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Memories of a Senior Scientist

Functions of mitochondria: from intracellular power stations to mediators of a senescence program

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Abstract. In 1950 s I started in science by showing that non-phosphorylating respiration is critical for survival of an animal at low temperature. Later, in the 1960 s and 1970 s, I took part in verification of Mitchell's chemiosmotic hypothesis postulating that (i) mitochondria transform energy of respiration to electricity and (ii) uncoupling of respiration represents discharge of this electricity by H^+ cycling. Fifteen years ago I turned to a specific kind of mitochondrial respiration which produces $O_2^{\cdot-}$, and I came to the conclusion that it plays an ominous role, killing mitochondria, cells, or even organisms. My present task is a "megaproject" with an ambitious goal of minimizing the damaging effect of $O_2^{\cdot-}$ and stopping senescence.

Non-phosphorylating oxidation and thermoregulatory uncoupling

Russians seem to differ from the great majority of the other people in their style of thinking. Thus neither my supervisor Professor S. E. Severin nor another professor of the Department of Animal Biochemistry of Moscow State University, V. A. Engelhardt, the pioneer of studies on oxidative phosphorylation, were very much surprised when I, a student of this department, tried to defend my degree in 1957 saying that biological function(s) of respiration might not be restricted to ATP synthesis. If such function(s) really exist, one can predict that they will be activated under conditions when heat rather than ATP is primarily needed for an organism. This suggestion was tested in my PhD thesis. Experiments performed together with my friend S. P. Maslov, a very talented zoologist, were done on pigeons that were shorn to avoid physical thermoregulation. It was found that the first exposure of such animals to –15°C with strong wind produced by a ventilator resulted in an exponential drop of the body temperature. If we interrupted the experiment 20 min after the beginning and put the pigeon back to room temperature, the animal survived. Surprisingly, the second cold exposure carried out a day later failed to induce pronounced hypothermia, so the pigeon survived in the cold for many hours. Respiration of the breast muscle mitochondria obtained after the first exposure to cold or before the second one proved to be coupled to phosphorylation, whereas after the second exposure it was almost completely uncoupled.

Similar relationships were revealed in experiments on mice. Moreover, in these animals it was shown that the artificial uncoupler, 2,4-*p*-dinitrophenol (DNP) prolongs the survival time on the first cold exposure. We coined the new phenomenon "thermoregulatory uncoupling" [1–3].

The work described above performed in 1958–1960 initiated our study on the mechanism of this effect. We paid attention to the fact that thermoregulatory uncoupling could be abolished by an *in vitro* addition of serum albumin. It was suggested that the action of the albumin is due to binding of free fatty acids whose level increased under cooling (at that time, uncoupling activity of fatty acids was already described by Pressman and Lardy and by Sholefield). In 1965, we published a paper showing that the fatty acid level is indeed increased under conditions of thermoregulatory uncoupling. Fatty acids could be extracted from the muscles of cold-exposed pigeons and added to mitochondria from non-exposed ones. As a result, uncoupling of respiration and phosphorylation occurred [3,4].

The role of fatty acids was later confirmed when Grav and Blix observed some uncoupling in skeletal muscle mitochondria from fur seals acclimated to cold under natural conditions, and this uncoupling could also be abolished by serum albumin [5]. More recently, Barre at al. [6,7] identified skeletal muscles as the major site of non-shivering thermogenesis in ducklings under cold exposure and revealed a fatty acid-mediated thermoregulatory uncoupling in skeletal muscle mitochondria of these birds. However, real progress in understanding the mechanism of the phenomenon was made when another tissue was studied, i.e. brown fat.

In the late 1960 s, it became clear that in some mammals there is a tissue specialized in thermoregulatory heat production. This is brown fat. Brown fat mitochondria proved to be well equipped to form heat rather than ATP. They have a high level of the respiratory chain enzymes, low level of H^+-ATP-synthase, and possess so-called uncoupling protein 1 (UCP1) discovered by Ricquier and Kader [8]. Uncoupling activity of UCP1 was shown to be mediated by fatty acids formed due to activation of triglyceride lipase under cold exposure of an animal. It was shown that fatty acids (i) come to the mitochondrial matrix via a fatty acyl CoA–fatty acyl carnitine system to be oxidized by the β-oxidation and respiratory chain enzymes; (ii) uncouple their own oxidation by means of UCP1 already present in the brown fat mitochondria; (iii) induce *de novo* synthesis of UCP1 by stimulating activity of the nuclear gene encoding this protein (reviewed in [9]).

The brown fat studies, in spite of obvious progress in solution of the problem of the thermoregulatory uncoupling mechanism, failed to answer the question of what happens in other tissues where an additional amount of oxygen is consumed in response to cold in spite of the absence of sufficient concentration of UCP1. In mammals, brown fat amounts to too small a portion of the total body weight to be responsible for all the cold-induced increase in O_2 consumption. In birds, where thermoregulatory uncoupling was discovered, there is no brown fat at all. Nevertheless, this uncoupling was fatty acid dependent like that in brown fat. This forced me to search for protein(s) that are similar but not identical to UCP1 and can catalyze uncoupling by fatty acids in skeletal muscles.

Structurally, UCP1 belongs to the family of mitochondrial anion carriers. Especially impressive similarity was revealed between UCP1 and the ATP/ADP antiporter. These proteins were found to be composed of three similar domains showing obvious homology in primary, secondary, and tertiary structures. They form dimers and possess a purine nucleotide-binding site (one per dimer) (reviewed in [9]). This is why I suggested that the ATP/ADP antiporter might be responsible for the fatty acid-induced uncoupling in skeletal muscle mitochondria.

In the 1980 s, E. Mokhova and coworkers in my group found that carboxyatractylate, the most potent and specific inhibitor of the ATP/ADP antiporter, recouples rat skeletal muscle mitochondria uncoupled by fatty acids [10]. Later the effect was reproduced in ATP/ADP antiporter proteoliposomes [11]. In other groups, it was found that in yeast mitochondria a mutation in the ATP/ADP antiporter strongly lowers uncoupling efficiency of fatty acids [12] and deletion in the antiporter gene abolishes the recoupling action of carboxyatractylate on the fatty acid uncoupling [13]. Further studies carried out in my group by Samartsev et al. revealed that the aspartate/glutamate antiporter can also be involved in fatty acid uncoupling. This is apparently also the case for the phosphate, dicarboxylate, and some other anion carriers (for review, see [3]).

To explain these relationships, I put forward the suggestion that UCP1 and the mentioned anion carriers facilitate electrophoretic export of fatty acid anions from the internal to external leaflet of the inner mitochondrial membrane. On the external membrane surface, the fatty acid anions are assumed to be protonated to return with proton to the internal leaflet by diffusion through the lipid part of the membrane. On the internal surface, protons are released to the mitochondrial matrix, the corresponding fatty acid anions being regenerated [3,9,14].

Mitochondrial electricity

After discovery of the phenomenon of thermoregulatory uncoupling, I faced the problem of determining its molecular mechanism. However, I recognized very soon that there was no chance to solve the problem of uncoupling before the mechanism of *coupling* of respiration and phosphorylation had become clear.

In the early 1960 s, the traditional point of view was that respiration is coupled to phosphorylation in the same way as for already well-studied glycolysis, where uncoupling does not occur even under severe cooling of an animal. Glycolysis is, in fact, organized in such a way that a non-phosphorylating process is mechanistically impossible.

In 1961 Peter Mitchell published a hypothesis postulating that, in contrast to the always phosphorylating glycolysis, respiration is primarily non-phosphorylating. As to respiratory phosphorylation, it was assumed to be supported by transmembrane difference in electrochemical potential of H^+ ($\Delta\bar{\mu}_H+$), which in turn is generated across the inner mitochondrial membrane by respiration [15]. Mitchell's scheme explained uncoupling as an increase in the H^+ leakage through this membrane. The latter explanation seemed to be confirmed in 1966 when Lehninger and coworkers showed that the classical uncoupler DNP decreases electric resistance of bilayer phospholipid membrane (BLM) [16]. This observation initiated experiments carried out by my and E. A. Liberman's groups where effects of uncouplers were compared in BLM and mitochondria.

In fact, the data showing a decrease in BLM resistance induced by DNP might be explained by some occasional damage to BLM caused by DNP, since resistance of the BLM, initially extremely high, was known to be lowered by quite different factors disturbing the regular lipid bilayer structure. On the other hand, if Mitchell's scheme was right, then (i) not only DNP but all of the numerous uncouplers described in the bioenergetic literature should decrease the resistance, (ii) sensitivities to uncouplers in mitochondria and BLM should correlate with each other, and (iii) the resistance decrease in BLM should be due to specific H^+ conductance.

All these predictions proved to be right. Sixteen different synthetic uncouplers strongly decreased the BLM resistance. The $C_{1/2}$ values in BLM and mitochondria were found to correlate. It was H^+ conductance that was responsible for the effect on the BLM. Experiments with tetrachlorotrifluoromethyl benzimidazole (TTFB) were especially demonstrative. In this compound, there is only one H atom, and thus this one must be responsible for H^+ conductance due to dissociation of TTFBH to $TTFB^-$. It was shown that replacing this H^+ with $-CH_3$ completely abolished the activity of this very potent uncoupler in both mitochondria and BLM. I dubbed uncouplers operating in this way "protonophores". This word was my first contribution to the scientific English language [17].

Elucidation of the protonophorous mechanism of uncoupling directly confirmed one of the crucial postulates of the chemiosmotic hypothesis. The next piece of evidence in favor of this concept was obtained when we tested Mitchell's assumption that respiratory and photosynthetic electron carriers, as well as membranous ATPases, generate transmembrane difference in electric potentials ($\Delta\Psi$) [15]. To this end, we decided to find artificial ions penetrating biomembranes.

By definition, biomembranes should be impermeable for hydrophilic substances. Ions are hydrophilic due to dipoles of water molecules surrounding ionized atoms. Hydrophobic residues fail to make an ion penetrating since the resulting molecule tends to localize in the membrane/water interface, ionized group facing water and hydrophobic part immersed in the lipid layer of a membrane. To solve the problem, we tried ions where the charge of the ionized atom is delocalized over residues connected with this atom. I had a plan to recruit chemists to synthesize such molecules. However, Liberman could not wait – he searched through collections of organic compounds in Moscow research institutes, being sure that substances we badly needed were getting dusty on a shelf of a chemical laboratory. And he proved to be right. Soon we had at our disposal several synthetic organic ions of the required structure. They were first tested with BLM. The prediction was that a trans-BLM concentration gradient of a penetrating ion should generate a Nernst diffusion potential of 60 mV per ten-fold concentration difference. For cations and anions, the compartment with higher ion concentration should be charged negatively and positively, respectively. Such ions were actually found. The most demonstrative were the tetraphenyl phosphonium cation (TPP^+) and the tetraphenyl borate anion (TPB^-). These two ions differ by only a single central atom which was charged positively in TPP^+ and negatively in TPB^-. In BLM, both of them proved to be competent in generating Nernstian $\Delta\psi$ but they were of opposite directions.

Then the same ions were tested in mitochondria. This was done by my Lithuanian coworker and friend Antanas Jasaitis who worked at that time in my department. It was shown that energization of mitochondria by respiration or ATP hydrolysis initiates oppositely directed fluxes of penetrating ions, cations and anions moving into and out of mitochondria, respectively. The ion fluxes were completely abolished

by protonophores. This observation was later extended to inside-out submitochondrial particles and bacterial chromatophores where cations moved out of the vesicle whereas anions were taken up. In the case of chromatophores, not only respiration and ATP hydrolysis but also light could be the energy source.

These data proved convincing for the bioenergetic community, who accepted the idea of electric current generators inlaid in the coupling membranes. It was important that we played with *artificial* ions. For such ions (in contrast to natural ions like K^+) it would be hardly realistic to expect existence of any carriers in the mitochondrial membrane. Our observation was favorably accepted by the audience. The data were immediately published in *Nature* [18]. David Green entitled one of the sections of his review "Skulachev ions" (Sk^+ and Sk^- for penetrating cations and anions, respectively) [19].

Discovery of mitochondrial electricity was followed by 30 years of studies related to this exciting phenomenon. I was involved in investigations into the molecular mechanisms of generation, transmission, and utilization of electric energy in living cells. Our experiments resulted in several findings. Among them one can mention (i) verification of $\Delta\psi$-forming activity of respiratory and photosynthetic chain redox proteins and H^+-ATP-synthase in proteoliposomes [20]; (ii) elaboration of a method of high time resolution for direct measurement of electrogenic activity of $\Delta\bar{\mu}_{H}+$ generators [21]; (iii) discovery of a new function of mitochondria, namely intracellular electric cables [22]; (iv) identification of bacteriorhodopsin as a light-dependent $\Delta\bar{\mu}_{H}+$ generator (nonchlorophyll photosynthesis) [23,24] and as a bright light sensor in halobacteria [25,26]. We took part in description of one of the most elegant inventions of biological evolution – the molecular electric motor rotating bacterial flagellum [27]; in discovery of a "Sodium World" [28,29], a novel type of membrane bioenergetics based on circulation of Na^+ instead of H^+ and the use of $\Delta\bar{\mu}_{Na}+$ instead of $\Delta\bar{\mu}_{H}+$ as a membrane-linked convertible energy currency [30], etc. (for review, see [14]).

Reactive oxygen species (ROS)

In all the cases listed in the preceding paragraph, respiration was found to play a positive role useful for the organelle, cell, and organism. And in all these cases the final reaction product of respiration was shown to be inoffensive H_2O. In mammals, at least 98% of oxygen consumption results in formation of water. However, careful analysis always revealed a residual consumption of O_2 resulting in formation of $O_2^{\cdot-}$ or H_2O_2 that could be transformed into reactive oxygen species (ROS), i.e. very aggressive poisons, such as $OH^{\cdot}$, $HO_2^{\cdot}$, $HONOO^{\cdot}$, and some others. Originally, biochemists considered the residual O_2 consumption as a chemical rather than a biological phenomenon. It was assumed that O_2, a small and penetrating oxidant, directly oxidizes some O_2-sensitive intermediates of electron transport, which results in $O_2^{\cdot-}$ formation. If this were the case, ROS generation would be an inevitable price for aerobiosis.

However, it became quite clear later that sometimes formation of $O_2^{\cdot-}$ or H_2O_2 is catalyzed by enzymes which are, in fact, specialized in carrying out such processes. First of all, I mean production of $O_2^{\cdot-}$ by a NADPH-oxidizing respiratory chain localized in the outer membrane of phagocytes. FAD and autoxidizable cytochrome *b* serve as electron carriers. The chain is oriented across the membrane so that NADPH is oxidized and $O_2^{\cdot-}$ is formed on the inner and outer membrane surfaces, respectively. Extracellular ROS generated by this mechanism are used by phagocytes to kill bacteria. Thus, ROS were selected during evolution as a biological weapon applied by macroorganisms as an antibacterial tool [31]. However, this is hardly an invention of Metazoa. As Malatesta and coworkers reported, the terminal oxidase of the bacterium *Pseudomonas nautica* reduces O_2 to H_2O_2. The released H_2O_2 could be employed to fight against cells of other species. Catalase sequestered in the *P. nautica* cytosol may protect this bacterium from the harmful effects of H_2O_2 generated by its respiration [32].

The above reasoning cannot be applied to cases where the ROS-producing enzyme is localized *inside* the cell so that ROS formed are released into the cytosol or intracellular organelles. This is the case for monoamine oxidase of the outer mitochondrial membrane, cytochrome P450 of the endoplasmic reticulum, some respiratory enzymes localized in peroxisomes, etc. Cytosolic xanthine oxidase belongs to the same group. This is an enzyme oxidizing hypoxanthine and xanthine by O_2 to uric acid, $O_2^{\cdot-}$, and H_2O_2. Xanthine oxidase is formed from xanthine dehydrogenase which employs NAD^+ as oxidant. To acquire the oxidase activity (i.e. to reduce O_2 instead of NAD^+), xanthine dehydrogenase should undergo either limited proteolysis or oxidation of its sulfhydryl groups [33]. The latter can be done by ROS. Thus, xanthine oxidase forms ROS which can, in turn, cause formation of new portions of xanthine oxidase from xanthine dehydrogenase. In other words, a poison stimulates its own formation. Such an autocatalysis should result in a burst of ROS generation inside the cell. To understand such paradoxical relationships, I addressed myself to the phenomena of programmed

cell death. Really, a burst in ROS formation might be considered a mechanism of cell suicide.

At the end of XIX century, the great German biologist August Weismann put forward a paradoxical idea that death due to aging was invented by evolution as an adaptive mechanism [34]. In the 1990 s, I revisited the Weismann concept [35] in connection with the discovery that programmed death mechanisms (apoptosis and necrosis) are inherent in cells of multicellular organisms. In particular, it was found that it is apoptosis that is responsible for purification of tissues from defective or infected cells, for cell elimination during ontogenesis, for suicide of homeless cells or cells of the immune system producing antibodies to their own proteins, etc. (for review, see [36]).

In 1996, an event occurred which initiated quite a new wave of mitochondrial research. Kroemer and coworkers [37] reported that in the mitochondrial intermembrane space a protein is hidden which causes apoptosis when released from mitochondria to the cytosol. The novel protein was called *apoptosis-inducing factor* (AIF). In the same year, Wang and coworkers reported that cytochrome *c* is also a pro-apoptotic protein [38]. It was found that many apoptotic stimuli cause cytochrome *c* release from the mitochondrial intermembrane space to the cytosol where *apoptotic protease-activating factor* 1 (Apaf-1) is present. The cytochrome *c*–Apaf-1 complex combines with several molecules of procaspase 9 in an ATP-dependent fashion. This results in conversion of procaspase 9 to active caspase 9, a protease, which, when formed, attacks procaspase 3 so that active caspase 3 appears. The latter hydrolyzes a group of proteins occupying key positions on the metabolic map or responsible for structural organization of the cell.

The question arose why the mitochondrial intermembrane space is used by the cell to hide suicide proteins like AIF or cytochrome *c*. In 1996 I suggested that the answer may be found if we take into account ROS [39].

In 1992 my coworker Dr. D. B. Zorov mentioned a possibility that the mitochondrion might possess a mechanism of its programmed death, namely the so-called permeability transition pore (PTP) [40]. The PTP can be formed in the inner mitochondrial membrane due to oxidation (or any other modification) of a cysteine SH-group in the ATP/ADP antiporter. Oxidation results in conversion of the antiporter to a nonspecific high conductance channel permeable to substances of molecular mass < 1.5 kDa. In fact, the mitochondrion with open PTP will perish just as a ship with open Kingstons. PTP opening results in collapse of $\Delta\Psi$, making impossible electrophoretic import of precursors of mitochondrial proteins and their proper arrangement in the inner membrane. Thus, the repair processes cease in the PTP-bearing mitochondrion.

It is noteworthy that the mitochondrion does not require any extramitochondrial proteins to open the PTP when [ROS] rises. This means that death of the mitochondrion, induced by its own ROS, can be regarded as suicide. I proposed that such an event called mitoptosis is actuated to purify the mitochondrial population from ROS-overproducing organelles [41].

PTP opening, besides initiating mitoptosis, has one more consequence, namely swelling of the matrix because of the appearance of an osmotic disbalance between matrix and intermembrane space. Swelling, in turn, causes disruption of the outer mitochondrial membrane [39]. As a result, cytochrome *c*, AIF, and some other pro-apoptotic proteins sequestered in the intermembrane space are released into the cytosol. If this happens in a single mitochondrion, the concentrations of pro-apoptotic proteins appear to be too small to initiate apoptosis. However, apoptosis is initiated if many mitochondria in the same cell commit suicide.

Continuing this line of reasoning, we came to the problem of what happens if many cells in one and the same organ start dying by means of apoptosis. It is obvious that this should result in the death of an organism if the organ in question is of vital importance. Hence, massive apoptosis might be used as a mechanism of biochemical suicide of an organism. Let us call such a phenomenon "phenoptosis" by analogy with mitoptosis and apoptosis as programmed death of the mitochondrion or the cell [35,36].

For unicellular organisms, phenoptosis represents programmed death of the cell. The simplest examples of phenoptosis can be found in bacteria. Here altruistic programmed death was shown to occur (i) to prevent expansion of some phage infections in a bacterial population, (ii) to purify this population from those cells whose genome or some other key systems are damaged, (iii) to optimize the bacterial cell concentration in the medium ("quorum sensing"), etc. (for review, see [42]).

In 2002, an interesting example of phenoptosis was described in a unicellular eukaryote. My son Fedor Severin and Dr. Antony Hyman working in Dresden studied vegetative-to-sexual reproduction switch, a typical response of yeast to deterioration of ambient conditions. This process is known to be mediated by production of pheromones that stimulate agglutination of haploid cells of opposite mating types. However, higher pheromone concentrations kill these cells [43].

Later Fedor joined us in Moscow to continue the above line of studies. It was shown that amiodarone mimics the pheromone effect by activating that stage of the phenoptotic cascade which consists of a strong increase in intracellular [Ca^{2+}]. The experiments were done on yeast growing in a sugar-containing medium. Under such conditions, yeast uses glycolysis to form ATP. As to respiration, it is slow and partially uncoupled (most probably to avoid ROS formation). Amiodarone was shown to induce strong increase in the maximal respiration rate measured in the presence of an uncoupler. Without uncoupler, respiration became sensitive to oligomycin as if an endogenous uncoupling was abolished. Activation of coupled respiration was accompanied by a strong increase in $\Delta\psi$ and [ROS]. The burst of ROS entailed decomposition of elongated mitochondria into spherical organelles, their swelling, and appearance of other features inherent in the late apoptosis of cells of multicellular organisms [44]. It is remarkable that pheromone- or amiodarone-induced phenoptosis was prevented by antioxidants as well as by small amounts of an uncoupler decreasing mitochondrial $\Delta\psi$ and ROS production, just as was previously shown in our experiments on isolated mitochondria [45]. This looks as if hyperpolarization of mitochondria which were initially uncoupled is specifically initiated to produce ROS and kill the yeast cell by these ROS, a paradoxical situation when energy-coupled respiration is needed for suicide rather than for life.

Our study of the phenomenon of ROS generation under hyperpolarization conditions revealed that $O_2^{\cdot-}$ is formed when electrons are passed from Complex III to NAD^+ via Complex I (so-called reverse electron transfer in the respiratory chain) [45]. Independently, it was found in several laboratories that the lifespan of mammals and birds is longer the lower is the rate of ROS generation by mitochondria during the reverse electron transfer [46–48]. The naked mole-rat proved to be the only obvious exception to this rule. This rodent of mouse size lives almost 10 times *longer* than the mouse in spite of the fact that its mitochondria produce ROS *faster* that mouse mitochondria [48]. The explanation of this paradox is that the naked mole-rat was the only "non-aging" animal among the species studied: *for mole-rats the probability of death did not depend upon the age of the animal* [49].

These and other numerous pieces of indirect evidence (for reviews, see [50,51]) suggest that intramitochondrial ROS are somehow involved in the aging process. Therefore, I decided to attack the problem of aging by lowering mitochondrial [ROS].

Megaproject "SkQ": in search of a tool to abolish senescence

To construct a mitochondria-targeted antioxidant, we used the same approach as Michael Murphy who applied an Sk^+ (decyltriphenyl phosphonium cation) as a carrier for ubiquinone that was covalently attached to Sk^+ with the decyl linker [52]. The idea to employ Sk^+ as molecular electric locomotive was put forward in our group in 1970 [53] soon after the discovery of penetrating ions [18]. We have confirmed the data of Murphy et al. on the antioxidant activity of MitoQ but have found that this activity turns to prooxidant with a rather small increase in the MitoQ concentration [54]. Therefore, we decided to start a search for antioxidants stronger than MitoQ and with a broader window between the anti- and prooxidant effects.

A project using penetrating ions in medical practice was started in 2003. It was initiated owing to grants from the "Paritet" charitable foundation (now "Volnoe Delo") created by Mr. O. V. Deripaska, a member of the Moscow State University Board of Trustees.

We turned our attention to *plastoquinone*, an electron carrier acting instead of ubiquinone in photosynthetic electron transport chains in chloroplasts of plants and cyanobacteria. Evolution of plants resulted in a situation where ubiquinone involved in the mitochondrial respiratory chain was substituted by plastoquinone in the chloroplast electron transport chain of the same plant cell, possibly just because of the better antioxidant properties of plastoquinone, as shown in chemical experiments on model systems by our group. In fact, an oxygen-generating chloroplast is under much stronger oxidative stress than a mitochondrion which takes up oxygen, lowering thereby [O_2]. As compared with ubiquinone, plastoquinone has methyl groups instead of methoxy groups, whereas the methyl group of ubiquinone is replaced by a hydrogen atom. We have synthesized plastoquinonyl decyltriphenyl phosphonium, a compound where the ubiquinone moiety is replaced by plastoquinone. The new substance was named SkQ1 where Sk is for penetrating cation and Q is for plastoquinone. Whereas the anti- and prooxidant concentrations of MitoQ differed less than twofold (300 and 500 nM), this difference for SkQ1 was shown to be increased up to 32-fold (25 and 800 nM) [54].

This result showed that we had found a very effective antioxidant specifically addressed to mitochondria and not complicated by prooxidant effect within a large range of concentrations. In this connection, we suggested that Mr. Deripaska change the grant to an investment project aimed at creation of a new type of drugs and biotechnology products on the basis of SkQ.

The proposal was accepted, and the investment project was started in the spring of 2005. Searching for the best antioxidant among cationic derivatives of quinones, a number of substances were synthesized by our chemists Drs. G. A. Korshunova, N. V. Sumbatyan, and L. S. Yaguzhinsky. The new compounds were first tested for their ability to penetrate the model membranes. As Dr. I. I. Severina showed, a gradient of SkQ across a BLM generated a nearly Nernstian diffusion potential. Uptake of SkQs by mitochondria was monitored with a hydrophobic cation-sensitive electrode. Addition of a $\Delta\psi$-discharging uncoupler initiated partial release of SkQs to the medium. As further analysis made by Dr. M. Yu. Vyssokikh revealed, it is cardiolipin that is first oxidized under conditions of $OH^{\cdot}$ generation by Fe^{2+} + ascorbate in heart mitochondria. The amount of cardiolipin was strongly decreased after Fe^{2+} + ascorbate treatment, the effect being significantly prevented by 100 nM SkQ1 [54]. Even lower [SkQ1] was shown by Dr. E. N. Mokhova et al. to lower peroxidation of lipids in mitochondria during their *in vitro* aging without added prooxidants. In the next part of the study, cell cultures were investigated. Here we started with an experiment showing intracellular localization of SkQ. To this end, SkQR1, a fluorescent SkQ derivative, was applied. SkQR1 was found to be specifically accumulated by mitochondria of HeLa cells, showing the same intracellular localization as mitochondria-targeted jellyfish yellow fluorescent protein, YFP. In a further study, we asked whether SkQs possess antiapoptotic and antinecrotic effects when cell death is induced by ROS. As experiments by Dr. B. V. Chernyak et al. showed, SkQs prevented ROS-linked cell death. Just 0.2 nM SkQ1 almost completely abolished apoptosis of human fibroblasts induced by 400 μM H_2O_2. Dissipation of $\Delta\psi$ by uncoupler prevented the antiapoptotic effect of such a low SkQ1 concentration [54].

In a final set of experiments, *in vivo* effects of SkQ1 and SkQR1 have been studied. It was found that SkQ1 increases the median lifespan of the fungus *Podospora*, the crustacean *Ceriodaphnia*, the fruit fly *Drosophila*, and mice (in the latter case, by a factor of 2, data by Dr. V. N. Anisimov et al. [55]). In mammals, the effect of SkQ1 on aging was shown to be accompanied by inhibition of development of cataract, retinopathy, glaucoma, balding, turning gray, osteoporosis, involution of thymus, disappearance of estrous cycles in females, chromosome aberrations, peroxidation of lipids and proteins, induction of β-galactosidase, etc. With drops containing 250 nM SkQ1, vision is restored to 67 of 89 animals (dogs, cats, and horses) that became blind because of retinopathies (data by Dr. V. V. Neroev et al.) [56]. Moreover, SkQ1 pretreatment of rats decreased ROS- or ischemia-induced heart arrhythmia (Dr. V. I. Kapelko) [57]. SkQs strongly reduced damaged area in myocardial infarction or stroke and prevented death of animals from kidney ischemia (Dr. O. I. Pisarenko, Dr. D. B. Zorov et al.) [57]. In mutant mice without p53, 5 nmol SkQ1/kg per day inhibited appearance of lymphomas to the same degree as a million-fold higher dose of conventional antioxidant N-acetyl cysteine (Dr. B. P. Kopnin et al.) [56]. Thus, SkQ1 looks promising as tool to treat senescence and age-related diseases [51, 55–58].

Two striking features were inherent in the SkQ effects, i. e. extremely effective low concentrations of SkQ and very pleiotropic character of its action. The first feature can be explained if one takes into account values of electrophoretic driving force for its translocation across membranes and very high hydrophobicity of this compound. Assuming electric potential on the plasma and inner mitochondrial membranes as 60 and 180 mV, respectively, and lipid/water distribution coefficient as 13,000:1, one can predict that SkQ1 can accumulate in the inner leaflet of the inner mitochondrial membrane up to *130 million times* as compared to the extracellular medium. As to pleiotropic effect, the simplest explanation might be that (i) senescence is programmed being, in fact, the last stage of ontogenesis [50,59] and (ii) SkQ effectively interferes with execution of such a program [51].

The data described above were obtained as a result of cooperation of more than 300 scientists from 40 research groups in Moscow State University, institutes of the Russian Academy of Sciences and Medical Academy of Sciences in Moscow, St.-Petersburg, Novosibirsk, Rostov State University, Wenner-Gren Institute in Stockholm and the R. W. Johnson Medical School in New Jersey. The final result will consist of completing preclinical trials of SkQ1 and preparation for its clinical trials which will start in this year. The "supergoal" of the "megaproject" consists of prevention of senescence of humans and animals. However, the first aim will be treatment of age-related blindness by drops of SkQ solution, a branch of the project where the most impressive results have been already achieved in five different species of mammals, from rats to horses.

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