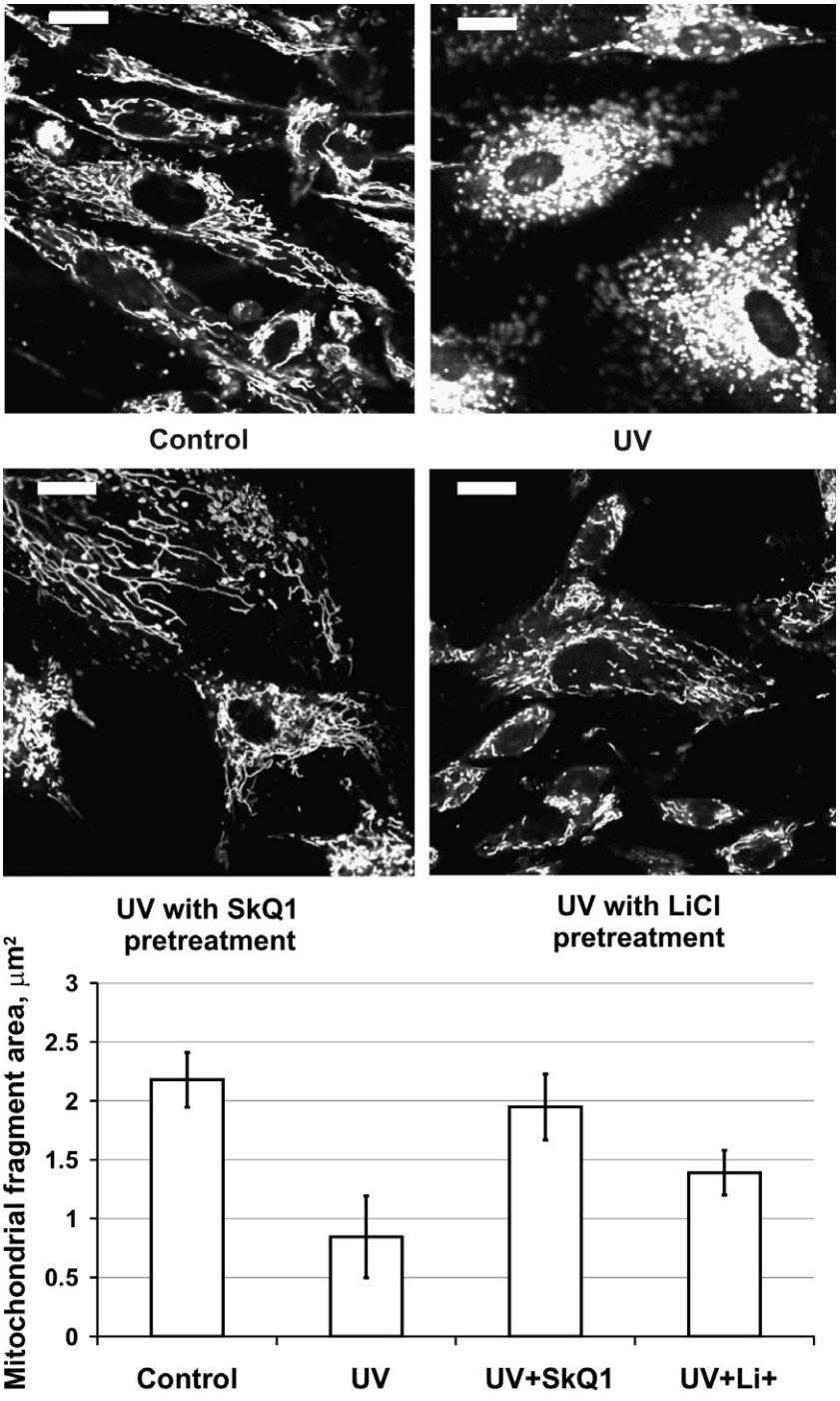

Fig. 5. Mitochondrial fragmentation in 60-s UV-exposed fibroblasts. TMRE (200 nM) staining. In control cells, mitochondria keep filamentous shape while after UV exposure mitochondria apparently swell and undergo fragmentation. The cells pretreated with 120 nM SkQ1 (5 days) are fully and with 3 mM LiCl partially protected from UV-induced fragmentation. Bar, 20 μm. Diagram illustrates alterations of average mitochondria fragments size (area occupied by a single mitochondrial fragment) under these particular conditions.

However, using the model of UV-induced apoptosis, we found a divergence of the signaling between apoptotic induction and mitochondrial fragmentation. Specifically, the mitochondria-targeted antioxidant, SkQ1 protects mitochondrial reticulum from UV-induced fragmentation but it is incapable of preventing further cell death, making the step of mitochondrial fragmentation not essential in the apoptotic cell death cascade. This is in contrast to ischemia/reoxygenation-induced cell death, which is tightly coupled with mitochondrial fragmentation.

Similarly, the pretreatment of cells with the inhibitor of GSK-3β, Li^+ before UV exposure results in preservation of mitochondrial filamentous structure but practically did not have any influence on the cell fate 1 day after UV-irradiation. Moreover, UV-induced cell death was proceeded by an apoptotic mechanism and it was not influenced by Li^+-pretreat-

ment. Basing on this observation, we conclude that signaling induced by SkQ1 or Li^+ is essentially directed to support the structure of mitochondrial reticulum but did not effect downstream UV-induced apoptotic reactions. Interestingly, that under moderate UV exposure causing the death of only about 20–25% cells, we found a significant protection of cell survival by both mitochondrial antioxidant, SkQ1 and GSK-3 inhibitors. Apparently, the mechanisms of a cell death caused by a moderate and strong UV may be different with the involvement of different signaling pathways. In cell signaling resulting in cell death there might be few parallel mechanisms running after the induction of apoptosis with fewer proceeding with structural alterations of mitochondrial shape. For some, the mitochondrial fragmentation is an obligatory step for apoptotic progression and which abrogation may result in the abrogation of cell death. For others, the mitochondrial fragmentation is either not essential or it occurs as a consequence of the apoptotic program and having no influence on the final result. Probably, under ischemia/reoxygenation only mitochondrial fission-dependent cascades are turned on while UV initiates additional, possibly parallel, or downstream of mitochondrial fragmentation signaling mechanisms.

Another explanation of the above relationships consists in that weak apoptotic signals require their amplification by mitochondria, which appear to be indispensable for the cell death in such cases. However, this amplification is no more needed when the signals become significantly strong. Thirty seconds UV (20% cell death) and 60-s UV (80% cell death) can be regarded as weak and strong signals, respectively. As to ischemia/reoxygenation it should be apparently acquired to be the weak signal. Mechanistically, the situation may well be explained, e.g., by findings observed when TNF-induced apoptosis was studied. In this case, a weak death signal (low [TNF]) can be executed via system including caspase 8 → Bid → mitochondria → caspase 9 → caspase 3, whereas the strong one (high [TNF]) can represent more direct activation of caspase 3 by caspase 8 with no mitochondria involved [31]. Similar explanation can be done for data of Murphy's group who failed to observe protection effect of another mitochondria-targeted antioxidant, 10-(6-ubiquinonyl)decyltriphenyl-phosphonium, on the UV-induced cell death [32].

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