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Mini Review

Cationic antioxidants as a powerful tool against mitochondrial oxidative stress

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ABSTRACT

This review describes evidence that mitochondrial reactive oxygen species (mROS) are of great importance under many physiological and pathological conditions. The most demonstrative indications favoring this conclusion originate from recent discoveries of the *in vivo* effects of mitochondria-targeted antioxidants (MitoQ and SkQs). The latter compounds look promising in treating several incurable pathologies as well as aging.

A year ago, Dr. Guy Brown (Cambridge, England) and Dr. Vilmante Borutaite (Kaunas, Lithuania) published an article entitled "There is no evidence that mitochondria are the main source of reactive oxygen species in mammalian cells" [1–4]. I agree with the authors that at present really there is no direct proof that these organelles usually produce the quantitatively major portion of ROS *in vivo*, but the title, nevertheless, might be misleading for readers especially if they are rather far from this field of research. In this review, I would like to show that mROS are of great importance for the fate of the mitochondrion, the cell, and even the organism.

First of all, it is firmly established that at least some ROS are generated by mitochondria. Even if this amount in the liver is only 15% of the total ROS production as assumed by Brown and Borutaite [1], it does not mean that mROS are of negligible importance for other tissues and for the liver itself. Moreover, the liver cell can hardly exemplify all the cell types in mammals. The liver cell is always in its working regime, so the resting State 4 when mROS production rate is maximal [2–5] is not typical for these cells. These cells contain large amounts of endoplasmic reticulum housing certain ROS-releasing enzymes (cytochrome P450 and some others) as well as peroxisomes [6]. On the other hand, skeletal muscle cells and the majority of neurons are often in the resting State 4. Low respiration rate as well as high membrane potential is inherent in this State [3,6]. Both these properties are favorable for mROS production [3,5]. Low respiration rate results in increase in $[O_2]$ in mitochondria, which in turn accelerates mROS formation. High membrane potential inhibits forward electron transfer and stimulates reverse electron transfer in Complexes I and III. Under these conditions, the semiquinone form of CoQ is stabilized in center *o* of Complex III (an event favorable for superoxide formation), and moreover mROS generation is somehow initiated in Complex I [3,5].

Considering the probable contribution of mitochondria to ROS generation in the cell, one should take into account that in the majority of mammalian cells the inner mitochondrial membrane occupies very much higher areas (more than a thousand m^2 in an adult human) than any other intracellular membrane. This membrane houses large amounts of four respiratory chain complexes. Three of them (Complexes I–III) are known to be competent in ROS generation at least under certain conditions [7]. The highest rate of mROS formation in State 4 by intact mitochondria is observed at millimolar succinate concentrations. For long time, such concentrations were regarded as non-physiological. However, quite recently it was found that a burst of succinate formation from glutamine and glutamate occurs, for example, under inflammatory conditions [8]. My colleague Dr. D.B. Zorov and coworkers [9] developed a concept assuming that in the cell some elevation of [ROS] can initiate further increase in ROS production ("ROS-induced ROS release"). The first piece of experimental evidence in favor of this assumption was obtained by Zorov when studying ROS level in cardiomyocytes on a fast time scale. A similar phenomenon was described later in our group by Dr. B.V. Chernyak and

Abbreviations: $\Delta\psi$, transmembrane electric potential difference; MitoQ, 10-(6′-ubiquinonyl) decyltriphenylphosphonium; mROS, mitochondrial reactive oxygen species; ROS, reactive oxygen species; SkQs, plastoquinonyl conjugates with penetrating cations; SkQ1, 10-(6′-plastoquinonyl) decyltriphenylphosphonium; SkQR1, 10-(6′-plastoquinonyl) decylrhodamine 19.

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coauthors [10] in fibroblasts, but in this case the effect took 2 h to develop. A small amount of H_2O_2 added to fibroblasts was decomposed in minutes, but it initiated a chain of events that later resulted in a large increase in [ROS] and apoptosis. Both the secondary burst of ROS and cell death were prevented by mitochondria-targeted antioxidant SkQ1 (see below), indicating that mROS are at least somehow involved in the scenario occurring after adding H_2O_2. Evidence exists that H_2O_2 can activate NADPH oxidases to make more $O_2^{\cdot-}$.

It should be stressed that at least three properties of mitochondria make mROS especially dangerous. (1) Mitochondrial DNA is less protected against attack by $OH^{\cdot}$ and other ROS than nuclear DNA, first of all since the former is much closer to a source of ROS (respiratory chain) than nuclear DNA. $OH^{\cdot}$ is so short-lived that it is quite unrealistic to imagine that this poison can travel from the interior of mitochondria to nuclear DNA. (2) The inner mitochondrial membrane maintains much higher $\Delta\psi$ than any other membrane in the cell. This membrane must be very well organized as an insulator to be resistant to electrical breakdown. Any damage to this organization by ROS results in H^+ leakage through the inner mitochondrial membrane and dissipation of respiratory energy. (3) Cardiolipin is an obligatory component of the inner mitochondrial membrane. In contrast to other phospholipids, it contains not two but four fatty acid residues, and they are all polyunsaturated (other phospholipids have only one unsaturated fatty acid, which often contains only one double bond). It is not surprising, therefore, that cardiolipin is oxidized by ROS much sooner than any other phospholipid [11]. Cardiolipin sometimes forms dimers integrated in protein complexes inside the membrane (e.g., Complex III). In the dimer, there are eight fatty acid residues. If all of these are linoleates (often the case for cardiolipins), this means that 16 double bonds in close vicinity to each other are present in the cardiolipin dimer. This situation makes probable initiation of a chain reaction of cardiolipin peroxidation when one of its linoleates is attacked by $OH^{\cdot}$. This is why mROS are much more dangerous for the cell than ROS generated in any place other than the interior of mitochondria [12].

It is hardly surprising therefore that just *mitochondrial* ROS are involved in initiation of programmed death phenomena from organelle to organism [6,13]. Suicide of malfunctioning mitochondria (mitoptosis [13]) is usually mediated by mROS. In particular, this may happen as a result of opening of permeability transition pores in the inner mitochondrial membrane, an event causing elimination of $\Delta\psi$ and efflux of NAD(P)(H) and glutathione from the matrix of the mitochondria. Organelles lacking $\Delta\psi$ are recognized by autophagosomes and digested (mitophagy) [6,13,14]. This mechanism allows the cell to purify the mitochondrial population from an isolated malfunctioning (e.g., mutated) mitochondrion. However, in a situation when all the mitochondria lose $\Delta\psi$ (e.g., when uncoupler or respiratory inhibitor appears inside the cell), a more radical mitoptotic mechanism occurs. The cell gathers the majority of its mitochondria close to the nucleus, surrounds them with a membrane, and expels the formed "mitoptotic body" from the cell [6,15–17]. If mitoptosis fails to quickly eliminate mitochondria with opened pores, the mitochondrial matrix swells, an event resulting in breakdown of the outer mitochondrial membrane and release of intermembrane proteins of mitochondria to the cytosol. Among them there are proapoptotic proteins (e.g., cytochrome *c*), which meet in the cytosol their partners or targets to initiate cell suicide (apoptosis) [13]. Remarkably, disappearance of an organ during ontogenesis (e.g., tail of tadpole) is also mediated by a ROS, namely H_2O_2 [18].

For a long time, it has been generally accepted by botanists and specialists in invertebrates that many plants and invertebrates can die due to operation of certain mechanisms of programmed death. More recently, such events were described also among fungi, unicellular organisms, and vertebrates including mammals [6,13,19]. In 1997, I coined the word "phenoptosis" to indicate various kinds of programmed death of organisms [20]. In the same, paper I suggested that aging is a particular case of slow phenoptosis initiated by mROS (see also [19,21–23]). Later, Lambert et al. [24] showed that the maximal life span of 11 species of mammals and birds shortened in parallel with the increase in mROS generation during reverse electron transfer via Complex I of the respiratory chain. Only one animal species was an exception to the rule. This was the naked mole-rat *Heterocephalus glaber*, an African rodent of mouse size that lives at least 10 times longer than a mouse while producing mROS much faster. However, the naked mole-rat was the only "*non-aging*" animal among the studied species [6]. The probability of its death is low and independent of age [25]. An explanation for the naked mole-rat paradox consists in that in its genome, there is a mutation interrupting a phenoptotic (aging) program at a stage occurring *after mROS*, so mROS are still generated but fail to initiate aging [6].

An increase in [mROS] with age was quite recently demonstrated *in vivo* in mitochondria of *Drosophila* by Murphy and colleagues, who used a penetrating cation as a ROS probe [26]. In another group, it was shown that H_2O_2 generation by skeletal muscle mitochondria of 23-year-old humans was three times lower than that of 67-year-old humans. The difference was abolished by rotenone, an inhibitor of Complex I [27]. These relationships might be explained by a decrease in the level of SIRT3, a deacetylase, stimulating (a) a mitochondrial system of glutathione reduction and (b) superoxide dismutase 2 [28], i.e. two key antioxidant mechanisms of mitochondria. Apparently, some enzymes other than Complex I may be also involved in age-dependent increase in [mROS] in certain tissues under some conditions. A 7.5-fold increase in monoamine oxidase A in the outer heart muscle mitochondrial membrane of 24-month-old rats compared to 1-month-old animals has been reported [29].

It was shown that decrease in mROS decelerates aging. This effect was achieved in three different ways:

(1) Dr. P. Rabinovitch and coworkers succeeded in targeting catalase to the mitochondrial matrix. To this end, an 11 amino acids sequence that targets catalase to peroxisomes was replaced by the 25 amino acid mitochondrial address of ornithine transcarbamylase. The modified gene was incorporated into the genome of the mouse embryos. The mice that developed from these embryos lived longer than the control animals [30]. The longevity was due to an increase in healthspan. In particular, aging of skeletal muscle was decelerated. An aging-linked elevation of $[H_2O_2]$ caused by increased production of mROS was prevented just as such consequences of oxidative stress as increase in mutagenesis of mitochondrial DNA, stimulation of carbonylation of mitochondrial proteins, decrease in respiration rate, and decrease in number of mitochondria in myocytes [31]. Moreover, effects of aging on the heart, systemic inflammation, and probability of incidents of some kinds of cancer were retarded [32,33]. In progeric mice with a mutation in the proofreading domain of mitochondrial DNA polymerase ("mutator mice"), the lifespan was dramatically increased by the mitochondria-targeted catalase, whereas development of several traits of aging was retarded. Nucleus-addressed catalase failed to show the effects of the mitochondria-targeted enzyme [30].

(2) Pelicci and colleagues reported that knockout of the gene encoding p66shc protein increased lifespan of mice by 30%. In mitochondria, this protein cooperates with cytochrome *c* in organizing a bypass of cytochrome oxidase, producing $O_2^{\cdot-}$ instead of H_2O [34–37].

(3) In our group, it was shown that synthetic cationic mitochondria-targeted antioxidant plastoquinonyl decyltriphenylphosphonium (SkQ1) prolongs lifespan of fungi, plants, crustaceans, insects, fishes, and mammals [11,12,38,39]. In "mutator mice", the effects of SkQ1 were similar to those of mitochondria-targeted catalase. The antioxidant increased both the lifespan and the healthspan (experiments performed in Prof. B. Cannon's group by Dr. I.G. Shabalina and coauthors [in preparation]). In several mammalian species, SkQ1 retarded development of a large number of age-related pathologies [11,12]. Experiments on animals suffering from eye diseases were especially demonstrative. Drops containing 250 nM SkQ1 retarded development (and certain cases even reversed) symptoms of cataract, glaucoma, macular degeneration, uveitis, and dry eye syndrome in old mice, rats, rabbits, cats, dogs, and horses [11,12]. In the case of dry eye syndrome clinical trials showed that three-week therapy with SkQ1 resulted in disappearance of symptoms of this pathology (previously regarded as incurable) in 60% of patients [40,41]. SkQ1-containing drops Visomitin have been available in Russian drugstores since July, 2012. Clinical trials of SkQ1 as a medicine against age-related cataract and glaucoma are close to being completed in the some hospitals of Moscow and St. Petersburg. SkQ1 and its Rhodamine derivative SkQR1 proved to be effective in prevention of not only slow phenoptosis (aging), but also acute phenoptosis (quick programmed death of an organism during or immediately after a severe crisis) [6,13]. These crises included ischemia/reperfusion of the kidney [13,42–44], rhabdomyolysis [42], septic shock resulting from pyelonephritis [45], stroke [13,44], and heart muscle infarction [13,44].

Recently, Dr. I.I. Severina and her colleagues in our laboratory carried out a study to optimize the structure of mitochondria-targeted quinone derivatives. Two parameters were taken into account, i.e. efficiency of the studied compounds in prevention of (i) malondialdehyde formation by isolated mitochondria in the presence of ascorbate and Fe^{2+} and (ii) apoptosis of human fibroblasts initiated by added H_2O_2 [46,47]. In the first series of experiments, all the derivatives contained decyltriphenylphosphonium as the penetrating cation attached to the sixth position of the following quinone derivatives: benzoquinone (SkBQ); 3-methylquinone (toluquinone, SkTQ); 2,3-dimethylquinone (plastoquinone, SkQ1); 2,3,5-trimethylquinone (SkQ3); 5-methyl,2-methoxyquinone (DMMQ); 5-methyl,2,3-dimethoxyquinone (MitoQ). Chronologically, MitoQ was the first mitochondria-targeted cationic CoQ derivative successfully applied by Murphy, Smith, and coworkers to suppress mROS [48–52]. Our results show that antioxidant activity increases in the series SkBQ < MitoQ < DMMQ = SkQ3 < SkQ1 < SkTQ. This means that removal of the methyl and methoxy groups from CoQ increases its activity up to that of SkTQ, which has no methoxy groups and only one methyl group. However, removal of the last methyl group (SkBQ) strongly decreased the antioxidant properties. Thus, SkTQ is the optimal antioxidant, at least for the isolated mitochondria and the cultured cells. In the second experimental series, we varied the cationic residue of the SkQ, where the charged ionized atom is screened by bulky hydrophobic substituents. As shown in Dr. B.V. Chernyak's group, decyltriphenylphosphonium can be successfully replaced by synthetic Rhodamine 19 (in SkQR1) and Rhodamine B (in SkQR4) or the natural penetrating cations berberine (in SkQB) and palmatine (in SkQP). All of them were active in the tested systems, SkQR1 being slightly more efficient [53].

In general, the efficiency of antioxidants of the SkQ family is quite remarkable. Recently, Chernyak and coworkers in our group found that 20 pM SkQ1 arrests the rhabdomyosarcoma cell cycle at G/M stage and induces apoptosis of these cells. Dodecyltriphenylphosphonium, a penetrating cation lacking the quinone residue, was inactive, whereas the non-targeted antioxidants Trolox and N-acetylcysteine can replace SkQ1 but at very much higher concentrations (0.1 and 1 mM, respectively)[46]. *In vivo* studies performed in our group by B.P. Copnin and colleagues [11] showed that SkQ1 (5 nmol/kg body weight per day) increases by 30% the lifespan of $p53^{-/-}$ mice usually dying due to lymphomas. N-acetylcysteine reproduced such an effect, but at 1.2×10^6 times higher dose.

Simple calculation indicates that the concentration of SkQ1 in the inner leaflet of the inner mitochondrial membrane should be very much higher than in the extracellular aqueous solution. When electrophoretically entering the cell, SkQ1 as a monovalent penetrating cation should be accumulated in cytosol and the intermembrane space of mitochondria by a factor of 10, assuming $\Delta\psi$ on the plasma membrane is 60 mV (cell interior negative). Further accumulation by factor 1000 occurs when SkQ1 enters, again electrophoretically, the mitochondrial matrix, which is 180 mV more negative than the intermembrane space. Then, the membrane/water distribution coefficient, which is about 10^4 for SkQ1, should be taken into account [12]. Hence, the magnification of the SkQ1 concentration in the inner leaflet of the inner mitochondrial membrane compared with the extracellular space will be $10 \times 10^3 \times 10^4 = 10^8$. In fact, it may be even higher since the SkQ1 cation forms a complex with cardiolipin anion, which is apparently the prime target of the antioxidant activity of SkQs [54]. One more feature increasing the efficiency of SkQs is that they are *rechargeable* antioxidants, since their inactive oxidized forms are reduced (i.e. reactivated) by the center *i* of Complex III [54].

The very high efficiency of SkQs allows the use of extremely low doses of these antioxidants for *in vivo* treatments, which decreases the probability of adverse side effects. Concerning possible overdose, such effects are not excluded, first of all, due to the fact that SkQs, like the great majority of antioxidants, become prooxidants when their concentration is increased. Fortunately, the window between anti- and prooxidant activities for plastoquinone and its derivatives is very large (from 30 to 1000 times). For MitoQ, a CoQ derivative, the window is much smaller [11,54]. Nevertheless, even MitoQ was shown to have favorable *in vivo* effect in treating animals suffering from several oxidative stress-related pathologies, namely cardiac ischemia/reperfusion injury [55], negative consequences of nitroglycerin therapy [56], hypertension [57], sepsis [58,59], doxorubicin toxicity [60], diabetes [61], cocaine toxicity [62], insecticide toxicity in rats [63], and hepatic stenosis in ethanol-consuming rats [64]. For SkQs, this list is even longer (which is hardly surprising since their antioxidant activities are higher and the therapeutic windows are much larger than for MitoQ). It includes, as already mentioned, such pathologies as rhabdomyolysis, sepsis caused by pyelonephritis, stroke, myocardial infarction, arrhythmia, lymphomas in $p53^{-/-}$ rats, uveitis, dry eye syndrome, glaucoma, cataract, macular degeneration, etc.

Effects of SkQs and MitoQ cannot be explained by the assumption that they operate as scavengers of non-mitochondrial ROS, stimulating antioxidant functions of mitochondria ("mitochondria as an intracellular sink for ROS" [1]). Being a small hydrophilic anion, $O_2^{\cdot-}$ cannot penetrate a membrane. The ROS species $OH^{\cdot}$ and $HO_2^{\cdot}$ are so aggressive and short-lived that their translocation from cytosol to the mitochondrial interior is highly improbable. As to H_2O_2, a long-lived penetrant, it does not interact with quinols.

It is absolutely amazing that Brown and Borutaite ignore in their paper [1] the well-established effects of both MitoQ and SkQs. For SkQs, this might be due to the fact that Dr. Brown works in Cambridge, which is too far from Moscow where SkQs were described, and Dr. Borutaite in Kaunas, which is apparently even

farther now. But what about MitoQ, which was described just in Cambridge? I agree with Brown and Borutaite's conclusion that we cannot yet precisely estimate what percent of ROS is produced by mitochondria *in vivo*. However, therapeutic effects of cationic derivatives of quinol clearly demonstrate the great importance of mROS under many physiological and pathological conditions.

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