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Bioenergetic aspects of apoptosis, necrosis and mitoptosis

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In this review I summarize interrelations between bioenergetic processes and such programmed death phenomena as cell suicide (apoptosis and necrosis) and mitochondrial suicide (mitoptosis). The following conclusions are made. (I) ATP and rather often mitochondrial hyperpolarization (*i.e.* an increase in membrane potential, $\Delta\Psi$) are required for certain steps of apoptosis and necrosis. (II) Apoptosis, even if it is accompanied by $\Delta\Psi$ and [ATP] increases at its early stage, finally results in a $\Delta\Psi$ collapse and ATP decrease. (III) Moderate (about three-fold) lowering of [ATP] for short and long periods of time induces apoptosis and necrosis, respectively. In some types of apoptosis and necrosis, the cell death is mediated by a $\Delta\Psi$-dependent overproduction of ROS by the initial (Complex I) and the middle (Complex III) spans of the respiratory chain. ROS initiate mitoptosis which is postulated to rid the intracellular population of mitochondria from those that are ROS overproducing. Massive mitoptosis can result in cell death due to release to cytosol of the cell death proteins normally hidden in the mitochondrial intermembrane space.

Keywords: apoptosis; bioenergetics; mitochondria; mitoptosis; necrosis; reactive oxygen species.

Abbreviations $\Delta\Psi$: electric potential difference across the inner mitochondrial membrane; AIF: apoptosis-inducing factor; Apaf-1: apoptotic protease-activating factor 1; CsA: cyclosporin A; JNK: c-Jun NH_2 terminal kinase; MitoQ: 10-(6′-ubiquinolyl)decyltriphenylphosphonium; PTP: permeability transition pore; RNS: reactive nitrogen species; ROS: reactive oxygen species; TNF: tumor necrosis factor α.

The mitochondrion, a Janus organelle

For many years we believed that the major function of mitochondria as intracellular power stations is of crucial importance for aerobic cell *life*. However, in 1996–1997 it became clear that the same organelles are also required for programmed cell *death*. It was elucidated that two mitochondrial intermembrane proteins, *i.e.* apoptosis inducing factor (AIF)[1] and cytochrome *c*,[2–4] play key roles in apoptosis. Later, some other pro-apoptotic proteins were identified in the same compartment of mitochondrion (for review, see [5]). This is why mitochondrial studies of apoptosis were mainly focused on the function of this organelle as the source of components which initiate the apoptotic cascade when they are released from the mitochondrion. On the other hand, another function of mitochondria in apoptosis has been revealed. It consists of supply of apoptosis with membrane potential and ATP. As a result, a rather complicated pattern of mitochondrial involvement in programmed cell death phenomena was elucidated. The situation became even more complex when it was shown that malfunctioning of mitochondria, usually resulting in strong *decrease* in the cellular ATP level, initiates apoptosis. Finally, it was reported that an *increase* in membrane potential and [ATP] represents one of initial steps of apoptosis at least in some cases.

In this paper I shall review the present state of the art in studies on multifaceted relationships between programmed death phenomena and bioenergetic mechanisms.

ATP-dependent steps of apoptosis

In various cell types, it has been found that apoptosis requires ATP.[6–11] Severe (but not complete) depletion of ATP resulted in that certain typical apoptotic stimuli initiated necrosis instead of apoptosis,[7–11] whereas complete ATP exhaustion gave rise to a third type of cell death differing from both apoptosis and necrosis ("energy catastrophe").[11] This indicates that not only apoptosis but also necrosis requires some ATP.

ATP, or better dATP, proved to be a cofactor in formation of Apaf-1-cytochrome *c* complex (the apoptosome) carrying out conversion of inactive procaspase 9 into active caspase 9, an obligatory step in cytochrome *c*-dependent apoptosis.[12,13] Moreover, those steps of apoptosis that are mediated by protein kinases depend by definition upon ATP, which is used as the phosphoryl donor for phosphorylation of some proteins involved in apoptosis (for reviews, see[14,15]). In particular, it was shown that the key *anti-apoptotic* protein Bcl-2 *is*

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inactivated by phosphorylation mediated by *c*-Jun NH_2 terminal kinase (JNK).[16,17] The same kinase phosphorylates and *activates pro-apoptotic* proteins Bad,[18] Bim[19] and Bmf.[19] Moreover, JNK somehow stimulates formation of truncated pro-apoptotic protein Bid.[20] In turn, JNK is activated by apoptotic stimuli via a cascade of other protein kinases. For example, in *Drosophila* the following chain of events was revealed[21]:

TNF–like inducer of apoptosis
→ plasma membrane receptor of inducer ● ● ●
→ JNKKKK → JNKKK → JNKK → JNK ● ● ●
→ apoptosis.

On the other hand, in some regulatory contexts JNK plays an anti-apoptotic role.[22,23] Besides JNK, some other protein kinases, *i.e.* PKC, DAP, PKB (Akt), p38, etc., are also involved in pro- or anti-apoptotic mechanisms (for refs., see [11,14,15]).

Intracellular migration of mitochondria represents another apoptosis-linked ATP-dependent process. It was reported that several types of apoptosis (in particular, the TNF-induced one) are accompanied with decomposition of extended mitochondrial profiles to small roundish mitochondria ("the thread-grain transition").[24] In many cases of apoptosis, such a transition was shown to be required for the cell death.[25,26] The roundish mitochondria were shown to move from the cell periphery to the nucleus (for refs., see [26,27]). Such a movement proved to be a result of the following chain of events:

TNF → TNF receptor ● ●● → p38 → kinesin kinases
→ kinesin inactivation by means of phosphorylation of its light chain.

This stops the kinesin-driven movement of mitochondria to the cell periphery and results in their translocation to the nucleus since dynein, *i.e.* ATPase responsible for mitochondrial movement in the opposite direction, remains active.[28] To realize all these events, ATP is required at protein kinase steps and as the energy source for dynein-mediated movement of organelles. It is significant that the thread–grain transition precedes release of cytochrome *c* and other proteins from mitochondria. This means that such a transition followed by translocation of mitochondria to the nucleus might be regarded as a way to deliver these proteins to the nucleus where final apoptotic events take place. In certain cases, appearance of mitochondria inside the nucleus has been described.[24]

In conclusion of this section, it should be stressed that the source of ATP required for apoptosis to proceed is not critical. It can be respiratory as well as glycolytic phosphorylation.[9]

Mitochondrial membrane potential-dependent apoptosis

If it is respiratory phosphorylation that produces the apoptosis-supporting ATP, mitochondrial membrane potential ($\Delta\Psi$) plays the role of an intermediate on the way from respiratory chain electron transfer to ATP. However, the functions of $\Delta\Psi$ in apoptosis are not restricted to such an obvious role.

Membrane potential is usually involved in those types of apoptosis that are mediated by Ca^{2+}.[29,30] In these cases Ca^{2+} comes to the cytosol from the endoplasmic reticulum or from the extracellular medium to be electrophoretically (*i.e.* in a $\Delta\Psi$-driven fashion) accumulated in the mitochondrial matrix. This results in opening of the so-called permeability transition pore (PTP) in the inner mitochondrial membrane, which becomes permeable for low molecular mass substances normally responsible for osmotic balance between the matrix and the intermembrane space. The PTP is impermeable for proteins, which now become of crucial contribution to the above balance. Protein concentration in the matrix is higher than in the intermembrane space so water goes to the matrix, inducing its swelling. This gives rise to disruption of the outer mitochondrial membrane, which has much smaller area than the inner one. Such an event entails release to cytosol of pro-apoptotic proteins hidden in the intermembrane space.[31] Thus, the following apoptotic cascade is actuated :

Apoptotic stimulus ● ●● → cytosolic [Ca^{2+}] ↑
→ matrix [Ca^{2+}] ↑→ PTP → swelling of matrix
→ disruption of outer mitochondrial membrane
→ release of mitochondrial pro–apoptotic proteins from mitochondria → apoptosis

For several years, such mechanism of release of pro-apoptotic proteins was considered as the only one. However, quite recently some indications have been obtained that the above scheme describes necrosis more often than apoptosis. As for apoptosis, the intermembrane proteins are assumed to be released with no PTP involved. This seems to occur due to an increased permeability of the outer mitochondrial membrane for proteins by means of pro-apoptotic Bax and Bak. However, at least in some cases apoptosis is mediated by the PTP (for discussion, see below).

High membrane potential stimulates ROS production

Apparently, $\Delta\Psi$-driven mechanisms of apoptosis and necrosis are also related to processes mediated by reactive oxygen and nitrogen species (ROS and RNS, respectively), first of all by $O_2^{-\bullet}$, H_2O_2, $OH^\bullet$ and $ONOO^-$. Superoxide anion ($O_2^{-\bullet}$) is the primary mitochondrial ROS. When formed, $O_2^{-\bullet}$ is

alternatively converted (i) to H_2O_2 (the superoxide dismutase reaction) and then to $OH^{\bullet}$ (the Fenton reaction), or to (ii) $ONOO^-$ by reacting with $NO^{\bullet}$. In both cases, the final products, *i.e.* $OH^{\bullet}$ or $ONOO^-$, are extremely aggressive oxidatnts.[32]

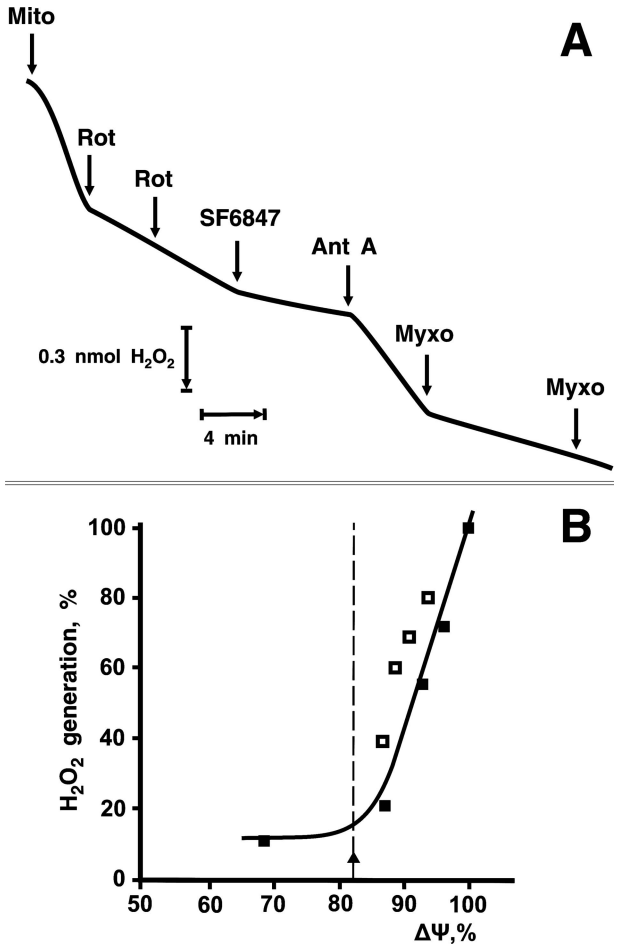
It is well established that ROS and RNS operate as necessary transients in various death programs.[33] In many cases, mitochondria-produced ROS are involved.[5,33] In mitochondria, there are several reactions potentially competent in ROS (RNS) formation. In certain cases, monoaminoxidase of the outer mitochondrial membrane, an enzyme oxidizing monoamines by O_2 in such a way that H_2O_2 is formed, plays an important role (see, *e.g.*[34]). However, this process, being involved in initiation of ROS generation, can hardly be responsible for production of large amounts of ROS, usually accompanying apoptosis or necrosis, due to low concentrations of the substrates (monoamines). It seems to be much more realistic to assume that it is the respiratory chain that is the major source of ROS in mitochondria since amount of electrons passing along the chain is enormously large compared to any other redox system in the cell, so large quantities of ROS could be obtained even if a small portion of this electron flow results in formation of $O_2^{-\bullet}$ rather than H_2O.

We measured the contribution of various ROS-generating reactions in H_2O_2 production by intact rat heart mitochondria oxidizing succinate in State 4 (the resting state where rate of respiration is limited by absence of the energy acceptor, ADP).[35,36] It was found that (i) succinate stimulated very strongly the H_2O_2 production, (ii) rotenone arrested a 80% of this stimulation, an effect which correlates with inhibition of the reverse electron transfer from succinate to NAD^+, (iii) subsequent addition of uncoupler arrested 18% more of the H_2O_2 generation, (iv) an uncoupler added before rotenone inhibited the H_2O_2 production by about 95%, (v) antimycin A added after uncoupler strongly stimulates formation of H_2O_2, (vi) myxothiazol abolished the antimycin A stimulation.

Like the reverse electron transfer, the succinate-supported H_2O_2 generation showed a very steep dependence upon the $\Delta\Psi$ value. The slight decrease in $\Delta\Psi$ accompanying the State 4-State 3 transition, initiated by adding ADP, caused (a) $\Delta\Psi$ decrease by about 18% and (b) more than 90% inhibition of the H_2O_2 production. The same effects on $\Delta\Psi$ and generation of hydrogen peroxide could be produced by low concentration of uncoupler or of malonate, a succinate dehydrogenase inhibitor. Similar inhibition of the ROS formation was obtained by adding NAD-linked substrates, glutamate and malate, to succinate-oxidizing mitochondria.

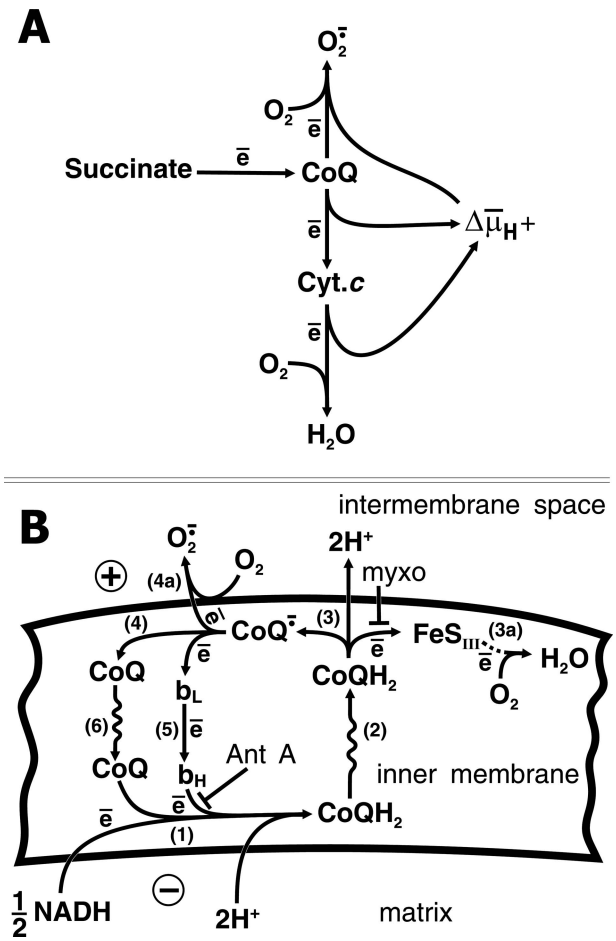
The above data (some of them are shown in Figure 1) were recently confirmed by Vinogradov and Grivennikova[37] who investigated tightly coupled heart muscle submitochondrial particles. They found that in State 4 the particles produce superoxide at highest rate when succinate is oxidized, the process being sensitive to rotenone and to an uncoupler that lowered $O_2^{-\bullet}$ formation by a factor of 10. NADH oxidation was accompanied by a two-fold lower $O_2^{-\bullet}$ formation, which was decreased two-fold more by adding rotenone or an uncoupler.

Figure 1. H_2O_2 production by succinate-oxidizing rat heart mitochondria. (From Korshunov *et al.*[35]). (A) additions: Mito, mitochondria (1 mg protein/ml); Rot, 4 μM rotenone; SF6847, 1 nM 3,5-di(*tret*-butyl)-4hydroxybenzylidenemalononitrill (an uncoupler); Ant. A, 1 μM Antimycin A; Myxo, 2 μM myxothiazol. (B) $\Delta\Psi$, safranin O response. 100%, the State 4 conditions. The $\Delta\Psi$ level was decreased by adding various concentrations of SF6847 (black squares), malonate (white squares) or ADP (triangle).



All these relationships can be explained by the assumptions that (a) reverse electron transfer via complex I is the main (80%) mechanism of the H_2O_2 production in the succinate-oxidizing State 4 mitochondria, (b) 15% of the H_2O_2 originates from operation of the *Q*-cycle in State 4, (c) all other ROS-generating reactions in mitochondria collectively contribute only 5% to the total H_2O_2 formation. As was shown by Kunz and coworkers,[38,39] the redox potential of a complex I component responsible for the $O_2^{-\bullet}$ formation is about -0.3 V. Therefore, it is not surprising that some energy is required to reduce $O_2^{-\bullet}$ by succinate (redox potentials of pairs $O_2^{-\bullet}/O_2$ and succinate/fumarate are -0.3 V and $+0.03$ V, respectively. The necessary energy is supplied in the form of protonic potential ($\Delta\bar{\mu}_{H^+}$)

Figure 2. $O_2^{-\bullet}$ formation by Complexes I (A) and III (B). For explanations, see the text.



produced by the forward electron transfer from succinate to O_2 via Complexes III and IV (Figure 2A). It seems probable that it is half-reduced FMN of complex I ($FMNH^{\bullet}$) that is attacked by O_2 to form $O_2^{-\bullet}$.[38] The radical $FMNH^{\bullet}$ is indeed an excellent electron donor for processes of one-electron reduction like $O_2 \rightarrow O_2^{-\bullet}$. However, under *in vivo* conditions $O_2^{-\bullet}$ formation by Complex I should be limited by effects of NADH and NAD^+, which inhibit to different degree this reactions. Apparently, interplay of [NADH] and [NAD^+] affects the $FMNH^{\bullet}$ level and, hence, the rate of $O_2^{-\bullet}$ production. One might suggest that [NADH]/[NAD^+] ratio is more favorable for higher [$FMNH^{\bullet}$] when succinate, rather than NADH or NAD^+-linked substrates, is used as the electron donor.

Another mechanism responsible for the $O_2^{-\bullet}$ generation is related to Complex III. Here semiubiquinone anion ($CoQ^{-\bullet}$) serves as the most probable one-electron O_2 reductant (Figure 2B). $CoQ^{-\bullet}$ formed in center *o* near the outer surface of the inner mitochondrial membrane is oxidized by heme b_L which is in turn oxidized by heme b_H. The latter reaction is electrogenic, being directed across the membrane. This is why high $\Delta\Psi$ arrests this reaction and b_L remains always reduced. As a consequence, $CoQ^{-\bullet}$ cannot be oxidized by b_L and becomes a target for O_2 to form $O_2^{-\bullet}$.[40] Therefore, it is not surprising that the State 4 conditions are favourable for formation of $O_2^{-\bullet}$ not only by Complex I but also by Complex III, whereas uncouplers abolishing $\Delta\Psi$ strongly inhibit this process. The above scheme also accounts for stimulation of the Complex III-linked ROS production by antimycin A (inhibitor of heme b_H oxidation in center *i*) and removal of such a stimulation by myxothiazol (inhibitor of $CoQ^{-\bullet}$ formation in center *o*).[40]

Thus, two major mechanisms of $O_2^{-\bullet}$ generation by the respiratory chain are $\Delta\Psi$-dependent. This accounts for observations that ROS-induced animal cell apoptosis often includes at an early stage a $\Delta\Psi$ increase.[41–50] This phenomenon was recently analyzed in detail in yeast (see the next section).

Paradoxical role of mitochondria in yeast phenoptosis

In the yeast *Saccharomyces cerevisiae*, programmed death has been described. Since this is a unicellular organism, such a phenomenon cannot be defined as apoptosis, a programmed cell death ridding a multicellular organism from unwanted or unnecessary cells. Rather, I would characterize it as being phenoptosis, or programmed death of an organism.[51,51a]

In yeast, H_2O_2,[52] acetic acid at low pH,[53] the plant antibiotic osmotin,[54] high level of yeast pheromone[55] or the drug amiodarone[56] promoted cell death which could be classified as phenoptosis. It was revealed that the cell death induced by acetic acid[53] or by expression of animal pro-apoptotic protein Bax[57] are associated with $\Delta\Psi$-supported ROS formation.

In a cooperation of Severin's and our groups,[56] it was recently demonstrated that *S. cerevisiae* aerobically grown on 2% raffinose utilizes glycolysis to produce ATP. As for respiration, it was of low rate and partially uncoupled, so the membrane potential level was low. It was not surprising within the framework of the schemes in Figure 2 that the concentration of ROS in such cells was negligible. Apparently, the life strategy of *S. cerevisiae* under conditions when a sugar is present in excess consists of minimizing respiration and $\Delta\Psi$ to avoid ROS formation. However, initiation of phenoptosis by pheromone or amiodarone was found to induce a fast and strong increase in rate of respiration and its energy coupling. As a result, the ROS level dramatically increased. This was due to operation of the Q-cycle since myxothiazol or a low amount of an uncoupler arrested the ROS increase. As to the $\Delta\Psi$-linked ROS generation in the initial span of the respiratory chain, it is impossible in *S. cerevisiae* lacking Complex I which is replaced by two non-coupled NADH-CoQ reductases (one more way to lower the level of the respiration-produced ROS). Interestingly,

both events, *i.e.* stimulation of respiration and an increase in its energy coupling, were mediated by elevated cytosolic [Ca^{2+}]. In yeast mitochondria, in contrast to animal mitochondria, cytosolic Ca^{2+} cannot be electrophoretically transported to the matrix and cannot induce the PTP. It seems likely that a Ca^{2+}-induced activation of an external NADH dehydrogenase on the outer surface of the inner mitochondrial membrane is a primary reason for respiratory burst at the initial step of the phenoptosis-induced mitochondrial events in *S. cerevisiae*. Such an activation was described for a homologous enzyme in plant mitochondria.[58] In our hands, addition of Ca^{+} to isolated yeast mitochondria resulted in a two- or three-fold stimulation of oxidation of external NADH, oxidation of pyruvate and malate being unaffected. As for an energy coupling increase, it might also be related to the cytosolic [Ca^{2+}] rise because in yeast, unlike animals, a nonspecific pore in the inner mitochondrial membrane is inhibited, rather than activated, by extramitochondrial Ca^{2+} ions.[59]

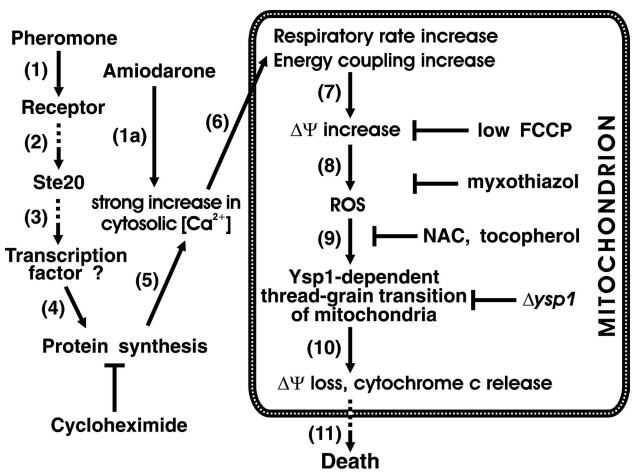
The Ca^{2+} release from an intracellular Ca^{2+} depot to cytosol seems to be the primary step of programmed death of yeast induced by amiodarone,[56] a anti-arrhythmic drug which is known to directly affect Ca^{2+} and Na^{+} ion channels in animal cells.[60] The situation is more complicated when pheromone is used to initiate phenoptosis. It was shown that pheromone caused a major [Ca^{2+}] increase in *S. cerevisiae*.[61] If pheromone concentration is low, this stimulates mating of α and *a* haploids, an effect related to a cytosolic Ca^{2+} increase. However, at high level of pheromone the cytosolic [Ca^{2+}] increase becomes so strong and occurs so long that the above-described mitochondrial changes develop. In line with such reasoning, we showed that pheromone-induced death required both the pheromone receptor and pheromone regulatory cascade components like a MAP kinases Ste20. The effect was apparently mediated by transcription factor(s) and required *de novo* protein synthesis.[56]

In any case, both pheromone- and amiodarone-initiated strong increase in membrane potential entailed a burst in ROS generation. Mild uncoupling caused by addition of a small amount of an uncoupler was shown to prevent this burst as well as the cell death. Such antioxidants as vitamin E and N-acetyl cysteine were of the same effect, whereas MitoQ, an antioxidant targeted to the mitochondrial matrix (see below), was ineffective.[56] The latter observation was in line with the assumption that ROS are formed in the Complex III center *o*, *i.e.* close to the outer surface of the inner membrane (see above, Figure 2B).

The next step of the yeast phenoptotic program consists of decomposition of filamentous mitochondria to small roundish organelles (the thread-grain transition). This process initiated by pheromone or amiodarone was found to be mediated by a protein of unknown function called yeast suicide protein 1 (Ysp1).[56] Mutation in the *ysp1* gene made the thread–grain transition impossible and prevented the cell death.[56] The final mitochondrion-related step of the yeast suicide cascade was swelling of mitochondria accompanied by $\Delta\Psi$ collapse and cytochrome *c* release. One may assume that in yeast, just as in higher animal cells, cytosolic cytochrome *c* mediates further events resulting in cell death. In fact, deletions in genes encoding two *S. cerevisiae* *c*-type cytochromes were shown to increase survival of pheromone- or amiodarone-treated yeast.[56] The problem is that in the *S. cerevisiae* genome there is no gene coding for Apaf-1, the animal cell target of cytosolic cytochrome *c*. It is not excluded that cytochrome *c* is needed for the respiratory and ROS bursts, *i.e.* events occurring upstream of cytochrome *c* release which might be a side effect of swelling of the matrix. In this case, it may be the yeast homolog of AIF[62] that mediates suicide signal transduction from mitochondria to the nucleus, causing decomposition of nuclear DNA.[56] The present state of knowledge concerning the phenoptotic cascade of *S. cerevisiae* is summarized in Figure 3.

Paradoxically, in yeast growing on a sugar medium mitochondria are required more for programmed death than for the life of the cell. Major bioenergetic function of mitochondria, *i.e.* ATP production, is apparently not important for raffinose-fermenting yeast since respiration is low and essentially uncoupled, so glycolysis acts as the ATP-regenerating process. However, when a suicide signal comes, mitochondrial energetics is activated to form the high $\Delta\Psi$ that is required to generate ROS in Complex III. To some degree, this situation resembles that in eosinophiles. In these cells, glycolysis is the energy source. As for mitochondria, they have low cyanide-resistant respiration non-coupled to ATP synthesis, but they are indispensable for amplification of apoptotic signals. A $\Delta\Psi$ on the mitochondrial membrane is generated by mitochondrial H^{+}-ATPase utilizing glycolytic ATP.[63]

Figure 3. Pheromone-induced phenoptosis of yeast *S. cerevisiae* growing on raffinose. (From Pozniakovsky *et al.*[56]).



Programmed cell death induced by a decrease in ATP level: Interrelations of apoptosis and necrosis

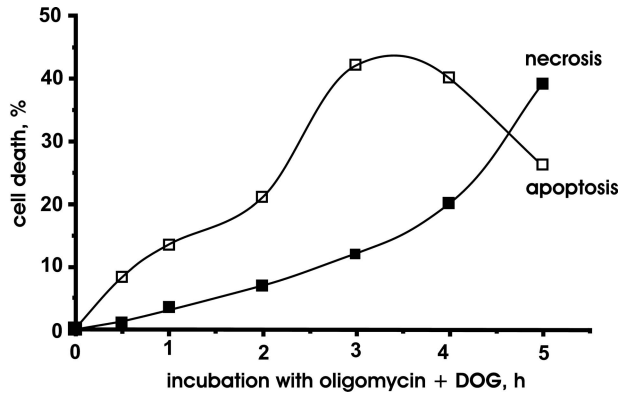
As already mentioned, ATP is required for several steps of apoptotic cascades. Moreover, some apoptoses are associated with an increase in $\Delta\Psi$ which is transient on the way from electron transfer to ATP synthesis. It is hardly surprising, therefore, that at an early step of apoptosis the intracellular ATP level rises.[11] On the other hand, lowering of this level was found to be a potent inducer of apoptosis. As has been shown in our group,[64] inhibition of respiratory phosphorylation by oligomycin combined with partial inhibition of glycolysis by a moderate deoxyglucose concentration results in about three-fold decrease in [ATP] in HeLa cells. Three hours later, deoxyglucose was excluded from the cell growth medium and the ATP level recovered. Nevertheless, the majority of cells committed suicide by apoptosis in 48 h. This means that transient lowering of ATP concentration is recognized by the cell as an apoptotic signal. Interestingly, lowering of the [ATP] by 1/3 does not induce cell death,[64] whereas transient more than 97% decrease in [ATP][65,66] or its three-fold lowering for a longer period of time (5 h instead of 3 h)[64] gave rise to necrosis (Figure 4).

The above findings raise an important question concerning interrelationships of apoptosis and necrosis. In the past, apoptosis as a programmed cell death was usually opposed to necrosis, which was believed to be a non-programmed death caused by such dramatic changes in crucial metabolic and structural cell parameters which are inconsistent with life. However, it is obvious that the ATP decrease-induced necrosis observed in our experiments could not be explained in such a way. (1) In this case, necrosis was arrested by adding a JNK inhibitor, *i.e.* it is under control by a protein kinase cascade.[65] (2) Even 5 h decrease in [ATP] by factor 3 *per se* can hardly be regarded as an event inconsistent with cell life since the great majority of the ATP-utilizing systems are still saturated by the residual ATP concentration which is still as high as about 1 mM (cf. μmolar K_m values for almost all ATP-dependent enzymes). Moreover, the very fact that necrosis requires small but measurable ATP level (see, *e.g.*, ref.[11]) also points to the conclusion that necrosis is, like apoptosis, programmed.

An example of the ATP decrease-induced cell death shown in Figure 4 illustrates a situation when *one and the same* unfavorable factor (here a transient and moderate [ATP] lowering) initiates either apoptosis or necrosis depending upon the time interval for operation of the factor in question. A similar situation was found in our group (unpublished data) when H_2O_2 was employed as the programmed death-inducing factor. Lower and higher H_2O_2 concentrations were shown to initiate apoptosis and necrosis, respectively. Apparently this is a general rule that apoptosis is replaced by necrosis when the strength of a damaging factor reaches a certain threshold.

To some degree, apoptosis and necrosis look like a private and a public suicide, respectively. In apoptosis, all the degradative processes developing during programmed cell death are isolated from the extracellular space by the outer cell membrane, which remains impermeable until formation of apoptotic bodies, *i.e.* fragments of the cell corpse. The bodies are engulfed by phagocytes who recognize them using phosphatidyl serine, which appears in the outer leaflet of the outer membrane of the apoptotic cell. On the other hand, necrosis is accompanied by disruption of the outer cell membrane integrity so all intracellular compounds are released to the intercellular space. Some of them attract phagocytes and initiate inflammation. This is a long-distance effect due to diffusion of compounds in question in the intercellular space. It does not require direct contact of a phagocyte with the dying cell.[33] Within the framework of the above concept, it seems quite reasonable that an increase in intensity of a damaging effect results in a switch from apoptosis to necrosis. A moderately damaged cell eliminates itself without attracting attention, while a strongly damaged one is "crying" that something is terribly wrong and dangerous for other cells in this tissue region. A massive apoptosis seems to be an intermediate case. This situation will be discussed in the next section.

Figure 4. Apoptosis is replaced by necrosis when duration of the ATP lowering period increases. (From Izyumov et al.[64]).



Long distance apoptotic cell killing mediated by intramitochondrial ROS and intercellular diffusion of H_2O_2

As a matter of fact, each apoptotic cell is a ROS generator. Superoxide radical is the primary ROS. It is converted by superoxide dismutase to hydrogen peroxide, a long-lived compound easily penetrating through membranes. H_2O_2 is a well known apoptotic inducer. This means that apoptotic cells might initiate apoptosis in healthy cells provided that the apoptotic cell-produced H_2O_2 level is sufficiently high and is maintained for sufficiently long time.

I have hypothesized that apoptogenic activity of H_2O_2 can be used by organisms to form a "dead zone" around a

virus-infected group of cells (as in a leaf of so-called hypersensitive tobacco plant infected by tobacco mosaic virus), thus limiting expansion of the infection.[67] Later some assumptions of this hypothesis were supported by Bakalkin and his coworkers[68] who described a clustering of dead cells in a monolayer culture during apoptosis. The clustering was shown to be prevented by catalase.

Our group recently suggested a new method to study such "bystander killing".[69] A cover-slip with intact HeLa cells growing on its surface (the recipient cells) was placed in a Petri dish side by side with another cover-slip on which TNF-pretreated HeLa cells were grown (the inducer cells). If the inducer cells were pretreated for 3 h with TNF, massive apoptosis was found to occur on not only on the inducer but also on the recipient cover-slip. Such long-distance killing was not inhibited by TNF-specific antibodies added in the beginning of co-incubation of the two cell samples, whereas the antibodies added to the inducer cells together with TNF prevented apoptosis. On the other hand, catalase suppressed apoptosis of the recipient but not the inducer cells, whereas aminotriazol, a catalase inhibitor, stimulated it.

Interesting results were obtained in experiments with 10-(6′-ubiquinolyl)decyltriphenylphosphonium (MitoQ). This compound was introduced by Murphy, Smith and their coworkers.[70,71] It is composed of ubiquinol and decyltriphenylphosphonium cation. As was previously shown in our group[72] (for review, see[73]), methyltriphenylphosphonium is a penetrating cation distributing across a membrane in a $\Delta\Psi$-dependent fashion according to the Nernst equation (60 mV $\Delta\Psi$ corresponds to 10-fold cation gradient). The $\Delta\Psi$ value on the inner mitochondrial membrane is about 180 mV (the matrix is negatively charged). This should result in a 1,000-fold accumulation of MitoQ in the matrix. Such an effect should strongly increase the matrix antioxidant capacity inasmuch as the ubiquinol moiety of MitoQ serves as an antioxidant which can be easily regenerated from the oxidized (ubiquinone-containing) MitoQ by accepting electrons from Complexes I or II.[70]

The *recipient* cells pretreated with very low (20 nm) MitoQ proved to be much more resistant to the killing by the inducer cells. Moreover, such a pretreatment of the *inducer* cells strongly inhibited their ability to initiate apoptosis in the recipient cells.[69] It is noteworthy that TNF apoptosis *per se* proved to be resistant to MitoQ[69,70] as well as to other antioxidants.[74] On the other hand, TNF was found to induce a burst of ROS production which seemed to be a side-effect unessential for this type of apoptosis since its suppression by antioxidants failed to stop the death program in the TNF-treated cells. Our finding elucidates a probable function of these ROS. At low TNF concentrations, they may be used for bystander killing of healthy cells surrounding a region of TNF-induced apoptosis.[69]

The role of intramitochondrial ROS in bystander killing requires further investigation. The simplest possibility is that hydrogen peroxide which we found to be released from the TNF-treated cells[69] is generated in the mitochondrial matrix. It remains possible, however, that intramitochondrial superoxide plays a role of primary ROS which initiate production of secondary ROS inside or outside mitochondria (so-called ROS-induced ROS release).[75] In any case, intramitochondrial ROS proved to be indispensable for both generation of the death signal (H_2O_2) by the bystander death-inducing cells and processing of this signal in the recipient cells. The latter is not surprising since MitoQ was shown to arrest apoptosis caused by added H_2O_2.[69,70]

Mitochondria are involved not only in apoptosis but also in necrosis

Quite recently, Green[76,77] suggested that both apoptosis and necrosis can be mitochondria-dependent but in two quite different fashions. In both cases, "death proteins" are postulated to be released from the mitochondrial intermembrane space. However, in apoptosis this occurs due to an increase in permeability of the outer mitochondrial membrane for proteins, the inner membrane remaining intact. This occurs because of formation of pores in the outer membrane by pro-apoptotic proteins Bax or Bak. As for necrosis, PTP is formed in the inner membrane so the outer one is broken due to the matrix swelling.

Historically, it was PTP that was proposed to be involved in apoptosis.[1,31] However, recent observations by Tsujimoto's[78] and Molkentin's[79] groups revealed that mitochondrial cyclophilin D, an enzyme involved in the PTP opening, is not required for several types of apoptosis (including that induced by TNF) but is critical for some kinds of necrosis.

However, in our hands TNF apoptosis in HeLa cells was sensitive to cyclosporin A, an inhibitor of mitochondrial cyclophilin D, and was accompanied by mitochondrial swelling, an event always observed after the PTP opening (see below, Figure 6).[74] Apparently, not only the level of the programmed cell death inducer but also some other factors determine what kind of the death pathway is employed by the dying cell. The situation is complicated by the fact that one and the same component can be involved in either apoptosis or necrosis (*e.g.*, JNK and some other protein kinases, mitochondria, ROS, etc.). This opens a possibility that a suicide process started as apoptosis can later be transformed to necrosis.

Mitoptosis

This term was coined to describe suicide of the mitochondrion.[80] Existence of such a process was suggested by Zorov *et al.* some years ago.[81] I have postulated that mitoptosis operates as a mechanism ridding the mitochondrial population in the cell from malfunctioning organelles (for example, from those which overproduce ROS). In this case, the following chain of events seems probable: ROS → PTP

opening → collapse of $\Delta\Psi$ → cessation of $\Delta\Psi$-dependent import of mitochondrial protein precursors → death of mitochondrion which cannot be repaired.[33,80] Each of these steps is experimentally verified.

(1). It is well known that ROS are the most potent inducers of PTP opening.[82]
(2). PTP opening inevitably entails dissipation of $\Delta\Psi$.[33]
(3). In the absence of $\Delta\Psi$, mitochondria fail to import from cytosol precursors of those mitochondrial proteins that are encoded by nuclear genes. As to those that are encoded by mitochondrial genes, they cannot be arranged in the proper way if $\Delta\Psi$ on the inner membrane is absent (for review, see[83]).
(4). It is obvious that a mitochondrion perishes if repair of its proteins becomes impossible.

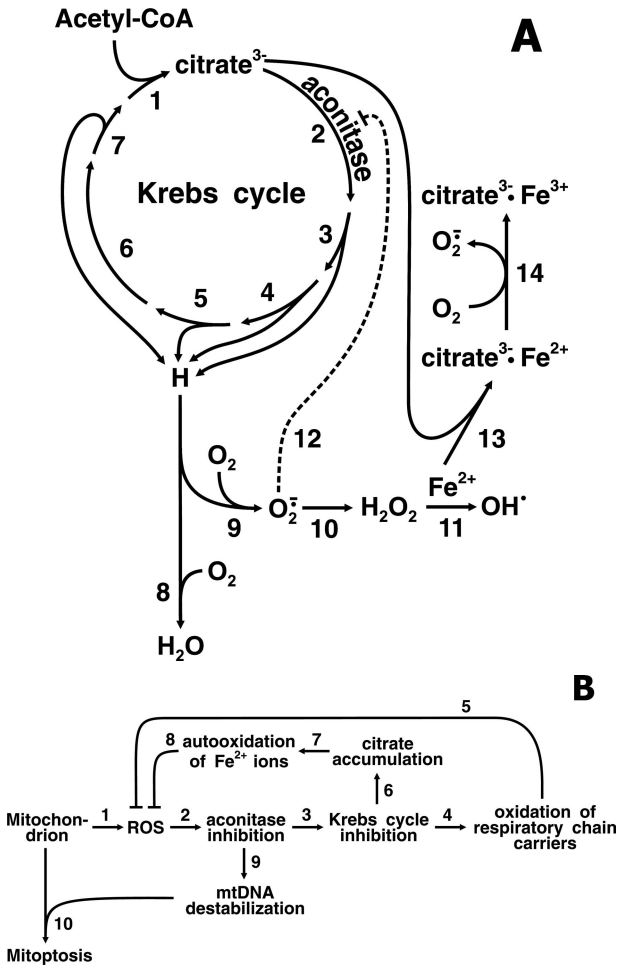
It is noteworthy that the above scheme of elimination of a mitochondrion does not require any extramitochondrial components and, hence, may be defined as mitochondrial suicide. It is initiated by ROS which are produced by this particular mitochondrion. In fact, this should occur if the mitochondrion generates more ROS than it can scavenge.

Inhibition of protein import in ROS-overproducing mitochondria as a mitoptotic mechanism could be supplemented by a ROS-induced degradation of mitochondrial DNA. This might be mediated by oxidative damage to aconitase. Aconitase is one of the most $O_2^{-\bullet}$-sensitive enzymes. It is inactivated by small amounts of $O_2^{-\bullet}$ due to an $O_2^{-\bullet}$-induced release of one of four Fe^{2+} ions, named $Fe^{2+}{}_a$, from the 4Fe–4S cluster present in active aconitase.[84] As Gardner and Fridovich suggested, inactivation of aconitase by $O_2^{-\bullet}$ may be a line of antioxidant defense, resulting in inhibition of the Krebs cycle in its very beginning, which entails cessation of electron supply to the respiratory chain and, hence, oxidation of all electron carriers including those which produce $O_2^{-\bullet}$.[85]

Another consequence of aconitase inhibition is accumulation of its substrate, citrate, which is a chelator for Fe^{2+}. When formed, the Fe^{2+}•citrate^{3-} complex, which is autoxidizable,[86] immediately reacts with O_2. As a result, even more stable Fe^{3+}•citrate^{3-} complex appears. In this way, the Fenton reaction ($H_2O_2 + Fe^{2+} \rightarrow OH^\bullet + OH^- + Fe^{3+}$) is prevented, and $OH^\bullet$, the most aggressive form of ROS, is not produced (Figure 5).[86] If, nevertheless, the intramitochondrial ROS level increases and inactivation of aconitase continues, this may result in an effect inconsistent with existence of the mitochondrion. I mean oxidative damage to mitochondrial DNA.

As was quite recently shown by Butow and coworkers[87] (for comments, see[88]), intact aconitase is a mitochondrial DNA-binding protein that stabilizes DNA and protects it from damage. The C382S or C445S aconitase mutants deficient in the iron-binding cysteines were found to lack the DNA-protecting activity. Thus, we might speculate that the $O_2^{-\bullet}$-inactivated aconitase cannot perform its mitochon-

Figure 5. Possible role of aconitase in the antioxidant defence of mitochondria. (A) Acetyl-CoA forms citrate (1) which enters the Krebs cycle in an aconitase-catalyzed fashion (2). The Krebs cycle (reactions 3–7) supplies the respiratory chain with reducing equivalents (H) which combine with O_2 to form H_2O (8) or $O_2^{-\bullet}$ (9). $O_2^{-\bullet}$ dismutates in such a way that H_2O_2 is a reaction product (10). H_2O_2 can be reduced to OH., accepting an electron from a Fe^{2+} ion (11). However, this process is assumed to be arrested due to inhibition of aconitase (12), which (i) blocks the H supply to the respiratory chain and (ii) results in accumulation of citrate. The latter binds Fe^{2+} to an autoxidable complex (13). Oxidation of the cytrate^{3-}•Fe^{2+} complex produces more stable cytrate^{3-}•Fe^{3+} complex and $O_2^{-\bullet}$ (14). Thus, $O_2^{-\bullet}$ is formed instead of much more toxic $OH^\bullet$. $O_2^{-\bullet}$ can be transformed to H_2O_2 which, however, cannot be transformed to $OH^\bullet$ since Fe^{2+} ions are lacking. (Modified from Skulachev).[86] (B) Interrelations of possible mechanisms of the aconitase-mediated antioxidant effects. Overproduction of ROS by a mitochondrion (1) results in inactivation of aconitase (2), which entails the following events. (a) Inhibition of the Krebs cycle (3) and, hence, oxidation of mitochondrial carriers (4) and, as a consequence, arrest of ROS production (5); (b) accumulation of citrate (6) and autoxidation of Fe^{2+} ions (7) which are required for the $OH^\bullet$ formation(8) (see Figure 5A for details); (c) destabilization of mitochondrial DNA (9) leading to mitoptosis, *i.e.* self-elimination of the ROS-overproducing mitochondrion (10).

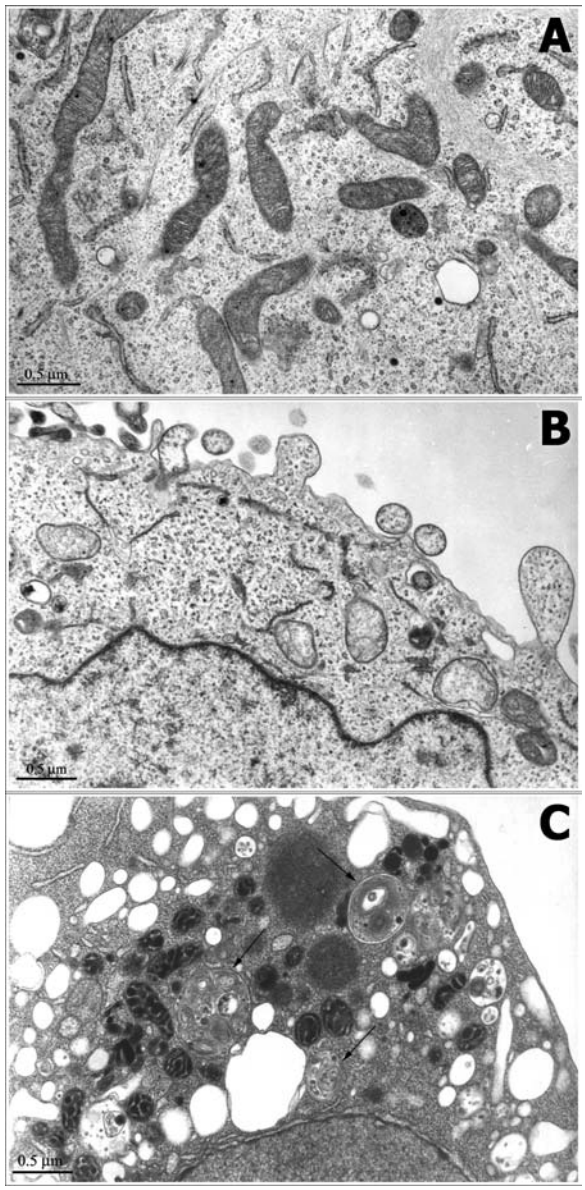


drial DNA-protecting function, DNA is damaged and the ROS-overproducing mitochondrion perishes.

To directly verify the above hypothesis assuming that ridding of mitochondrial population from the ROS-overproducing organelles is carried out by their self-elimination, one needs to obtain a ROS overproduction in a single mitochondrion and then to follow the fate of the organelle for many hours. It is much easier to force the entire population of mitochondria in the cell to overproduce ROS and see what happens with them. However, massive mitoptosis in a cell should initiate a programmed death of this cell since PTP opening results in the matrix swelling, disruption of the outer mitochondrial membrane and release of death proteins to the cytosol (see above). Nevertheless, sometimes it is possible to observe disappearance of mitochondria prior to the cell death. For instance, Lemasters and coworkers[89] have reported that addition of glucagon to a culture of hepatocytes results in a cyclosporin A-sensitive PTP opening (revealed by collapse of $\Delta\Psi$) followed by massive consumption of dead mitochondria by mitochondria-specific autophagosomes. In yeast, this process called by Lemasters *mitophagia*[90] requires Uth1p, a special protein localized to the outer mitochondrial membrane. When Uth1p is mutated, mitophagia (induced by paramycin or nutrient deprivation) was found to be suppressed, but autophagic degradation of cytosolic proteins occurred normally.[91] Importantly, Uth1p is one of four genes responsible for longer life of yeast under starvation conditions.[92,93]

To observe massive mitoptosis with no apoptosis entailed, one can block the apoptotic cascade downstream of mitochondria. Such an experiment was performed by Tolkovsky and coworkers.[94,95] Axonotomized sympathetic neurons deprived of neuronal growth factor were shown to die in a few days. However, the cells survive if a pan-caspase inhibitor was added a day after the grow factor deprivation. Electron microscopic study of such cells revealed that in the majority of the cells all the mitochondria disappear within 3 days.[94] Similar results were obtained in HeLa cells when apoptosis was induced by staurosporine in the presence of a caspase inhibitor.[95] In both cases, corpses of mitochondria were removed by autophagosomes. In our laboratory, this process was followed using an electron microscope by Bakeeva and Saprunova (Figure 6).[74] It was found that the TNF-induced apoptosis is accompanied by the thread-grain transition of mitochondria and their swelling followed by supercondensation to small electron dense particles which cannot respire and synthesize ATP, have no $\Delta\Psi$ and are engulfed by autophagosomes. The final steps of the process are fusion of autophagosomes with lysosomes and complete digestion of the mitochondrial material, which can be used as substrates for energy production. The precedent of the latter was described many years ago when it was found that during starvation *Tetrachymena* utilizes by mitophagia its mitochondria.[96] Quite recently, Thompson and his colleagues[97] have demonstrated that immortalized cells from bone narrow of Bax and Bak-deficient mice respond to

Figure 6. Effect of TNF on mitochondrial structure in HeLa cells. (A) no TNF. (B) TNF and emetine were added. An intermediate step of apoptosis. Mitochondria are swollen. (**C**) a terminal step of the TNF apoptosis. Mitochondria are supercondensed. Numerous autophagosomes are seen (arrows). (From Shchepina *et al.*[74]).



growth factor withdrawal by strong stimulation of autophagia instead of apoptosis observed in the wild type cells. Moreover, cells failed to take up nutrients to maintain cellular bioenergetics. Inhibition of autophagia killed the cell. Under these conditions, addition of methylpyruvate, an outer cell membrane-permeable pyruvate precursor, prolonged the cell survival.

A quite different mechanism of mitoptosis was recently disclosed in our group by Lyamzaev *et al.*[98] We studied how the cell responds to a mitochondrial malfunctioning other than ROS overproduction. Highly glycolytic monkey tumor kidney cells CV-1 or human carcinoma cells HeLa were

treated with an uncoupler and a respiratory chain inhibitor. As a result, the mitochondria could not respire and synthesize ATP. Instead they start to hydrolyze glycolytic ATP, which should entail an energy catastrophe. In fact, 50–70% of the cells died but others survived for some time, expelling their mitochondria to the extracellular medium. Details of this process proved to be the following.

(1) Mitochondrial network and mitochondrial filaments are disintegrated to numerous small roundish mitochondrial (the thread–grain transition). This event is not accompanied by cytochrome *c* release.
(2) Mitochondria are gathered near the nucleus, forming one or several clusters.
(3) The cluster(s) are surrounded by a single membrane.
(4) The resulting large vacuole-like vesicle filled with mitochondrial material (*mitoptotic body*) moves toward the cell periphery and is finally expelled from the cell.

In conclusion, I would like to give some advices of a bioenergeticist to students of the programmed death phenomena.

Lesson 1: Be careful with poisons!

To verify participation of an enzymatic process in the studied phenomenon, you very often employ specific inhibitors of the corresponding enzyme. However, when doing this, you should be sure that the selected poison is really specific for the enzyme in question. Unfortunately, many inhibitors are, in fact, bi- or even polyfunctional. This is why it is very desirable to try at least two inhibitors of one and the same process, differing in the mode of their action. If both of them are of similar effects, this means that you are really dealing with the studied reaction since it is rather improbable that side effects of two inhibitors coincide. On the other hand, if you don't apply such a double inhibitor approach, you may come to a wrong conclusion. This happened quite recently with De Vos *et al.*[99] who reported about mitochondrial fragmentation in the kidney cells CV-1, induced by inhibitors of mitochondrial functions.

The authors used oligomycin as a specific inhibitor of mitochondrial ATP-synthase, observed the rapid fission of mitochondrial filaments and concluded that functioning of ATP-synthase and/or oxidative phosphorylation were critical to support the thread-like mitochondrial morphology. Previously we obtained the same results[74] when we applied oligomycin in an attempt to dissect the specific effect of respiratory inhibitors (such as rotenone or myxothiazol) from inhibition of ATP synthesis. However, when we used aurovertin B, another effective inhibitor of ATP-synthase, no effect on mitochondrial morphology was observed. Oligomycin, in contrast to aurovertin, has a non-mitochondrial target in the cell, namely Na^+/K^+-ATPase of the plasma membrane. We found that ouabain, a specific inhibitor of Na^+/K^+-ATPase, caused rapid and complete fission of mitochondrial reticulum in the CV-1 cells. These data strongly indicate that ion homeostasis created by Na^+/K^+-ATPase is critical for support of the thread-like mitochondria in kidney cells, and inhibition of this enzyme with oligomycin underlies their thread-grain transition. Interestingly, in human endometrium carcinoma cell line HeLa neither oligomycin nor ouabain affected the mitochondrial network.

Another misleading conclusion was made by De Vos *et al.*[99] when they employed CsA as an inhibitor of PTP in the CV-1 cells. This compound specifically interacts with cyclophilins, including cyclophilin D, the mitochondrial member of this family, which is involved in the PTP opening. The other (non-mitochondrial) cyclophilins were also shown to tightly bind CsA. Such a complex is inhibitory for calcineurin, a key protein phosphatase involved in regulation of the immune response. This mechanism underlies a well-known immunosuppressive action of CsA. We applied CsA as an inhibitor of the PTP in an attempt to test the possible role of the PTP opening in ROS-induced fission of mitochondria and observed rapid fragmentation of filamentous mitochondria in CV-1 cells. As a control, we used a non-immunosuppressive analog of CsA, 4-MeVal-CsA, which is an effective inhibitor of cyclophilin D and the PTP opening. In this case, the rapid thread-grain transition did not occur. The same result was obtained with Sanglifehrin A, which is not structurally related to CsA but strongly inhibited cyclophilin D and the PTP opening, being ineffective in inhibiting of calcineurin. Sanglifehrin A did not affect mitochondrial morphology in CV-1 cells. These data indicated that calcineurin-dependent process(es), rather than PTP, are somehow involved in regulation of mitochondrial fission in the CV-1 cells. Again, as in the case of oligomycin, CsA did not cause fission of mitochondria in HeLa cells.

De Vos *et al.*[99] demonstrated that translocation of Drp-1 to mitochondria was critical for fragmentation of mitochondria induced by oligomycin and CsA in the CV-1 cells. Earlier it was shown that fragmentation caused by uncouplers[100] and by various apoptotic stimuli[26] also depends on Drp-1. These data indicate that the final events in fission of mitochondria could be similar for different initial signals. However, the role of mitochondrial H^+-ATP-synthase and the PTP in initiation of fission remains obscure.

Lesson 2: Be careful with mutants!

If you decided to use a mutant of a bifunctional protein involved in both bioenergetics and apoptosis, which is believed to be deprived of one of these functions, you should be sure that this function is really completely lost.

In 2001, Yu *et al.*[101] reported that the K72A mutant of cytochrome *c* is deprived of apoptogenic activity. In 2003, Kirpichnikov's, Newmeyer's and our groups[102] revisited this problem and found that, in fact, the mutant possesses some ability to induce apoptosis but at higher concentrations than the wild type cytochrome *c*. In 2005, Hao *et al.*,[103] using

the knock-in procedure, substituted the gene of the K72A cytochrome *c* for that of the wild-type protein. The authors described some phenotypic changes of the mutant mice, which, however, were much less pronounced than in the case of knocking-out of the gene encoding Apaf-1. They concluded that there is a cytochrome *c*-independent mechanism of the Apaf-1 activation in response to apoptotic signals. However, such a conclusion seems to be hardly valid since according to our data the K72A cytochrome *c* can completely replace the wild type in Apaf-1 activation when the mutant cytochrome *c* concentration was sufficiently high.[102] Quite recently, one more study was carried out by Kirpichnikov's and our laboratories[104] to compare apoptogenic activities of several mutated cytochromes *c* electrophoretically introduced into intact cells. As was found, the K72A mutant fully activated apoptosis at five-fold higher concentration than the wild type. On the other hand, the K72W mutant proved to be completely ineffective at concentration almost 20-fold higher than intact cytochrome *c*. Even more, three-fold excess of K72W over the wild type cytochrome *c* strongly inhibited apoptosis caused by intact cytochrome *c*. The K72W mutant, like K72A, proved to be fully active as the respiratory chain electron carrier. Thus, it is obvious that K72W is a very much better candidate for the knock-in experiment, whereas in the case of K72A one is dealing with a *decrease* in the cytochrome *c* apoptogenic activity rather than with the *complete arrest* of such activity. Respectively, the conclusion concerning the existence of a cytochrome *c*-independent Apaf-1-mediated pathway of apoptosis looks at present groundless.

It should be stressed that mistakes described in Lessons 1 and 2 could easily be avoided if the authors glance through such respectable journals as Oncogene and Biochemical Journal. From this, the third lessen can be deduced.

Lesson 3: Learn news by reading not only e-mails from good friends but also articles in good journals.

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