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Review

Prevention of cardiolipin oxidation and fatty acid cycling as two antioxidant mechanisms of cationic derivatives of plastoquinone (SkQs)

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ABSTRACT

The present state of the art in studies on the mechanisms of antioxidant activities of mitochondria-targeted cationic plastoquinone derivatives (SkQs) is reviewed. Our experiments showed that these compounds can operate as antioxidants in two quite different ways, i.e. (i) by preventing peroxidation of cardiolipin [Antonenko et al., *Biochemistry (Moscow)* 73 (2008) 1273–1287] and (ii) by fatty acid cycling resulting in mild uncoupling that inhibits the formation of reactive oxygen species (ROS) in mitochondrial State 4 [Severin et al. *Proc. Natl. Acad. Sci. USA* 107 (2009), 663–668]. The quinol and cationic moieties of SkQ are involved in cases (i) and (ii), respectively. In case (i) $SkQH_2$ interrupts propagation of chain reactions involved in peroxidation of unsaturated fatty acid residues in cardiolipin, the formed $SkQ^{\bullet -}$ being reduced back to $SkQH_2$ by heme b_H of complex III in an antimycin-sensitive way. Molecular dynamics simulation showed that there are two stable conformations of SkQ1 with the quinol residue localized near peroxyl radicals at C_9 or C_{13} of the linoleate residue in cardiolipin. In mechanism (ii), fatty acid cycling mediated by the cationic SkQ moiety is involved. It consists of (a) transmembrane movement of the fatty acid anion/SkQ cation pair and (b) back flows of free SkQ cation and protonated fatty acid. The cycling results in a protonophorous effect that was demonstrated in planar phospholipid membranes and liposomes. In mitochondria, the cycling gives rise to mild uncoupling, thereby decreasing membrane potential and ROS generation coupled to reverse electron transport in the respiratory chain. In yeast cells, dodecyltriphenylphosphonium (C_{12}TPP), the cationic part of SkQ1, induces uncoupling that is mitochondria-targeted since C_{12}TPP is specifically accumulated in mitochondria and increases the H^+ conductance of their inner membrane. The conductance of the outer cell membrane is not affected by C_{12}TPP.

Abbreviations: $\Delta\psi$, transmembrane electric potential; AAPH, 2,2′-azobis(2-amidinopropane)dihydrochloride; BLM, planar bilayer phospholipid membrane; Cart, carboxyatractyloside; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CTMA, cetyltrimethylammonium; C_{10}TPP, decyltriphenylphosphonium; C_{12}R1, decylrhodamine 19; C_{12}R4, decylrhodamine B; C_{12}TPP, dodecyltriphenylphosphonium; DCF, 2′,7′-dichlorodihydrofluorescein diacetate; DMQ, 3-demethoxy ubiquinonyl decyltriphenylphosphonium; DPQ, decylplastoquinone; FCCP, carbonylcyanide p-trifluoromethoxyphenylhydrazone; MDA, malondialdehyde; MitoQ, ubiquinonyl decyltriphenylphosphonium; ML, methyl linoleate; NAC, N-acetyl cysteine; ROS, reactive oxygen species; SkQ, a compound composed of plastoquinone or methylplastoquinone and decyl (or amyl) triphenylphosphonium, Rhodamine 19, or Rhodamine B; SkQ1, plastoquinonyl decyltriphenylphosphonium; SkQ3, 5-methylplastoquinonyl decyltriphenylphosphonium; SkQ5, plastoquinonyl amyltriphenylphosphonium; SkQR1, plastoquinonyl decylrhodamine 19; SkQR4, plastoquinonyl decylrhodamine B; TMRM, tetramethylrhodamine methyl ester; TPP, tetraphenylphosphonium

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1. Introduction

In 2003 we started an ambitious project with the goal of creating a small molecule that decelerates the senescence of organisms. Mitochondria-targeted antioxidants (cationic derivatives of plastoquinone) were chosen for this purpose. The investigation was based on the discovery of mitochondria-penetrating ions by groups of Dr. E.A. Liberman and of one of the authors (V.P.S.) in 1969–1970. Penetrating ions are hydrophobic molecules that can easily penetrate through membranes in spite of the presence of an ionized atom in their structures [1–4]. An example is the alkyltriphenylphosphonium ion. In this cation, the positive charge on phosphorus is strongly delocalized over the three phenyl residues. For this reason, water dipoles cannot be held on the cation and do not form an aqueous shell preventing penetration of the cation through the hydrophobic membrane core. In the same studies, it was found that the mitochondrial interior is the only intracellular compartment negatively charged relative to its surrounding (i.e. to the cytosol) [4,5]. Therefore, on entering the cell, penetrating cations will be specifically concentrated within mitochondria. This concentrating effect should be described by the Nernst equation, being 1000 fold if the mitochondrial $\Delta\psi$ is 180 mV [5].

Following this hypothesis, we suggested that penetrating cations can be used as "molecular electric locomotives" to target uncharged substances attached to these cations specifically to mitochondria [5,6].

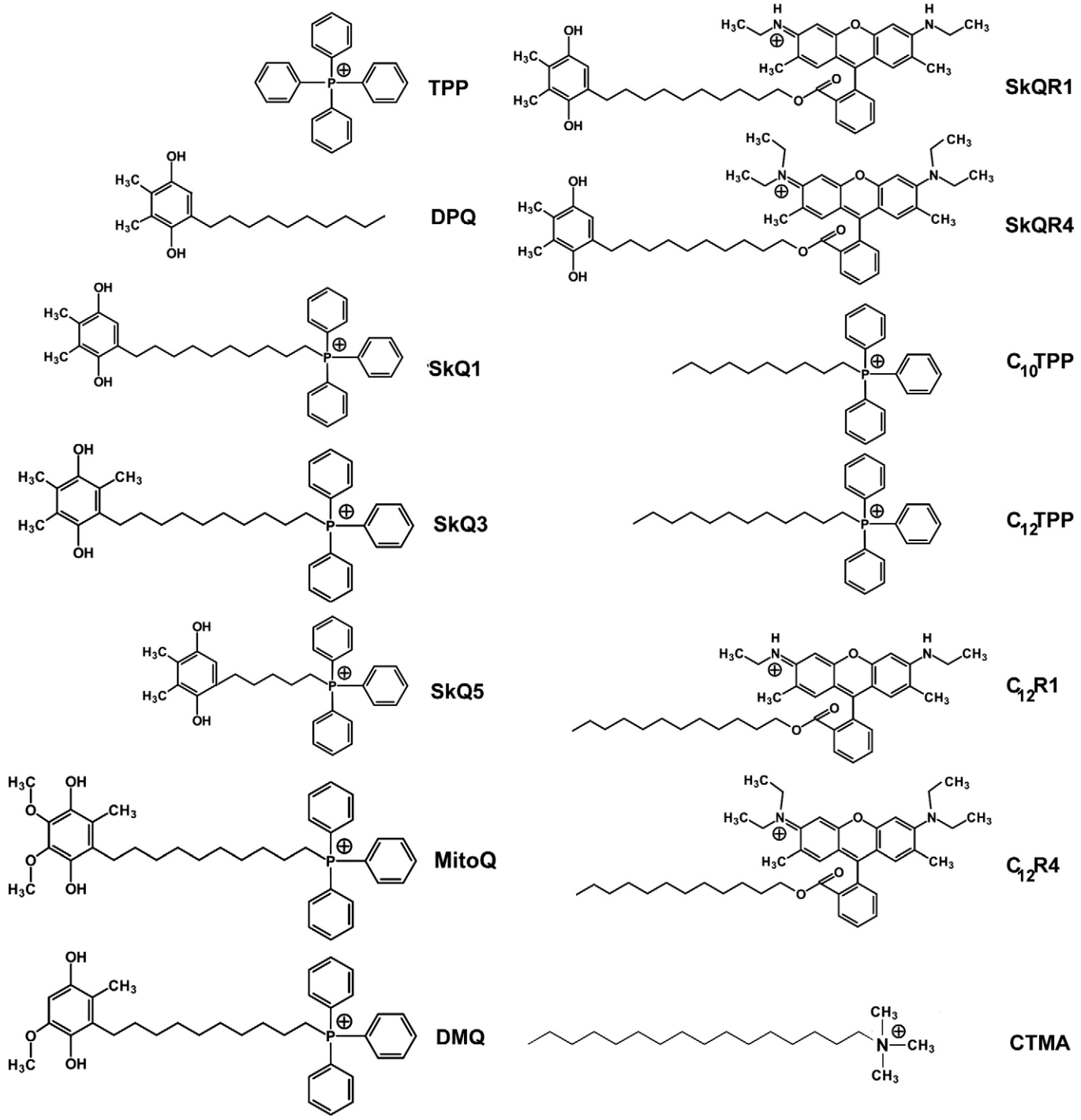

Fig. 1. Formulas of the studied penetrating cations. The quinone moieties are shown in their reduced forms.

This suggestion was successfully used by Dr. M.P. Murphy to deliver to mitochondria the rechargeable antioxidant MitoQ composed of ubiquinone and decyltriphenylphosphonium [7] (for review, see [8]). We confirmed Murphy's data that MitoQ increases the antioxidant capacity of these organelles. However, it was also found that the activity of MitoQ as an antioxidant changes to prooxidant when MitoQ concentration is increased ([9–11]; see also [12–14]). For example, at concentrations of about 0.5 to 1 μM, MitoQ protects mitochondria from oxidative stress, but 2 μM MitoQ stimulates H_2O_2 production by isolated rat heart mitochondria to a rate that appears to be higher than all recorded values described in the literature [9]. This is why the first goal of our project was to find a mitochondria-targeted antioxidant with antioxidant versus prooxidant window larger than that of MitoQ.

We synthesized decyltriphenylphosphonium conjugated to quinones other than ubiquinone, namely 3-demethoxyubiquinone, 5-methylplastoquinone, and plastoquinone [10]. They were all better antioxidants than ubiquinone, plastoquinonyl decyltriphenylphosphonium being the best [10,15]. This substance was named SkQ1, where Sk is for penetrating cation ("Skulachev ion", a term introduced by David Green [16], see also [17]) and Q is for quinone [10]. For $SkQ1H_2$ (the reduced form of SkQ1), it was found that it not only is a much better antioxidant than $MitoQH_2$ [10,11,18], it is also a less active prooxidant reacting with O_2 more slowly than $MitoQH_2$ does [10,11]. As a result, the antioxidant versus prooxidant window for isolated rat mitochondria was as large as 1000 (cf. MitoQ, where it was about 2) [10,11].

Experiments with BLMs and liposomes clearly showed that SkQ1 and SkQR1 (an SkQ1 analog with Rhodamine 19 instead of triphenylphosphonium as the cationic moiety) are cations easily penetrating phospholipid membranes [10,11,19]. With isolated mitochondria, it was found that SkQ1, like plastoquinone, can be reduced by heme b_H in center *i* of complex III, the reaction being sensitive to antimycin A. The oxidation of $SkQ1H_2$ was slower than the reduction of SkQ1 and was partially inhibited by myxothiazol. Thus, SkQ1 is an antioxidant that is recharged by the mitochondrial respiratory chain, and it exists in respiring mitochondria mainly in its antioxidant (reduced) form, $SkQ1H_2$ [10,11]. In isolated mitochondria, $SkQ1H_2$ specifically prevented oxidation of cardiolipin under conditions of oxidative stress induced by Fe^{2+} and ascorbate [10,11].

In intact human cells (HeLa and fibroblast cultures were studied) SkQR1 is targeted to mitochondria and protects the cells from undergoing H_2O_2-induced apoptosis (as well as necrosis caused by illumination in the presence of a photosensitizer) [10].

Several species were studied for geroprotective effect of SkQ1. Positive results were obtained with the fungus *Podospora anserina*, the crustacean *Ceriodaphnia affinis*, the insect *Drosophila melanogaster*, the fish *Nothobranchius furzeri*, and mice. In all these cases an increase in the median lifespan was observed (in mice, twofold) whereas the maximal lifespan was less affected [11,20]. Development of age-related diseases and typical traits of senescence was found to be slowed by very small amounts of SkQ1 [11,20–22]. All these observations could be regarded as evidence that SkQ1 inhibits a senescence program that is mediated by mitochondrial reactive oxygen species (ROS) [9,11].

This paper provides a review of our recent studies on elucidation of possible mechanisms of antioxidant action of SkQ. The data indicate that SkQ can operate as antioxidant in two quite different ways, i.e. (i) by direct quenching of ROS (first of all, radical intermediates of cardiolipin peroxidation) and (ii) indirectly, by mediating transport of fatty acid anions, resulting in mild uncoupling.

2. Antioxidant mechanism mediated by the quinone moiety of SkQ

In the experiments described below, penetrating cations (structures are shown in Fig. 1) and some related compounds were studied using detergent micelles, BLM, liposomes, isolated rat heart mitochondria, and human cell cultures.

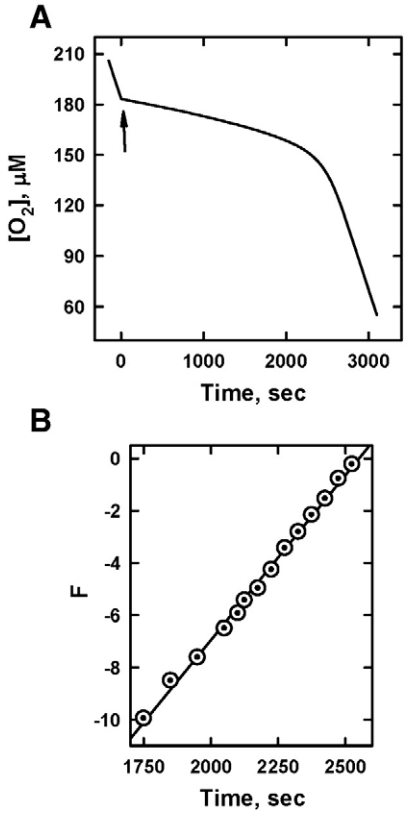

Fig. 2. Effect of 5 μM $SkQ1H_2$ on oxygen consumption resulting from peroxidation of 20 mM methyl linoleate in micelles of 50 mM Triton X-100. Incubation mixture, 3 mM AAPH (peroxidation initiator) and 50 mM phosphate, pH 7.4; 37 °C. A, [O_2] trace; arrow, addition of $SkQ1H_2$. B, plot of A in the coordinates of Eq. (4). (From Roginsky et al. [15]).

Peroxidation of the methyl ester of linoleate (ML) in Triton X-100 micelles was used as a kinetic testing model to compare antioxidant properties of cationic derivatives of ubiquinone (MitoQ), 3-demethoxyubiquinone (DMQ), plastoquinone (SkQ1), methylplastoquinone (SkQ3), an SkQ1 analog with pentane linker instead of decane (SkQ5), and α-tocopherol. The rate of methyl linoleate peroxidation was measured using polarographic monitoring of the O_2 level in the reaction mixture. Peroxidation was initiated by a water-soluble initiator, AAPH. The chain-breaking antioxidant activity of the studied compounds was characterized by the rate constant for the reaction of these compounds with the lipid peroxyl radical ($LO_2^{\bullet}$) formed from methyl linoleate:

$$LO_2^{\bullet} + QH_2 \rightarrow LOOH + QH^{\bullet} \qquad k_1 \qquad (1)$$

Table 1
Kinetic parameters characterizing the antioxidant activity of the reduced forms of mitochondria-targeted quinones and α-tocopherol in methyl linoleate (ML)-Triton X-100 micelles. Incubation mixture, 20 mM ML, 50 mM Triton X-100, 50 mM phosphate buffer, pH 7.4, 37 °C. Oxidation of ML was initiated by addition of 3 mM AAPH. Figures in brackets are the number of independent experiments. (For details, see Roginsky et al. [15]).

Quinol-containing compound	k_1/k_2	$k_1 \times 10^5$, $M^{-1} s^{-1}$
$SkQR1H_2$	3800 ± 500 (5)	2.3 ± 0.3
$SkQ1H_2$	3670 ± 280 (7)	2.2 ± 0.2
$SkQ3H_2$	2720 ± 210 (4)	1.6 ± 0,1
$SkQ5H_2$	2670 ± 180 (5)	1.6 ± 0,1
$DMQH_2$	1260 ± 85 (4)	0.76 ± 0.5
α-tocopherol	1170 ± 70 (4)	0.70 ± 0.04
$MitoQH_2$	970 ± 55 (6)	0.58 ± 0.03

which competes with the reaction of chain propagation of lipid peroxidation

$$LO_2^{\bullet} + LH \rightarrow LOOH + L^{\bullet} \qquad k_2 \qquad (2)$$

The non-inhibited oxidation of methyl linoleate in Triton micelles is a chain reaction whose rate R_0 was found to be proportional to [methyl linoleate] and the square root of [AAPH]. Such relationships are also characteristic of the lipid peroxidation in other microheterogeneous aqueous systems. They correspond to the "classic" kinetic scheme of lipid peroxidation chain reaction (for references, see [15]):

$$AAPH + LH + O_2 \rightarrow LO_2\bullet + P \quad k_0 \qquad (0)$$

$$LO_2\bullet + LH \rightarrow LOOH + L\bullet \qquad k_2 \qquad (2)$$

$$L\bullet + O_2 \rightarrow LO_2\bullet \qquad k_3 \qquad (3)$$

where LH, L$^{\bullet}$, and LOOH are methyl linoleate, its radical, and its peroxide, respectively; P stands for products of AAPH decomposition.

All the tested QH_2 displayed pronounced chain-breaking antioxidant activity as exemplified by Fig. 2. When $SkQ1H_2$ was added, the rate of O_2 consumption, R, dramatically decreased. As $SkQ1H_2$ was progressively oxidized due to reaction (1), R increased with time and eventually reached the level of non-inhibited methyl linoleate oxidation (R_0). As a result, a pronounced induction period in the O_2 uptake was observed (Fig. 2A).

$$F = \ln\frac{1 + R/R_0}{1 - R/R_0} - \frac{R_0}{R} = \frac{k_1 R_0}{2k_2[LH]}t + constant. \qquad (4)$$

Fig. 2A depicts the original [O_2] trace in the coordinates of Eq. (4). The plot of F versus time is a straight line as predicted by Eq. (4). The kinetic behavior of all the other QH_2 studied was qualitatively similar. The value of k_1/k_2 can be calculated from the slope of the straight line, using Eq. (4). The k_1/k_2, values are listed in Table 1. The absolute values of k_1 were calculated from k_1/k_2 assuming $k_2 = 60\ M^{-1}\ s^{-1}$ [15].

Table 1 shows that the antioxidant activity (k_1) of the studied quinols decreases in the series $SkQR1H_2 = SkQ1H_2 > SkQ3H_2 = SkQ5H_2 \gg DMQH_2 > \alpha$-tocopherol$> MitoQH_2$. Oxidized SkQ1 as well as C_{12}TPP lacking a quinol moiety were completely inactive as antioxidants. From these data, we conclude that (i) the quinol moiety is crucial for the antioxidant effect of the studied compounds; (ii) substitution of 2- and 3-methoxy residues by methyls and, to a lesser degree, removal of 5-methyl groups in the ubiquinol moiety is favorable for antioxidant activity, and (iii) shortening of the linker from C_{10} to C_5 is unfavorable. The latter effect might be explained by the fact that the initiator of the peroxidation chain reaction (AAPH in our model system and such radicals as OH• in mitochondria) attacks the C_{11} atom of linoleic acid residue in cardiolipin, an event resulting in formation of rather stable radicals LO_2• in position C_9 or C_{13} (Fig. 3). Molecular dynamics simulation (for method, see [31]) showed that SkQ1 can exist in two conformations which occupy positions in the phospholipid bilayer such that its quinone residue is near either C_9 or C_{13} of the fatty acid hydrocarbon chain (Fig. 4). SkQ5 (the SkQ1 analog where pentane substitutes for decane as a linker) is too short to reach C_{13} when its cationic moiety is in the water/lipid interphase (not show in the figures).

Then we studied the antioxidant activity of cationic quinones in isolated rat heart mitochondria. We found that they all prevent formation of malondialdehyde and other thiobarbituric acid-reactive (TBAR) substances in mitochondria treated with Fe^{2+} and ascorbate (Fig. 5A). The antioxidant activities of various quinones decreased like in lipid micelles (SkQ1 > SkQ3 > MitoQ). Cardiolipin was the first mitochondrial phospholipid oxidized under conditions of oxidative stress caused by Fe^{2+} and ascorbate, and this effect was strongly inhibited by SkQ1 (Fig. 5B). Both effects shown in Fig. 5 were absent when C_{12}TPP was used instead of SkQ1 (not show in the figures) [10].

The cationic group of our quinone derivatives was crucial for binding to cardiolipin. We found that all the studied hydrophobic cations, including C_{12}TPP, can displace N-nonyl acridine orange (NAO) from a complex with phospholipids (presumably with cardiolipin). These activities produced the following series: C_{12}TPP > SkQ1 > MitoQ, SkQ3. However, the most active was SkQR1, in which the triphenylphosphonium cation is replaced by the Rhodamine 19 cation. Its efficiency was 80 times higher than that of MitoQ (Table 2) [10]. This suggests that the cationic group of Rhodamine fits better than phosphonium to bind to the cardiolipin anion.

In this context, it should be mentioned that the majority of the fatty acid residues in cardiolipin are linoleic acid. In fact, at least three of the four fatty acids in each cardiolipin molecule are linoleates. Taking into account the excellent structural correspondence of positions of the SkQ quinone and the crucial C_9 and C_{13} carbons in cardiolipin linoleates (Fig. 4) and high affinity of positively charged moieties of SkQ to negative charged cardiolipin (Table 2), one can explain the high efficiency of SkQs in preventing cardiolipin peroxidation in mitochondria.

In further experiments human fibroblasts were stained with SkQR1 as a fluorescent dye. Consistent with our preceding study on HeLa cells [10], we found that also in fibroblasts, SkQR1 is specifically accumulated in mitochondria. The staining of fibroblast mitochondria took about 1 h, the process being strongly inhibited by protonophorous uncoupler FCCP

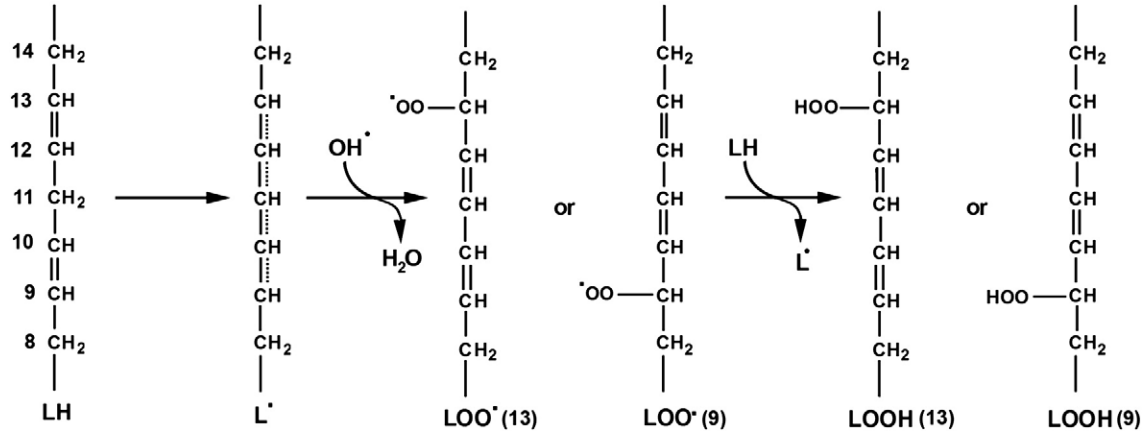

Fig. 3. Conversion of linoleate (LH) to its primary radical form (L$^{\bullet}$), peroxyl radicals (LOO$^{\bullet}$), and peroxides (LOOH). R$^{\bullet}$, a radical initiating the chain reaction of lipid peroxidation (OH$^{\bullet}$, $HO_2^{\bullet}$, L$^{\bullet}$, etc.).

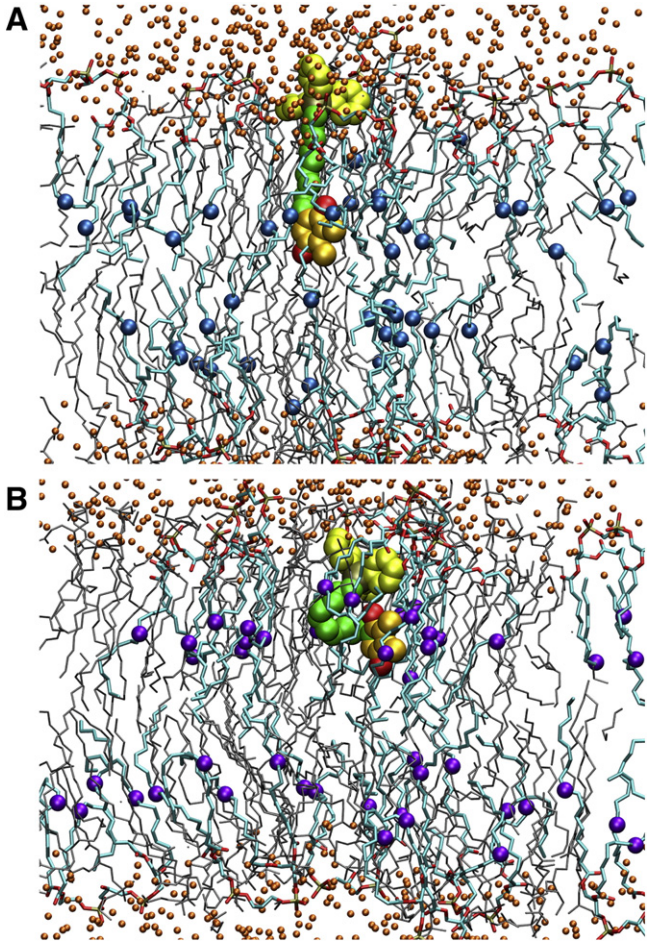

Fig. 4. Molecular dynamics modeling. Two stable conformations of SkQ1 in cardiolipin-containing lipid bilayer: (A) extended conformation; and (B) folded conformation. In cases A and B, the quinone moiety is located near C_{13} and C_9 carbon atom of the cardiolipin linoleate residues, respectively. Shown are the lipid hydrocarbon tails (thin gray sticks), the cardiolipin hydrocarbon tails (bulky cyan sticks), carbonyls and phosphates (red and brown sticks), SkQ1 (yellow spheres, phenyl rings; tan sphere, phosphorus; green spheres, decyl linker; orange and red spheres, quinone moiety), C_9 and C_{13} carbon atoms of cardiolipin (violet and blue spheres, respectively). The model system included a bilayer that contained 88 molecules of 1-palmitoyl 2-oleoyl phosphatidylethanolamine and 12 molecules of cardiolipin [1,3-*bis*(*sn*-3′-phosphatidyl)-*sn*-glycerol with $C_{18:2}$ aliphatic chains], 4074 water molecules, 24 counterions, and one SkQ1 molecule. The molecular dynamics were simulated with the CHARMM 27 force field. The calculations were performed using the SKIF Chebyshev supercomputer, Moscow State University. The system was energy minimized and equilibrated in a 10 ns-long molecular dynamics run, which allowed the bilayer area to relax; the area per lipid was 0.66 nm^2 (the experimental value for pure POPE reference bilayer is 0.64 nm^2). The structure of the system was analyzed using the 50 ns simulation. For other details of molecular dynamics calculation, see [31].

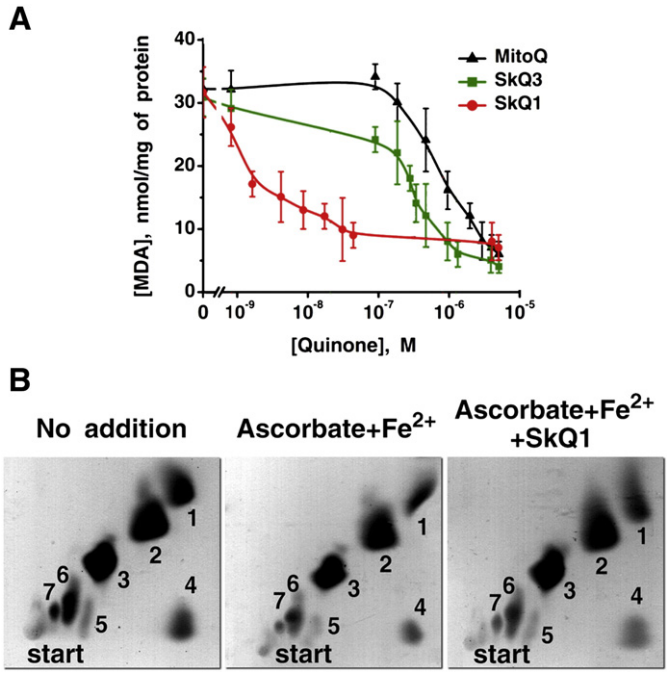

Fig. 5. SkQ1 prevents malondialdehyde formation and cardiolipin degradation in rat heart mitochondria under in vitro conditions of oxidative stress induced by Fe^{2+} and ascorbate. Mitochondria were incubated with 0.1 mM $FeSO_4$ and 10 mM ascorbate for 20 min. (A) MDA levels at different concentrations of SkQ1. (B) Amounts of different phospholipids under conditions as in (A). Where indicated, 100 nM SkQ1 was added. Silica gel-60 chromatogram of mitochondrial phospholipids. 1, cardiolipin; 2, phosphatidylethanolamine; 3, phosphatidylcholine; 4, phosphatidic acid; 5, phosphatidylserine; 6, phosphatidylinositol; 7, lysophosphatidylinositol. (From Antonenko at al. [10]).

(Fig. 6A). Oxidative stress induced in fibroblasts by adding H_2O_2 and monitored by an increase in 2′,7′-dichlorodihydrofluorescein diacetate (DCF) fluorescence was sensitive to both SkQ1 and SkQR1. Surprisingly, the antioxidant effect of these compounds was shown to require much longer time than their penetration into the cell, i.e. 24 h, 6 h being ineffective (Fig. 6B). Very low concentrations of SkQ1 or SkQR1 were effective in preventing H_2O_2-induced cell death ($C_{1/2}$ about 0.2 nM, Fig. 6C). As in the case of the DCF probe, a 24 h preincubation with SkQ was required. The non-targeted antioxidant Trolox (a water-soluble vitamin E derivative) was active only at much higher concentrations ($C_{1/2}$ was 10^5 times higher than for SkQ1) and, even at its optimal concentration it was less effective than SkQ1 (Fig. 6D).

It is known that long before cell death, H_2O_2 induces fragmentation of elongated mitochondria into numerous small spherical organelles ("the thread–grain transition" [24]). This effect was also prevented by pretreatment of cells with 2 nM SkQ1, but in this case a 2 h preincubation was sufficient (Fig. 7). One hundred micromolar Trolox or 5 mM NAC was required to be as effective as 2 nM SkQ1 [23].

The data described above show that SkQ1 and SkQR1 affect H_2O_2-treated mitochondria in two different ways, i.e. they prevent (i) "the thread–grain transition" and (ii) the H_2O_2-induced ROS burst and cell death. In both cases, the pretreatment of cells with SkQ was necessary. In case (i), it could be as short as 2 h, which corresponds to the time required for SkQ to enter the cell and then to reach mitochondria. In case (ii) the antioxidant effect was revealed after a 24 h preincubation with SkQ. Both effects of SkQ seem to be related to its antioxidant activity since they were reproduced with conventional antioxidants (Trolox and NAC) but only at very much higher concentrations than those of SkQ. The observed activity was clearly related to the quinone moiety of SkQ since C_{12}TPP was inactive [23].

Paradoxically, the fast antioxidant effect of SkQ had no obvious influence upon total DCF response of fibroblasts. Apparently, only intramitochondrial ROS were involved in case (i). Support for this assumption was obtained when we studied H_2O_2-induced translocation of Bax to mitochondria. This process is suppressed by a 2 h preincubation of fibroblasts with 2 nM SkQ1 [10]. Bongkrekic acid also

Table 2
Hydrophobic cations compete with N-nonyl acridine orange (NAO) for cardiolipin in rat heart mitochondria. K_a, association constant of NAO with mitochondria in the absence and in the presence of hydrophobic cations. (From Antonenko et al. [10]).

Cation-containing compound	K_a, M^{-1}
NAO	1.2×10^6
SkQ3	5.5×10^6
MitoQ	1.0×10^5
SkQ1	2.5×10^5
C_{12}TPP	1.0×10^4
SkQR1	8.0×10^4

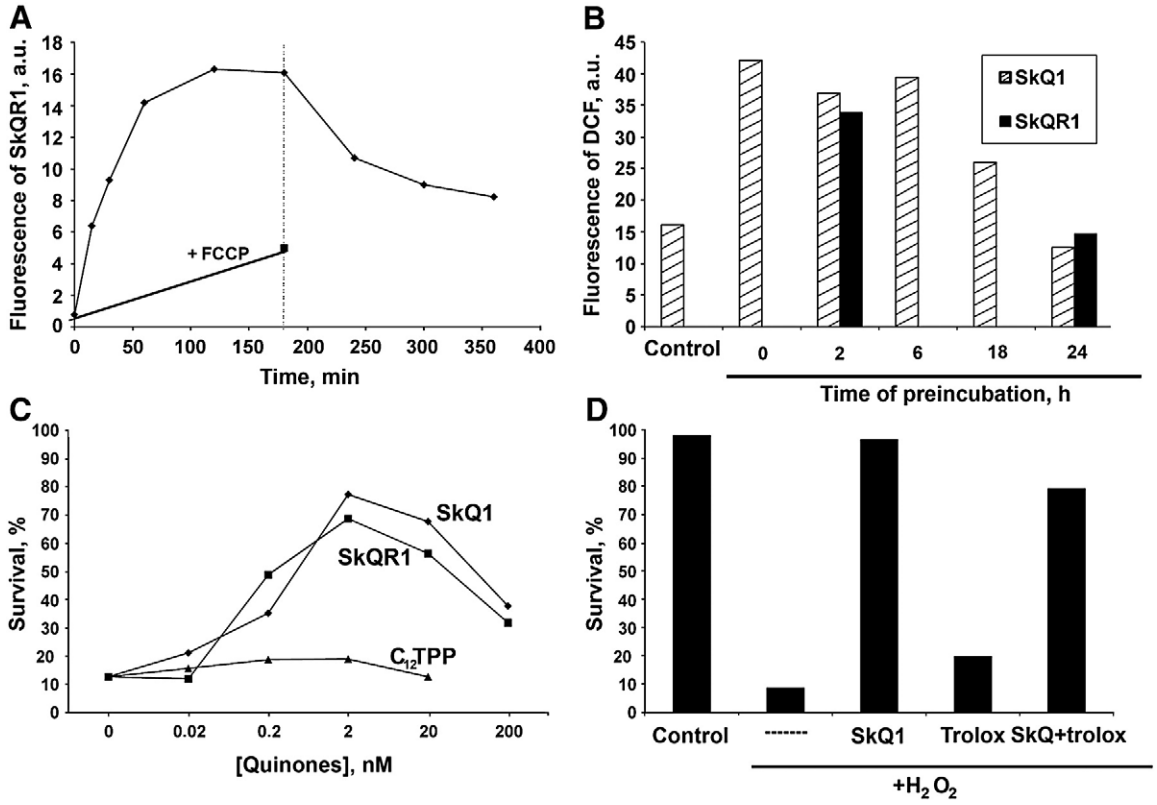

Fig. 6. Interaction of SkQ1 and SkQR1 with human fibroblasts. (A) Accumulation of SkQR1 in fibroblasts and SkQR1 release from these cells (before and after the dashed line, respectively). At zero time, 50 nM SkQR1 was added to the cell growth medium. After 3 h, the mixture was replaced by medium without SkQR1. Where indicated, 10 μM FCCP was added. (B) The cells were preincubated with SkQ1 or SkQR1 for the indicated period and then were treated with 0.6 mM H_2O_2 for 2 h. The intracellular ROS level was measured using DCF fluorescence. Control, without H_2O_2. (C) and (D) Effects of antioxidants on survival of human fibroblasts after treatment with 0.5 mM H_2O_2. Preincubation with antioxidants for 24 h, then H_2O_2 was added. Surviving cells were counted 18 h after the H_2O_2 addition. (D) Where indicated 0.2 mM Trolox was added 1 h before 2 nM SkQ1. (From Izyumov et al. [23]).

inhibited Bax translocation, whereas atractyloside induced the translocation even without added H_2O_2. A possible explanation for these facts is that Bax translocation requires an ATP/ADP antiporter conformation stabilized by atractyloside or oxidation of the antiporter SH-groups facing the mitochondrial matrix (for discussion on role of the ATP/ADP antiporter in formation of contact sites between two mitochondrial membranes and on the relation of these sites to Bax binding, see Refs. [23,26,27]). The 24 h preincubation with SkQ seems also to be related to the antioxidant properties of this compound since other antioxidants (Trolox and NAC) have similar effects. The very fact that these antioxidants were operating only at much higher concentrations than SkQ1 points to the mitochondrial localization of the ROS involved. Inefficiency of C_{12}TPP clearly shows that it was the plastoquinone moiety of SkQ is that responsible for the action of SkQ on total ROS level in fibroblasts and survival of these cells after H_2O_2 treatment [10].

Staining of fibroblasts with MitoTracker Green and TMRM is shown in Fig. 8. TMRM accumulates in mitochondria in a $\Delta\psi$-dependent manner. TMRM quenches the fluorescence of MitoTracker Green in those mitochondria that generate high $\Delta\psi$. If $\Delta\psi$ is low, mitochondrial [TMRM] is too small to quench the MitoTracker Green fluorescence (this fact was applied by Lemasters and Ramshesh [25] to identify de-energized mitochondria in hepatocytes). The majority of mitochondria have high $\Delta\psi$. However, in mitochondria localized at the cell periphery green fluorescence is seen, indicating that the mitochondrial $\Delta\psi$ level is low in this region of the cell [23]. SkQ accumulation in these

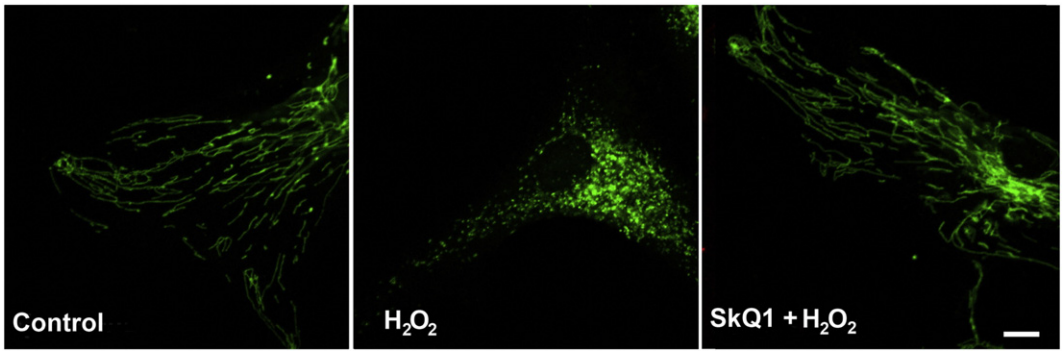

Fig. 7. SkQ1 prevents the H_2O_2-induced fragmentation of elongated mitochondria in human fibroblasts. The cells were preincubated with 2 nM SkQ1 for 2 h and then treated with 0.4 mM H_2O_2 for 3 h. After the treatment, the cells were stained with MitoTracker Green. Bar, 15 μm. (From Izyumov et al. [23]).

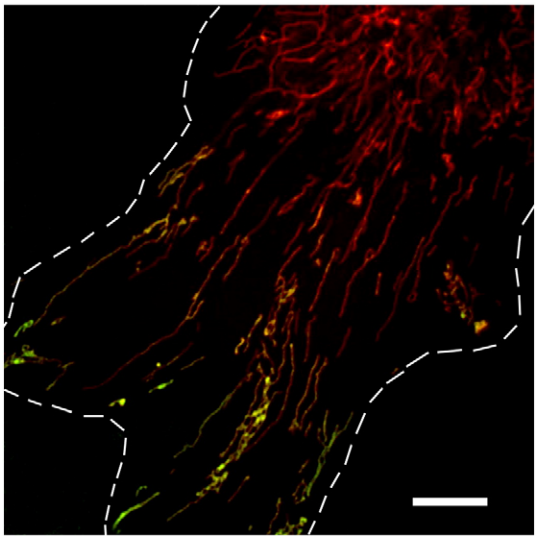

Fig. 8. Small portion of mitochondria in the peripheral regions of fibroblasts failed to maintain high $\Delta\psi$. The cells were incubated with 200 nM MitoTracker Green and 400 nM TMRM for 15 min. The $\Delta\psi$-dependent accumulation of TMRM resulted in quenching of the green fluorescence of MitoTracker. Bright green fluorescence is seen in mitochondria with decreased $\Delta\psi$. Dashed line, plasma membrane. Bar, 15 μm. (From Izyumov et al. [23]).

mitochondria should be slower and less than in mitochondria with high $\Delta\psi$. Assuming that low potential mitochondria produce larger amounts of ROS, we suggest that (a) these ROS induce the burst of ROS formation in the cell (the so-called ROS-induced ROS release [28]) and (b) ROS superproduction is responsible for damage to a minor portion of mitochondria resulting in $\Delta\psi$ lowering. These points can explain the mechanism of the slow effect of SkQ1 described above. SkQ1 slowly accumulating in damaged mitochondria lowers ROS production, which seals, step-by-step, their inner membrane that was leaky due to ROS-induced oxidation of lipids and proteins composing this membrane. A decrease in the ROS production lowers the total ROS level in the cell and prevents cell death.

An increase in ROS formation by mitochondria when $\Delta\psi$ is decreased was described in our [28] and other [29] groups. This effect should be distinguished from a ROS decrease on lowering of $\Delta\psi$ which is initially very high (State 4→State 3 transition or mild uncoupling) [30]. Possible involvement of SkQ in the latter phenomenon will be considered in the next section.

3. An antioxidant effect of the cation moiety of SkQ: fatty acid cycling

In the experiments described above, the effects of SkQ could not be reproduced with its derivative lacking quinone (C_{12}TPP). This was also the case in the majority of our *in vivo* studies. However, in certain cases, e.g. in studies on *Drosophila* lifespan [20] or on some plant systems (A.B. Vartapetian et al., in preparation), C_{12}TPP could effectively substitute for SkQ1. We investigated the possible mechanism of this phenomenon [31].

In rat heart mitochondria studied *in vitro*, it was found that not only SkQ1 but also C_{12}TPP decreases H_2O_2 production under State 4 conditions when reversed electron transport from succinate to NAD^+ occurs. However, the $C_{1/2}$ value for the effect of SkQ1 was 10–15-fold lower than for C_{12}TPP (Fig. 9A). Fat-free bovine serum albumin abolished the action of C_{12}TPP but did not influence the inhibitory effect of low [SkQ1] [31]. The effect of albumin suggests that endogenous fatty acids might be involved in the action of C_{12}TPP. To test this possibility, we added palmitate to the same experimental system (Fig. 9B–D). The palmitate completely inhibited H_2O_2 formation by mitochondria under the conditions of reverse electron transfer ($C_{1/2}$ for palmitate 1×10^{-6} M). Addition of 1×10^{-6} M C_{12}TPP lowered the $C_{1/2}$ value of palmitate fivefold (Fig. 9B). The effect of palmitate without C_{12}TPP was strongly reduced by carboxyatractyloside (Catr), an inhibitor of the ATP/ADP antiporter as previously shown in our group [32]. However, Catr was absolutely ineffective if 1 μM C_{12}TPP was added (Fig. 10C and D). This reminds us of Shönfeld's observation [33] that tetraphenylphosphonium (TPP) stimulates uncoupling activity of fatty acids. This stimulation, according to data of our group, is Catr-sensitive [34]. Such relationships are explained assuming that TPP facilitates translocation of fatty acid anions to nucleotide anion-binding positively charged amino acid residues of the antiporter, which are involved in the fatty acid cycling in the mitochondrial membrane [34–36]. The more hydrophobic C_{12}TPP is apparently competent in the transmembrane movement of fatty acid anions with no ATP/ADP antiporter or other proteins involved. This hypothesis was proved experimentally (see below, BLM experiments).

The mechanism of fatty acid uncoupling was studied in detail in yeast mitochondria [31,37]. It was found that SkQ1, SkQ3, and MitoQ, i.e. hydrophobic compounds containing, like C_{12}TPP, *delocalized* positive charge, can effectively substitute for C_{12}TPP for potentiating the $\Delta\psi$-lowering effect of palmitate-induced uncoupling (Fig. 9E). Cetyltrimethylammonium (CTMA), a hydrophobic cation with *localized* charge, was ineffective (Fig. 9F).

To directly show that proteins are not involved in the C_{12}TPP effect, it was studied in a bilayer planar phospholipid membrane (BLM) separating two solutions differing in pH value. In this system addition of a protonophore (FCCP) resulted in a transmembrane H^+ flux generating an electric potential difference across the BLM, the more acidic compartment being negatively charged. It was found (Fig. 10) that palmitate and a penetrating cation added together can effectively substitute for FCCP. The $\Delta\psi$ increased when pH values of the BLM-separated solutions are higher than pK of the fatty acid. At pH 4 in one compartment and pH 5 in the other compartment, $\Delta\psi$ was as small as 13 mV, whereas at pH 6 versus 7 (or 7 versus 8) it reached the theoretical Nernstian value approaching 60 mV [31].

More complicated relationships were revealed when SkQR1 and its quinone-lacking analog C_{12}R1 were studied. In these cases, $\Delta\psi$ with the expected sign was observed even without palmitate. But $\Delta\psi$ never reached theoretical Nernstian level and was absent in the acidic pH region. SkQR4 and C_{12}R4 failed to substitute for SkQR1 and C_{12}R1. The difference between the R1 and the R4 compounds is in the structure of their Rhodamine moieties (Rhodamines 19 and B, respectively, see above Fig. 1). The cationic group of Rhodamine 19 contains a dissociable proton, whereas that of Rhodamine B has no such H^+, it being replaced by an ethyl residue. It is not surprising, therefore, that derivatives of Rhodamine 19, but not of Rhodamine B, display some protonophorous activity. This activity of SkQR1 and C_{12}R1 was further increased by fatty acids.

The data described above clearly show that palmitate added together with hydrophobic penetrating cations (SkQ1, C_{12}TPP, etc.) can mediate $\Delta pH\rightarrow\Delta\psi$ transduction in a protein-free system like the BLM. We hypothesized that SkQ1 and C_{12}TPP operate in the BLM as carriers of fatty acid anions. This hypothesis was confirmed by experiments on liposomes. We found [31,37] that addition of SkQR1 caused a release of carboxyfluorescein (CF) anions from CF-loaded liposomes. The effect was absent when doxorubicin cation substituted for CF. Melittin, which induces an unspecific increase in the liposome membrane ion conductance, was effective with both CF- and doxorubicin-loaded liposomes. The effect of SkQR1 on CF-loaded liposomes was strongly inhibited by palmitate. This can be explained by the competition of the palmitate anion with the CF anions for the SkQR1 cation. Formation of a complex of C_{12}R1 and palmitate in an aqueous solution was directly proven by the experiment shown in Fig. 11. Micromolar palmitate changes the light absorption of C_{12}R1 and strongly diminishes its fluorescence [37].

The fatty acid anion transport by C_{12}TPP was modeled by molecular dynamics simulation. For the starting point, we placed C_{12}TPP and

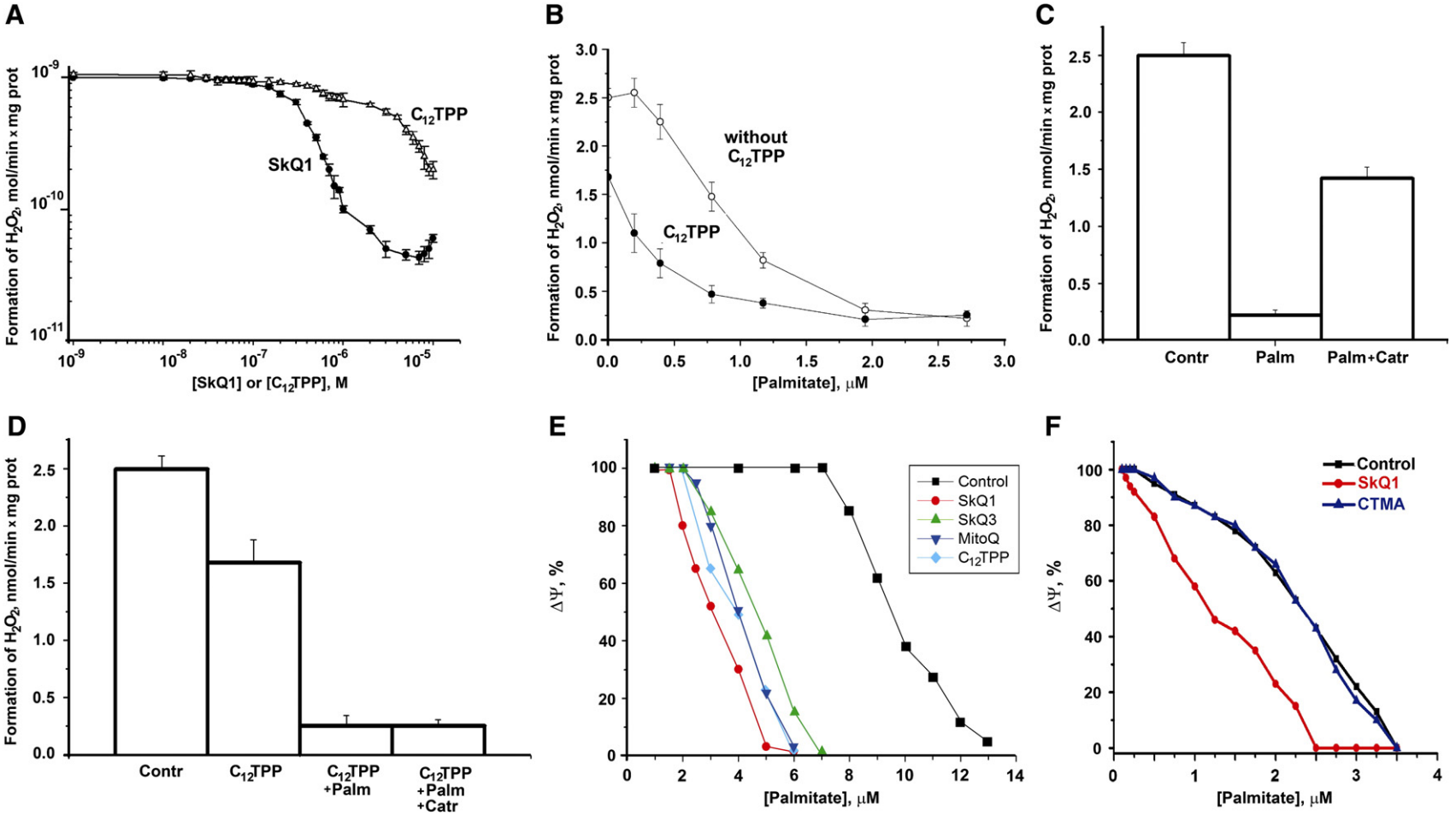

Fig. 9. Inhibition of H_2O_2 formation in isolated rat heart mitochondria (A–D), *Dipodascus (Endomyces) magnusii* mitochondria (E), and *Yarrowia lipolytica* mitochondria (F) by penetrating cations and palmitate. State 4 conditions using succinate as the respiratory substrate. (A) Comparison of the efficiencies of SkQ and $C_{12}TPP$ in inhibiting H_2O_2 formation. Incubation mixture, 250 mM sucrose, 1 mM EGTA, 10 mM MOPS-KOH (pH 7.4), 5 mM succinate, 2 μM Amplex Red, horseradish peroxidase (9 U), and rat heart mitochondria (0.15 mg protein/ml). (B) 1 μM $C_{12}TPP$ makes palmitate more efficient in inhibiting H_2O_2 formation. Condition as in (A). (C, D) Palmitate stimulates inhibitory effect of $C_{12}TPP$ on H_2O_2 formation in a Catr-resistant manner. Incubation mixture as in (A). Additions, 2 μM palmitate, 1 μM Catr, and 1 μM $C_{12}TPP$. (E) Incubation mixture, 0.6 M mannitol, 0.2 mM Tris–phosphate (pH 7.2), 0.5 mM EGTA, 20 mM succinate, and 10.5 μM safranin O, and *D. magnusii* mitochondria (0.5 mg protein/ml). Where indicated, 1.5 μM SkQ1, SkQ3, MitoQ, or 2.5 μM $C_{12}TPP$ was added. In I, no BSA was used during isolation of *Y. lipolytica* mitochondria. Additions, 0.25 μM SkQ1 or 1.1 μM CTMA. (From Severin et al. [31] and Sukhanova et al. [37]).

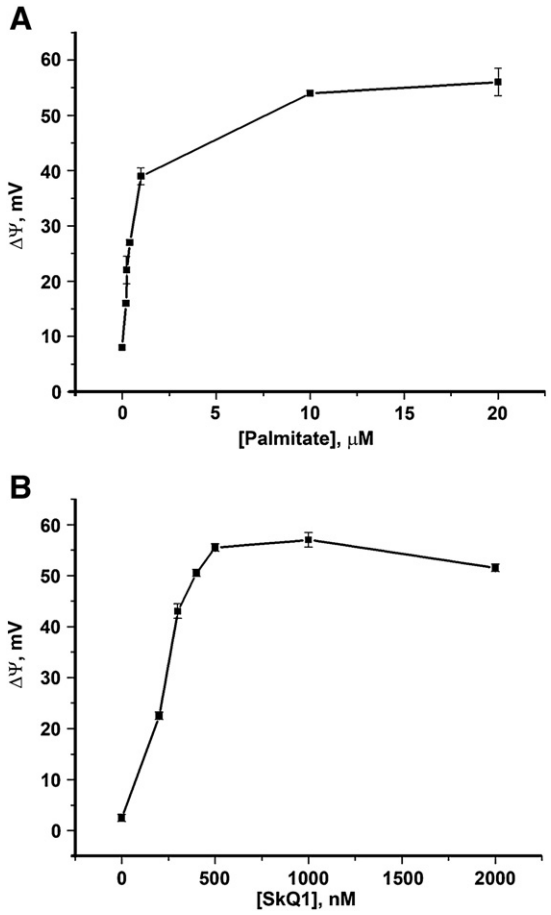

Fig. 10. Penetrating cations mediate protonophorous effect of fatty acids in BLM. Incubation mixture, 10 mM Tris–MES, 10 mM KCl. The pH was 7 in one compartment and 6 in the other compartment. When palmitate and SkQ1 were added, the more acidic compartment was negatively charged. (A) 1 μM SkQ1 is present in all cases; for [Palmitate], see abscissa. (B) 10 μM palmitate in all cases; for [SkQ1], see abscissa. (For details, see Severin et al. [31]).

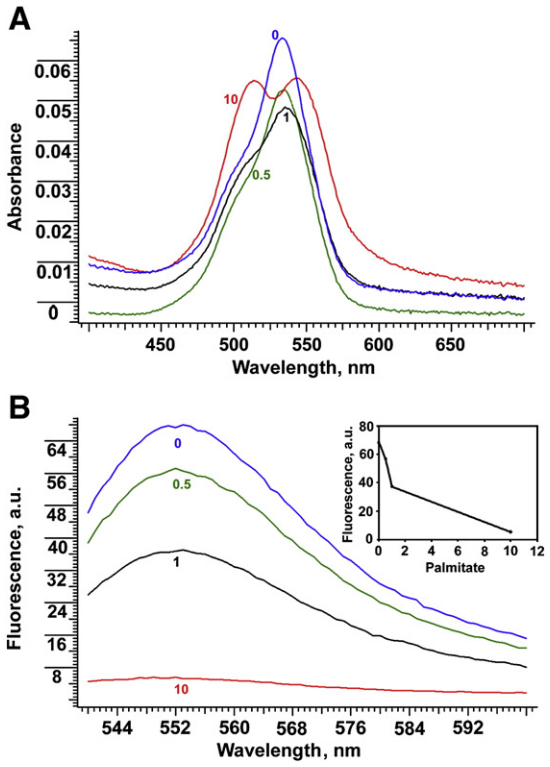

Fig. 11. Micromolar palmitate (palm) changes the light absorption spectrum of $C_{12}R1$ (A) and quenches its fluorescence in aqueous solution. Incubation mixture, 10 mM Tris–HCl, pH 7.4, 1 μM $C_{12}R1$ and various palmitate concentrations indicated by figures near curves, μM. (From Skulachev et al., in preparation and Rokitskaya et al. [37]).

palmitate ions near each other. After 25 ns equilibration, direct contact between the ions occurred. When adaptive biasing force (ABF) was applied to $C_{12}TPP$, its charged headgroup penetrated the lipid phase. The carboxyl group of palmitate followed the movement of $C_{12}TPP$ through the membrane core, usually remaining at a distance of 4–6 Å. In certain cases, however, the distance increased up to 8–10 Å (Fig. 12) [31].

The next question we addressed was whether the protonophorous effect of fatty acid/penetrating cation pair occurs in mitochondria *in vivo*. If an intact cell contains a certain level of non-esterified fatty acids, $C_{12}TPP$ should specifically increase H^+ conductance of the mitochondrial membrane, other membranes being unaffected since $C_{12}TPP$ is targeted to mitochondria. This prediction was verified by experiments on intact *S. cerevisiae* cells, which are known to contain large amounts of non-esterified fatty acids (e.g., [palmitate] was as high as 0.1 mM [37]). As shown in Fig. 13A, lowering of pH of the medium to 3.0 allowed FCCP to stimulate cell respiration only at low uncoupler concentrations. If the concentration was increased, the stimulation disappeared. However, both low and high concentrations of $C_{12}TPP$ caused a strong increase in the respiration rate (Fig. 13B). These relationships can be accounted for by assuming that high [FCCP] increased H^+ conductance not only of the mitochondrial but also of the plasma membrane. This should result in acidification of the cytosol if the pH outside the cell is low. Lowering of intracellular pH should, in turn, inhibit mitochondrial respiration [31]. $C_{12}TPP$ electrophoretically accumulates in mitochondria and, hence, in cooperation with endogenous free fatty acids, increases H^+ conductance of the mitochondrial (but not of the plasma) membrane. If this is the case, inhibition of respiration at $pH_{out} = 3.0$ could be achieved by adding a penetrating weak acid. Consistent with this suggestion, we found that acetate strongly inhibits respiration at pH 3.0 but not at pH 6.0 [31]. Collectively, these data demonstrate mitochondria-targeted uncoupling by $C_{12}TPP$ in living yeast cells.

The relationships described above are illustrated by the scheme shown in Fig. 14. According to this scheme, protonated fatty acid moves across a membrane by a flip-flop mechanism, transporting H^+ from the lower (more acidic) compartment to the upper (more alkaline) compartment (stage 1). This stage is fast [38] and does not require any carrier. In the upper membrane/water interface, the fatty acid is deprotonated (stage 2). The resulting fatty acid anion combines with $C_{12}TPP$ cation (stage 3). Then the $C_{12}TPP$ cation/fatty acid anion

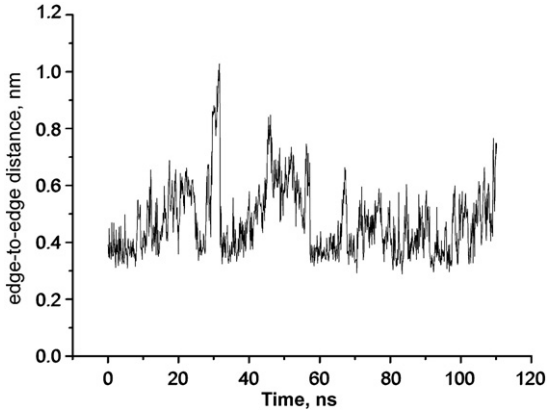

Fig. 12. Dynamics of the edge-to-edge distance between the $C_{12}TPP$ cation and palmitate anion during their translocation through a phospholipid membrane (a molecular dynamics simulation; for details, see Severin et al. [31]).

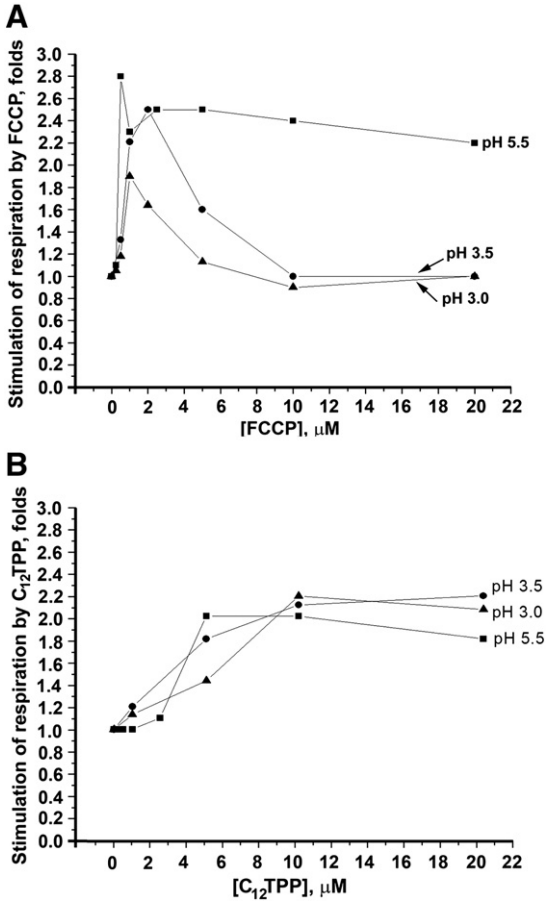

Fig. 13. Effects of FCCP (A) and C_{12}TPP (B) on respiration of *S. cerevisiae* cells at different extracellular pH values. Incubation mixture, 50 mM potassium phosphate and 0.005% glucose. (For details, see Severin et al. [31]).

pair diffuses across the membrane to the opposite (lower) interface (stage 4). The cycle is completed by protonation of the fatty acid anion in the interface facing the lower compartment (stage 5), accompanied by release of free C_{12}TPP cation (stage 6), which returns to the upper membrane surface (stage 7). It is stage 7 that is electrogenic, thus being responsible for $\Delta\psi$ generation, the upper (alkaline) compartment being positively charged.

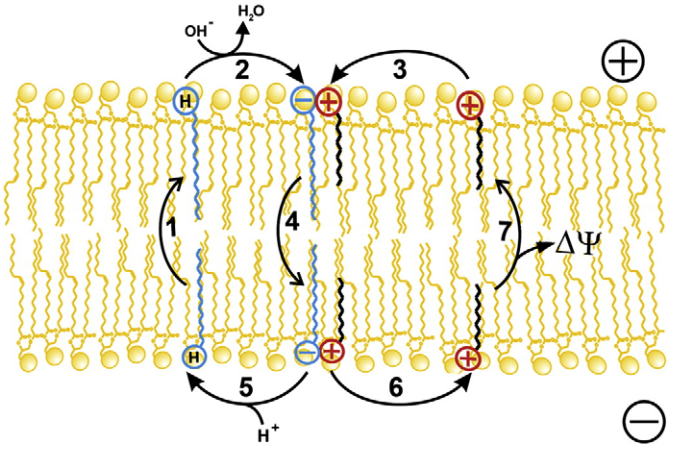

Fig. 14. Scheme illustrating mechanism of protonophorous fatty acid cycling mediated by penetrating cations. Symbols ⊖ and ⊕ represent anionic and cationic groups of fatty acid and C_{12}TPP, respectively; ⊕ represents a protonated carboxyl group of a fatty acid. The upper compartment is more alkaline than the lower one. (For details, see Severin et al. [31]).

The crucial stage 3 was directly proven when bleaching of SkQR1 by stoichiometric amounts of palmitate was shown in aqueous solution (Fig. 12). This stage as well as stage 4 was modeled by molecular dynamics calculations [31]. The remarkable result of this analysis is that during the transmembrane journey of the C_{12}TPP/palmitate ion pair the distance between the cation and anion moieties is not constant. Rather, it varies from 0.3 to 1.0 nm without irreversible decomposition of the pair. This phenomenon can be explained by very low dielectric constant in the hydrophobic membrane core. This means that direct contact of the cation and anion is not necessary to organize translocation of a penetrating cation/non-penetrating anion pair through a phospholipid membrane. Therefore, it seems possible that steric difficulties preventing ion pair formation in the aqueous phase can be insignificant in the hydrophobic region of a membrane. In fact, we deal here with new type of a positively charged carrier which facilitates transmembrane movement of an anion by means of a long-distance electric interaction with this anion in the hydrophobic membrane core.

Interaction of this type accounts for lack of specificity of the cation to the chemical structure of the transported anion. As mentioned above, SkQR1 is effective in transporting not only such carboxylate monanion as palmitate but also carboxyfluorescein, a compound of quite different structure containing three anionic groups. Perhaps certain biological effects of SkQ1 reproduced by C_{12}TPP are due to facilitating the transport of some hydrophobic anions (e.g., peroxides of fatty acids [44]).

Mild uncoupling is another possibility to explain the situation when both SkQ1 and C_{12}TPP appear to be effective. As found by our group, mitochondrial ROS generation shows very steep dependence on $\Delta\psi$ [30]. Thus, a slight decrease in $\Delta\psi$ ("mild uncoupling" [39]) results in many-fold decrease in the rate of ROS formation by mitochondria. This suggests that uncoupling by SkQ1 (or C_{12}TPP)/fatty acid ion pair mimics the mild uncoupling activity of UCP, ATP/ADP antiporter, or some other mitochondrial anion carriers also mediated by fatty acids [35,36].

It should be stressed that an effective mechanism of mild uncoupling must be organized in a way preventing its escalation to strong uncoupling when $\Delta\psi$ drops below the level critical for ATP synthesis. The mild uncoupling mechanism in its optimal version should only slightly decrease $\Delta\psi$, preventing ROS formation but leaving the resting $\Delta\psi$ at a level still sufficient to support operation of H^+-ATP-synthase. This requirement is fulfilled when the penetrating cation concentration increases. This increase should result in lowering of $\Delta\psi$, the driving force for accumulation of cations in mitochondria. Hence, a $\Delta\psi$ decrease lowers, say, the C_{12}TPP concentration in mitochondria, which in turn prevents further $\Delta\psi$ decrease. The idea of a "self-limiting mitochondrial protonophore" was discussed by Blaikie at al. [40] who conjugated dinitrophenol and triphenylphosphonium using propyl linker. The compound was accumulated by mitochondria but did not uncouple (most probably due to impermeability of its zwitterionic form). Now this problem is solved using much more hydrophobic C_{12}TPP and related compounds. These substances are promising tools for prevention of mitochondrial hyperpolarization (which has been assumed to be involved in aging, obesity, hypothyroidism, certain types of cancer, etc. [5,41–43]), without a risk of such a dramatic side effect as inhibition of oxidative phosphorylation. Comparing MitoQ, SkQ1, and C_{12}TPP as mild uncouplers, we conclude that C_{12}TPP looks like a better mediator of this effect. C_{12}TPP, in contrast to MitoQ and even SkQ1, lacks prooxidant activity at high concentrations (see Ref. [10] and Fig. 9A).

An interesting observation was made when Catr was tested in palmitate-treated mitochondria. Without C_{12}TPP, Catr prevented palmitate-induced decrease in H_2O_2 formation by mitochondria (Fig. 9C). A similar action of Catr was revealed when fatty acid uncoupling was potentiated by TPP [33]. However, the effect of C_{12}TPP was Catr-resistant (Fig. 9D). Apparently, C_{12}TPP, being much more hydrophobic than TPP, does not require assistance by ATP/ADP

antiporter to transport palmitate. This suggestion was confirmed by the observation that C_{12}TPP, but not TPP, facilitates fatty acid-mediated H^+ conductance in BLM (Fig. 10 and Ref. [31]).

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