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Functional Activity and Ultrastructure of Mitochondria Isolated from Myocardial Apoptotic Tissue

A. A. Tonshin*, V. B. Saprunova, I. M. Solodovnikova, L. E. Bakeeva, and L. S. Yaguzhinsky

Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, Moscow 119899, Russia; fax: (095) 939-0338; E-mail: yag@genebee.msu.su

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Abstract—Apoptosis in myocardial tissue slices was induced by extended incubation under anoxic conditions. Mitochondria were isolated from the studied tissue. A new method of isolation of mitochondria in special conditions by differential centrifugation at 1700, 10,000, and 17,000*g* resulted in three fractions of mitochondria. According to the data of electron microscopy the heavy mitochondrial fraction (1700*g*) consisted of mitochondrial clusters only, the middle mitochondrial fraction (10,000*g*) consisted of mitochondria with typical for isolated mitochondria ultrastructure, and the light fraction consisted of small mitochondria (2 or 3 cristae) of various preservation. The heavy fraction contained unusual structural elements that we detected earlier in apoptotic myocardial tissue—small electron-dense mitochondria incorporated in bigger mitochondria. The structure of small mitochondria from the light fraction corresponded to that of the small mitochondria from these unusual elements—"mitochondrion in mitochondrion". The most important functions of isolated mitochondria are strongly inhibited when apoptosis is induced in our model. The detailed study of the activities of the two fractions of the apoptotic mitochondria showed that the system of malate oxidation is completely altered, the activity of cytochrome *c* as electron carrier is partly inhibited, while succinate oxidase activity is completely preserved (complexes II, III, and IV of the respiration chain). Succinate oxidase activity was accompanied by high permeability of the internal membrane for protons: the addition of uncoupler did not stimulate respiration. ATP synthesis in mitochondria was inhibited. We demonstrated that in our model of apoptosis cytochrome *c* remains in the intermembrane space, and, consequently, is not involved in the cascade of activation of effector caspases. The possible mechanisms of induction of apoptosis during anoxia are discussed.

Key words: mitochondrion, apoptosis, ultrastructure, anoxia, hypoxia, cytochrome *c*, respiration, myocardium

Numerous studies have been devoted to apoptosis, but only a few deal with isolation and functional properties of mitochondria in apoptosis, mainly due to technical problems of isolation of mitochondria from cellular cultures that are used for the study of apoptosis in the majority of investigations. Here we should cite the works on isolation of mitochondria from apoptotic hepatocytes [1] and apoptotic tissue of the whole heart [2].

Earlier we [3] observed internucleosomal DNA fragmentation (one of the well-known markers of apoptosis [4]) after incubation of myocardial tissue slides under anoxic conditions for 72 h. The ultrastructure of cardiomyocytes in the studied myocardial slides corresponded to that of cardiomyocytes from slides incubated in the presence of TNF-α (tumor necrosis factor α), a widely used inducer of apoptosis. Under anoxic conditions, cardiomyocytes are characterized by the appearance of three morphologically different types of mitochondria. They share ultrastructural properties with mitochondria of cardiomyocytes from tissue incubated in the presence of TNF-α under aerobic conditions. The goal of this study was to isolate mitochondria from apoptotic myocardial tissue obtained in our experiment and separate these mitochondria into fractions, each containing mitochondria with specific ultrastructure. We also supposed to study the functional activity of the mitochondria and the content of cytochrome *c* in certain fractions.

MATERIALS AND METHODS

Induction of apoptosis. Hearts of decapitated adult rats (150-180 g) were isolated and ventricle tissue was quickly cut into pieces (1-3 mm^3) at 4°C in the following buffer: 0.3 M sucrose, 250 μM EDTA, 5 mM Tris, pH 7.4. Three to five grams of the resulting preparation was put into a 10-ml tube and the tube was filled with the same buffer that was ventilated beforehand with nitrogen for

* To whom correspondence should be addressed.

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30 min. This procedure eliminated 70-90% of the oxygen (the amount of residual oxygen was monitored using a Clark platinum electrode). The sample was quickly hermetically sealed. The measured earlier [3] respiration rate of the initial tissue slides on endogenous substrates allowed for the assessment of oxygen content in the hermetic tubes with samples (it should decrease to K_m value of cytochrome oxidase (<1 μM) in less than 5 min).

Isolation of mitochondrial fractions. After 48 h of incubation the tissue was taken from the tube, passed through a metal lattice (mesh diameter was 1 mm) and then disintegrated in a Potter homogenizer in the following buffer: 0.3 M sucrose, 250 μM EDTA, 5 mM Tris, pH 7.4. Then mitochondrial fractions were isolated by the method of successive differential centrifugation. The nuclear fraction was pelleted at 300*g*, the supernatant was used for succeeding sedimentation of the heavy fraction of mitochondria at 1700*g*, the middle fraction at 10,000*g*, and the light fraction at 17,000*g*. Centrifugations were carried out for 20 min for all fractions in a Beckman J2-21 centrifuge with JA-20 rotor (USA). Each mitochondrial fraction was resuspended in 1 ml of 0.2% BSA for 2 min, then the volume of suspension was adjusted to 30 ml with the isolation medium and pelleted again. The protein quantity in mitochondrial suspension was measured by the biuret method.

Electron microscopy. For electron microscopy the material was fixed with 2.5% solution of glutaraldehyde for 2 h at 4°C, then additionally fixed with 1% solution of OsO_4 for 1.5 h and dehydrated with ethanol solutions with increasing ethanol concentration (70% ethanol contained saturated solution of uranyl acetate). The material was embedded in epoxide resin Epon-812. Slides were cut on an LKB-III ultramicrotome and stained with lead according to Reinolds. The resulted preparations were analyzed and photographed in an HU-11B electron microscope (Hitachi, Japan).

Respiration of mitochondria. Respiration of mitochondria was registered by the polarographic method using a platinum Clark electrode in the following incubation medium: 0.25 M sucrose, 250 μM EDTA, 5 mM Tris, pH 7.4.

Determination of cytochrome *c/a* ratio in the fractions of isolated mitochondria. The relative content of cytochromes *c/a* in suspensions of the heavy and the middle mitochondrial fractions was measured by registration of differential spectrums of absorbance of cytochromes from mitochondria reduced with dithionite versus oxidized ones. Mitochondrial spectrums were registered on an Aminco DW2000 spectrophotometer. Concentration of mitochondria in the cuvette was 0.5-1 mg protein in 1 ml of medium. To obtain the reduced suspension several crystals of dithionite and KCN (0.5 mM) were added to the "sample" cuvette. To obtain the oxidized suspension rotenone (1 μM) and FCCP (carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone, 0.5 μM) were added to the "comparison" cuvette.

Statistics. To study the differences between cytochrome *c/a* ratios we used Student *t*-criterium with 5% level of significance.

Reagents. Tris from ICN (USA), EDTA from Serva (Germany), sucrose from Merck (Germany), dithionite, rotenone, glutamate, malate, succinate, FCCP from Sigma (USA), and KCN, $MgSO_4$ from Russian suppliers were used.

RESULTS AND DISCUSSION

Ultrastructure of mitochondria isolated from apoptotic myocardial tissue. We selected the conditions of fractionation that yielded three mitochondrial fractions from the apoptotic myocardial tissue by successive differential centrifugation. The heavy fraction was pelleted at 1700*g*, the middle at 10,000*g*, and the light at 17,000*g*. The heavy fraction contained approximately 15-30 mg of protein, the middle 2-4 mg, and the light 0.5-1 mg.

According to the data of electron microscopy, mitochondria from the fractions differed greatly in morphology. The heavy fraction (1700*g*) contained mitochondrial clusters united by well-developed inter-mitochondrial contacts (IMC), but not independent mitochondria [5] (Fig. 1). The structure of IMC was the same as in the intact tissue. Previous numerous attempts to isolate mitochondria united by IMC in isolated suspension of organelles have failed. Thus, it appears that induction of apoptosis leads to the increase in the structural resistance of IMC. It is important to note that inside some mitochondria of this fraction were localized small, electronically dense mitochondria. These structures, "mitochondrion in mitochondrion", were rather often observed in tissue slides in our model of apoptosis and were described in detail in our previous work [3].

The ultrastructure of mitochondria from the middle fraction pelleted at 10,000*g* (Fig. 2) corresponded to that of mitochondria isolated in sucrose solutions from intact heart.

The light fraction was pelleted at 17,000*g* and contained free small electronically dense mitochondria that were analogous to electronically dense mitochondria that we detected in the heavy fraction inside bigger mitochondria, a large quantity of small mitochondria in the swollen state, and a significant admixture of submitochondrial particles (SMP) (Fig. 3).

However, the ultrastructure of three mitochondrial fractions obtained from intact myocardial tissue (control) corresponded to that of mitochondria isolated in sucrose medium. These three fractions had identical ultrastructures; the light fraction did not contain swollen mitochondria and SMP; it consisted of mitochondria with normal ultrastructure that were slightly smaller than those in other fractions.

Respiration. We studied in detail the functional activity of the first two fractions. The detailed study of the third fraction was limited by the small quantity of material.

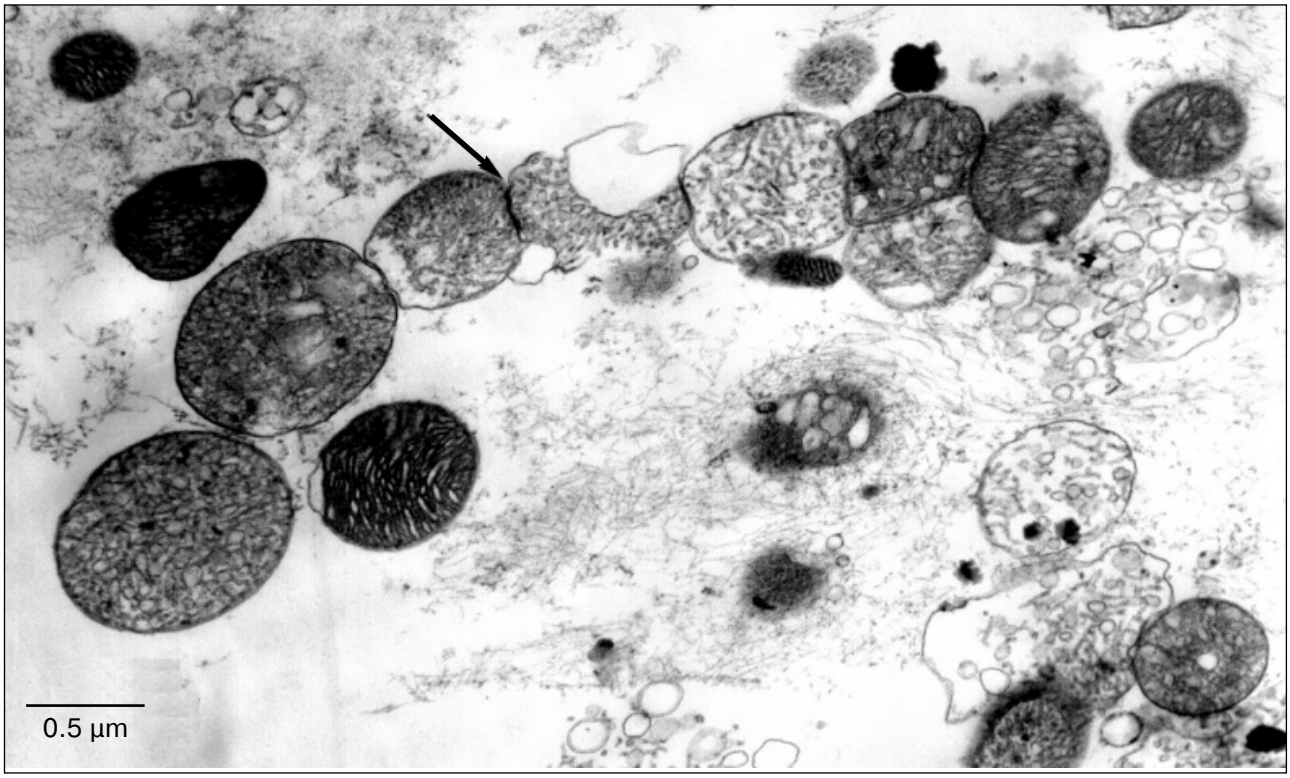

Fig. 1. Electron microscopic photo of mitochondrial cluster (fraction isolated at 1700*g*). An inter-mitochondrial contact (IMC) is indicated by the arrow.

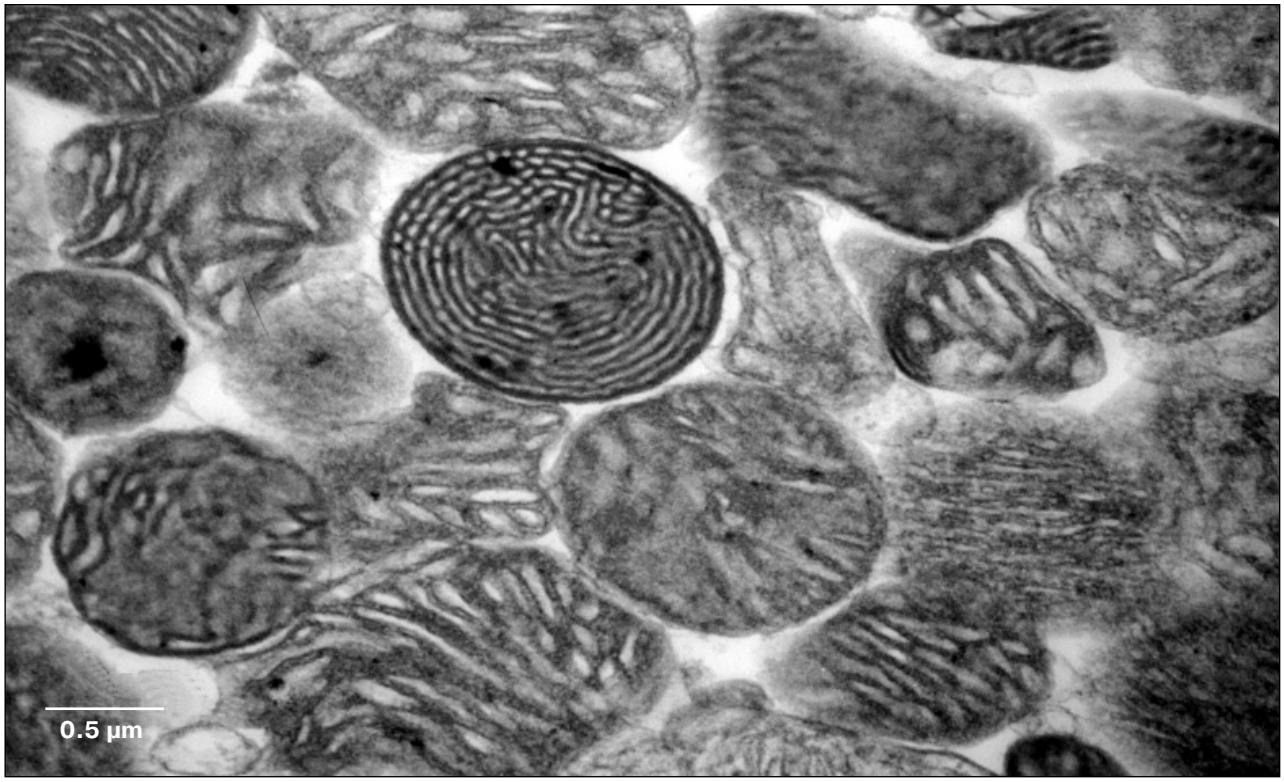

Fig. 2. Electron microscopic photo of mitochondrial fraction isolated at 10,000*g*.

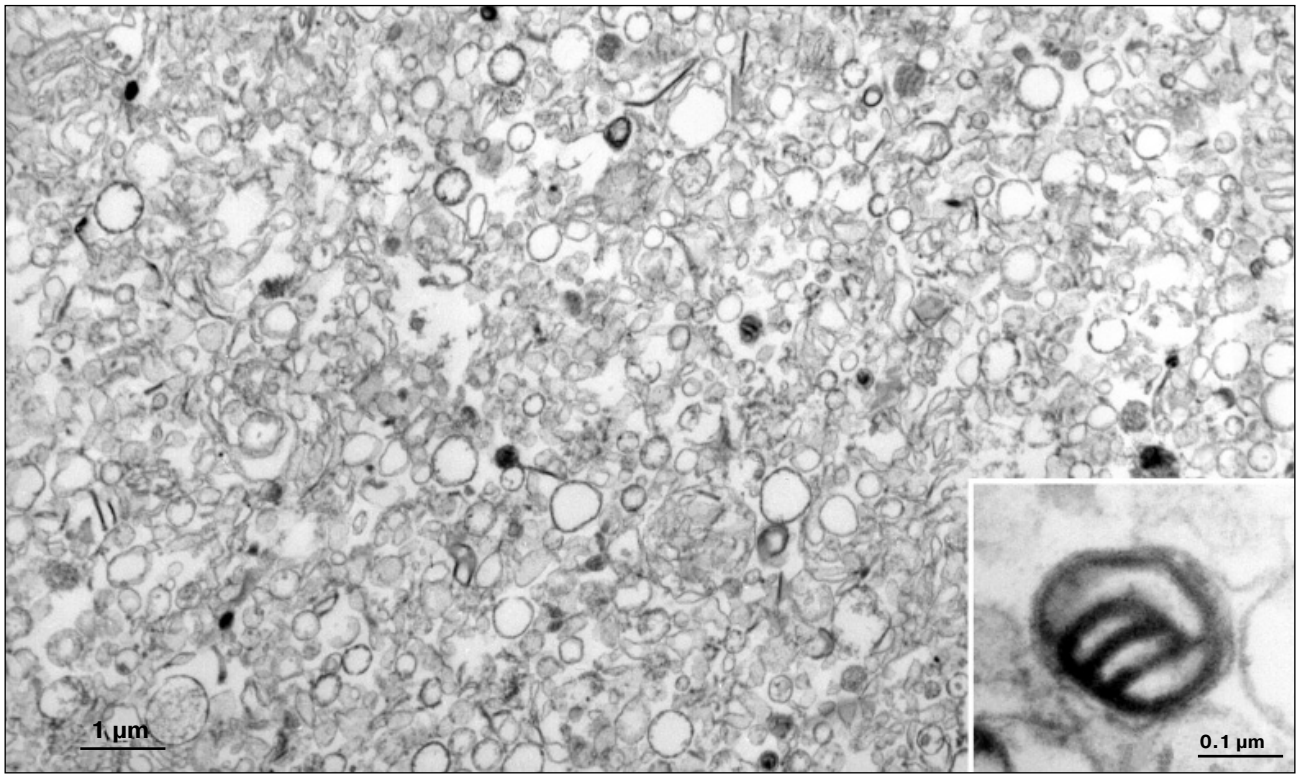

Fig. 3. Electron microscopic photo of small electron-dense mitochondria, small swollen mitochondria, and submitochondrial particles (SMP) (fraction isolated at 17,000*g*).

Figure 4 (the beginning of polarogram A) demonstrates that NADH oxidase activity is absent in the heavy fraction: addition of malate and glutamate does not stimulate respiration. An analogous effect was observed in the middle (Fig. 4, polarogram B) and the light (data not shown) fractions.

These results indicate large changes occurring in the respiration system of mitochondria during apoptosis under anoxic conditions. It is evident that the functions of the citric acid cycle and/or complex I in these preparations are completely inhibited. At the same time, succinate oxidase activity of mitochondria is maintained in apoptosis (Fig. 4). Table 1 shows that the maximal respiration rates of both middle and heavy fractions in apoptotic mitochondria during succinate oxidation vary from one experiment to another, but they are comparable (with each other and with the respiration rate in the control mitochondria isolated from an intact heart). The data provide evidence to conclude that in our experimental conditions mitochondrial succinate oxidase (it includes complexes II, III, and IV) remains rather active. Table 1 also demonstrates that the specific rates of respiration of the light and heavy mitochondria are of the same order. It was indicated above that ultrastructure of the light mitochondrial fraction is not homogenous (Fig. 3). High specific respiration rate of this fraction indicates that the majority of membranes in it are represented by mitochondrial membranes, and not by the products of destruction of cellular or microsomal membranes. The study of phosphorylation function of mitochondria isolated from the apoptotic tissue showed that the system of ATP synthesis under conditions of succinate oxidation is altered or strongly inhibited in all three fractions. Phosphorylation substrates (ADP and phosphate) do not stimulate respiration either in the absence (data not shown) or in the presence (Fig. 4, Table 1) of Mg^{2+}. Respiration rate does not increase when the uncoupler (FCCP) is added to mitochondria in all three fractions. The results (Table 1) indicate that all mitochondrial types from apoptotic myocardial tissue are initially separated. A decrease in potential on the mitochondrial membrane during apoptosis was described by Kroemer in 1997 [6]. The decrease in $\Delta\Psi$ was also observed during apoptosis in mitochondria from liver [1].

Determination of the ratio between quantities of cytochromes *c/a* and of cytochrome *c* activity in the apoptotic mitochondria. Cytochrome *c* is a multifunctional

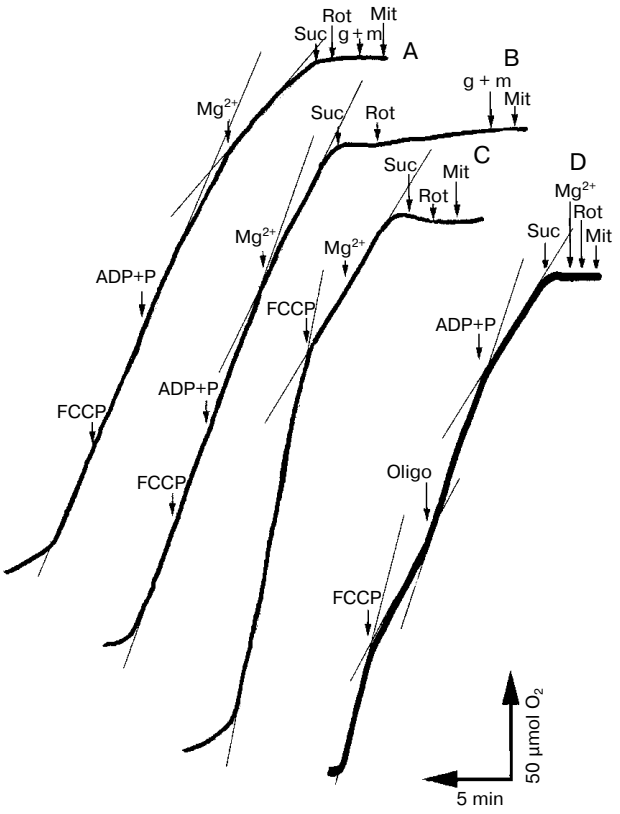

Fig. 4. Polarographic monitoring of respiration rates of mitochondria from heavy (A), middle (B), and control (C) fractions (D is control fraction where Mg^{2+} and P_i are initially in the incubation medium). Additives: ADP (200 μM) + P_i (1 mM); uncoupler FCCP (0.2 μM). See Table 1 for absolute respiration rates. Suc, succinate; Rot, rotenone; Mit, mitochondrion; Oligo, oligomycin; g + m, glutamate plus malate.

molecule and has at least two functions: on one hand, it is a carrier of electrons within the electron transport chain; on the other hand, it can migrate from the intermolecular space of a mitochondrion to cytosol and interact with APAF-1 factor and procaspase 9 in the presence of dATP [7], thus forming an apoptosome. Within this complex, cytochrome *c* induces a part of the cascade of apoptotic reactions. Types of apoptosis without cytochrome *c* involvement have also been described [8-12]. Measurement of cytochrome *c* content in mitochondria isolated from apoptotic tissue allow estimation whether this cascade of apoptotic reactions includes formation of apoptosomes, or activation of the effector caspases occurs without the involvement of cytochrome *c*.

The study of cytochrome *c* functions in mitochondria included two stages: determination of the relative cytochrome *c* content and measurement of cytochrome *c* activity as electron carrier in the respiration chain.

The relative cytochrome *c/a* content in the first two fractions was determined spectrophotometrically by the ratio between absorbance at 550 and 630 nm (in the maximums of absorbance for cytochromes *c* and *a*, respectively). Since cytochrome *a* is tightly attached to the internal membrane, the decrease of this ratio should strongly indicate that cytochrome *c* is partly released from the intermembrane space of mitochondria. The spectrum of the heavy fraction of the apoptotic mitochondria (with the most significant amount of protein) and the spectrum of the control mitochondria are almost identical (Fig. 5). The same results were obtained with mitochondria from the middle fraction (Table 2). We did not study the light fraction because it contained a very small amount of pro-

Table 1. Absolute respiration rates of apoptotic and control mitochondria (μmol O_2/min per mg protein)

Experiment number	Fraction	Succinate (5 mM)	$MgSO_4$ (1 mM)	ADP (200 μM) + P_i (1 mM)		FCCP (0.2 μM)
1	light	44	48	48		48
	middle	34	55	55		55
	heavy	17	30	30		30
2	middle	45	64	64		64
	heavy	10	15	15		20
3	middle	22	30	30		30
	heavy	22	43	43		43
4	control	24	24	—		71
5	control	22	22	—		44
6	control (Mg^{2+} and phosphate initially in the incubation medium)	22	—	87		87
7	control (Mg^{2+} and phosphate initially in the incubation medium)	21	—	38	+ Oligo (1 μg) 28	62

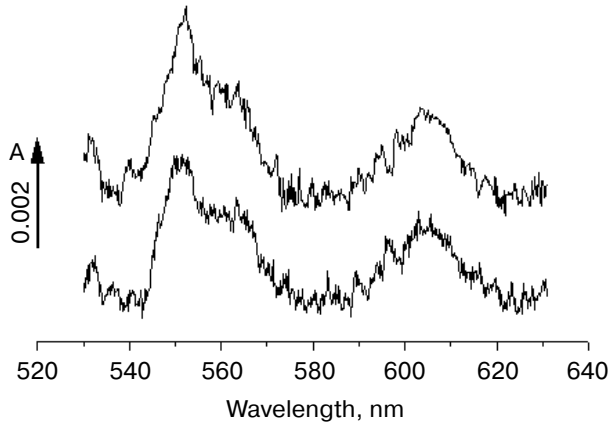

Fig. 5. Spectrums of cytochromes from apoptotic (lower spectrum) and control (upper spectrum) mitochondria. Cytochromes *a*, wavelength 605 nm; cytochromes *b*, 562-566 nm; cytochromes *c*, 550 nm.

tein. In addition, according to the data of electron microscopy, it contains a significant amount of SMP. In our model, during induction of apoptosis the content of cytochrome *c* in mitochondria does not vary significantly. Therefore, it appears that under our conditions apoptosis occurs without the involvement of cytochrome *c*.

According to the data in the literature [13, 14], Mg^{2+} increases the activity of cytochrome *c* in the external pathway of electron transfer, which includes cytochromes b_5 and *c*. The increase in cytochrome *c* activity occurs through its elevated desorption from the external surface of the internal mitochondrial membrane. On the other hand, the data of studies [15, 16] indicate that Mg^{2+} stimulates the release of cytochrome *c* induced by tBid. Here we demonstrated that respiration of both middle and heavy fractions of mitochondria is rather effectively stimulated by addition of Mg^{2+} (Fig. 4, Table 1). Thus, although in our model cytochrome *c* is not released from mitochondria, its activity is reduced. Mg^{2+} increases cytochrome *c* activity as electron carrier leading to the increase in respiration rate. It is important that addition of Mg^{2+} leads to the increase in respiration only in mitochondria isolated from apoptotic tissue. Addition of Mg^{2+} to the intact (control) mitochondria has no influence on respiration rate (Fig. 4, Table 1). Consequently, the observed effect of partial inhibition of cytochrome *c* in our experiments correlates with the induction of apoptosis.

Here we continue the work with a new model of apoptosis, where it is induced in myocardial tissue slides under conditions of extended anoxia [3]. We isolated mitochondria from apoptotic myocardial tissue and separated them into fractions according to morphologic criteria. The ultrastructure of the isolated mitochondria has several peculiarities. In particular, we registered formation of small electron-dense mitochondria inside bigger ones (Fig. 1). Free electron-dense mitochondria were also observed in the tissue [3] and could be separated from the other mitochondria by differential centrifugation (Fig. 3).

This picture differs greatly from morphological changes observed in apoptotic mitochondria in liver cells [1, 17] and in culture of adult cardiomyocytes [18], where the closed membrane structures containing cytoplasmic elements of mitochondria have been detected. Analogous structures were observed during apoptosis in plants [19].

The results obtained in this study together with the data from the literature suggest the most likely stages of induction of apoptosis in our system.

A. Decrease in pH appears to be one of the first stages of apoptosis induction under conditions of anoxia. It was shown [20] that shift of pH induced by activation of glycolysis is an essential condition for induction of apoptosis during hypoxia without re-oxygenation. Under our conditions pH decreased from 7.4 to 6.8. However, when the quantity of buffer with respect to tissue weight was reduced (when pH decreased to 5.9), we did not observe the internuleosomal DNA fragmentation, and electron microscopy revealed classical necrosis [3].

B. Induction of effector caspases without participation of cytochrome *c*. Here we showed that cytochrome *c* is not released from the intermembrane space and, consequently, it cannot be involved in the cascade of activation of effector caspases. Data from the literature indicate the existence of several mechanisms of apoptosis activation without involvement of cytochrome *c* [8-12]. At least two pathways of activation of effector caspases without

Table 2. Correlation between absorption of cytochromes *c/a* in suspension of apoptotic and control mitochondria at $\lambda = 550$ and 603 nm, respectively

Fraction	Experiment 1	Experiment 2	Experiment 3	Mean value	Standard deviation
Heavy	1.61	1.65	1.82	1.7	0.1
Middle	1.45	1.59	1.67	1.6	0.1
Control	1.64	1.75	1.96	1.8	0.2

cytochrome *c* are possible under conditions of acidosis (acidification of cytosol) that occurs during anoxia. According to [21], the first pathway is induced by the increase in calcium concentration in cytosol during acidosis. Apoptosis with involvement of caspase 12 may be induced under these conditions [22, 23]. Caspase 12 activates effector caspases without participation of cytochrome *c* [12]. The second pathway [8] suggests that cytochrome *c* independent apoptosis induced by hypoxia occurs only in the presence of the substrate for glycolysis (glucose). Caspase 8 was involved in apoptosis induction; overexpression of caspase 8 inhibitor, v-FLIP, prevented apoptosis.

The detailed study of the activities of two fractions of apoptotic mitochondria demonstrated that the system of malate oxidation is fully altered in them. It was shown that cytochrome *c* is not released from the intermembrane space, but its activity as electron carrier is partly inhibited. At the same time, the activity of the succinate oxidase system (complexes II, III, and IV of the respiration chain) is almost completely preserved. When succinate oxidase is functioning the addition of the uncoupler does not stimulate respiration. This effect provides evidence indicating that the potential on the membrane is very low; consequently, ATP synthesis in mitochondria should be inhibited or strongly reduced.

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