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Induction of Apoptosis in Rat Myocardium under Anoxic Conditions

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Abstract—The effect of anoxic incubation of small slices of isolated rat hearts on respiration, internucleosomal DNA fragmentation, and mitochondrial ultrastructure was investigated. Anoxic incubation for 72 h induced apoptosis accompanied by internucleosomal DNA fragmentation and changes in respiration and mitochondrial ultrastructure. The mitochondrial population was characterized by morphological heterogeneity. In a significant part of the mitochondrial population there were signs of mitochondrial swelling and appearance of electron-dense mitochondria. Anoxia also induced the appearance of an atypical (and previously unknown) population of small electron-dense mitochondria. They were characterized by unusual localization inside electron-light mitochondria. Under anoxic conditions the inner mitochondrial membrane formed electron-dense ordered structures. All changes described here reflect two opposing processes occurring in mitochondria: apoptotic destruction and compensatory processes responsible for maintenance of mitochondria.

Key words: apoptosis, anoxia, cardiomyocytes, myocardial infarction, mitochondria, electron microscopy

Programmed cell death, also known as apoptosis, is one of the most timely subjects of modern science. Apoptosis is one of the most important mechanisms of morphogenesis and defense against cancer; however, under certain condition it promotes the development of pathological processes in the body. Massive cell apoptosis under septic shock, myocardial infarction, and stroke may be fatal for human beings [1]. Cell cultures are the most popular object for studies of programmed cell death [2-4]. Using various cell cultures, several factors the triggering apoptotic process have been recognized [1]. Ultrastructural changes typical for apoptosis include fragmentation of cell cytoplasm and nucleus, increase of electron density of organelles, and formation of apoptotic bodies [5]. Specific ultrastructural signs are the most convincing evidence of occurring apoptosis [6, 7].

Studies of ultrastructural changes in mitochondria during apoptosis attract much interest. The interrelationship between ultrastructure and function of mitochondria was elucidated in the 1970s [8]. Now good evidence exists that mitochondria play a key role in the early stages of apoptosis.

Mitochondria are known as the major power stations of the cell, supplying various energy-consuming processes with ATP. Besides the main two-electron reduction of O_2 by cytochrome oxidase, many mitochondrial processes are accompanied by one-electron reduction of O_2 (by ubiquinone and other coenzymes of the respiratory chain) to superoxide anion. The latter together with hydrogen peroxide are important factors triggering apoptosis [9]. Superoxide anion and some other proapoptotic agents such as NO [3] and Bcl-2 proteins [10, 11] open a nonspecific mitochondrial pore. This results in release of mitochondrial proteins involved in the cell death program into the cytoplasm [9].

Little is known about specific ultrastructural mitochondrial changes underlying apoptotic process. Tumor necrosis factor α (TNF-α) discovered by Old et al. [12] was shown to trigger simultaneously several apoptotic pathways; it causes drastic changes in the mitochondrial ultrastructure. For example, incubation of L929 fibrosarcoma cell culture with TNF-α for 6 h was accompanied by a sharp increase of mitochondrial electron-density in 84% of the cells; changes in 12% of cells were interpreted by the authors as condensation and breakdown of mitochondrial cristae [2].

Myocardial infarction is a widespread disease caused by the damaging effects of anoxia or hypoxia. Massive apoptosis is considered as the causal reason of death in this disease [9]. The effects of anoxia are usually studied in cardiomyocyte cultures [3] rather than in *in situ* system(s). It should be noted that cardiomyocytes in

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cell culture significantly differ in ultrastructure and functional features from cardiomyocytes of native myocardium. The development of massive post-infarction cell apoptosis leading to death of individuals is typical for the heart of adults. Although morphological changes are the most accurate criterion of apoptosis, studies on the role of the programmed cell death in the development of cardiovascular system lesions often ignore characterization of ultrastructural changes of apoptotic myocardium. There are a few studies using sinus node biopsy material [13, 14]. Photographs presented in those papers clearly indicated the development of late stages of apoptosis characterized by formation of apoptotic bodies, myofibril collapse, and increase of electron-density of mitochondria due to matrix condensation. However, earlier stages of the development of apoptosis in the myocardium of operated patients were not investigated.

The effect of anoxia followed by reperfusion of the isolated heart with saline was accompanied by ultrastructural changes typical for necrosis: cardiomyocyte swelling, cytoplasmic membrane breaks, swelling and damage of intracellular organelles [6]. These data conflict with the general notion that apoptosis is the main process responsible for death of cardiomyocytes surviving after acute myocardial ischemia [15, 16].

In the present study the post-infarction state of myocardial tissue was modeled using anoxic incubation of isolated heart slices. After incubation at 20°C for 72 h, the respiration of slices, mitochondria ultrastructure, and internucleosomal DNA fragmentation were investigated. In parallel experiments we also investigated the effect of TNF-α on mitochondrial ultrastructure of aerobically incubated heart slices. These studies demonstrated the development of apoptosis under our experimental conditions.

MATERIALS AND METHODS

Tissue preparation. Hearts of rats weighing 150-180 g were used in the experiments. After decapitation of the animals, the hearts were quickly removed. Ventricular tissue was immediately cut into small slices in medium containing 300 mM sucrose, 0.25 mM EDTA, 5 mM Tris, pH 7.4 (at 4°C). Anoxic conditions were achieved by passing nitrogen through the sample; this removed about 95% of the oxygen. According to calculation, hermetic sealing resulted in significant reduction of oxygen concentration to ≤1 μM due to its consumption over the first 5-15 min.

Incubations. Isolated pieces of heart ventricles were incubated at 20°C for 72 h. For evaluation of possible contribution of pH decrease to structural–functional changes in cardiomyocytes, parallel incubations were carried out at pH 5.9. Cardiomyocyte morphology was studied after 72 h. Electrophoresis of DNA was carried out at 24, 38, and 72 h after the beginning of anoxic incubation.

The effect of TNF-α on cardiomyocytes was evaluated after the incubation of heart ventricle slices in the buffer with TNF-α (15 ng/ml) for 6 h. Samples were divided into two parts which were incubated under anoxic or aerobic conditions. Samples incubated aerobically in the absence of TNF-α for 6 h were used as one of the controls.

Electron microscopy. For the electron microscopy studies, material was fixed with 3% glutaraldehyde in buffer, pH 7.4, at 4°C for 2 h and then fixed with buffered 1% osmium tetroxide for 1.5 h. The material was dehydrated in alcohol solutions (50-80.96% alcohol) with increasing alcohol concentrations (70% alcohol was saturated with uranyl acetate). The material was embedded into Epon-812 epoxide resin and sections were prepared using an LKB-III microtome. Sections were stained with lead by the method of Reynolds. The resulting preparations were investigated and photographed using a HU-11B electron microscope (Hitachi, Japan).

DNA isolation and electrophoresis. After termination of the experiment, ventricular tissue in a plastic tube was frozen in liquid nitrogen. Under a layer of nitrogen the tube was destroyed and its content was dispersed in a mortar containing liquid nitrogen and lysed in the buffer solution (0.05 M Tris-HCl, 25 mM EDTA, 1% SDS, pH 7.4) at room temperature. Lysates were deproteinized by shaking with chloroform. Nucleic acids were isolated from the aqueous phase (separated by centrifugation) by adding 2.5 volumes of 96% ethanol. The resulting sediment was dissolved in TE-buffer (0.05 M Tris-HCl, 5 mM EDTA, pH 7.5) and RNase A solution was added to the mixture (final concentration 100 μg/ml). The mixture was incubated at 37°C for 20 min and subjected again to deproteinization with chloroform. DNA was sedimented by adding 2.5 volumes of cold ethanol, redissolved, and stored at –20°C.

DNA electrophoresis was carried out in 1% agarose gel in Tris-acetate buffer (0.04 M Tris-acetate, 2 mM EDTA, pH 8.0) containing ethidium bromide (0.5 μg/ml) for 1.5 h at 4-5 V/cm.

Respiration was registered polarographically using a Clark type platinum electrode. The respiration rate of myocardial slices was studied in the earlier described medium (see the section "Tissue preparation") pH 7.4; we measured respiration on endogenous substrates and using succinate as the respiration substrate. Thirty-forty milligrams tissue was placed into polarographic cell. Changes of respiration were studied after 2, 29, 52, and 72 h of anoxic incubation. The respiration rate was expressed per mg wet tissue.

Chemicals. The following chemical were used in the study: Tris from ICN (USA), EDTA from Serva (Germany), RNase A from Sigma (USA), sucrose from Merck (Germany); agarose and ethidium bromide were from Amresco (USA).

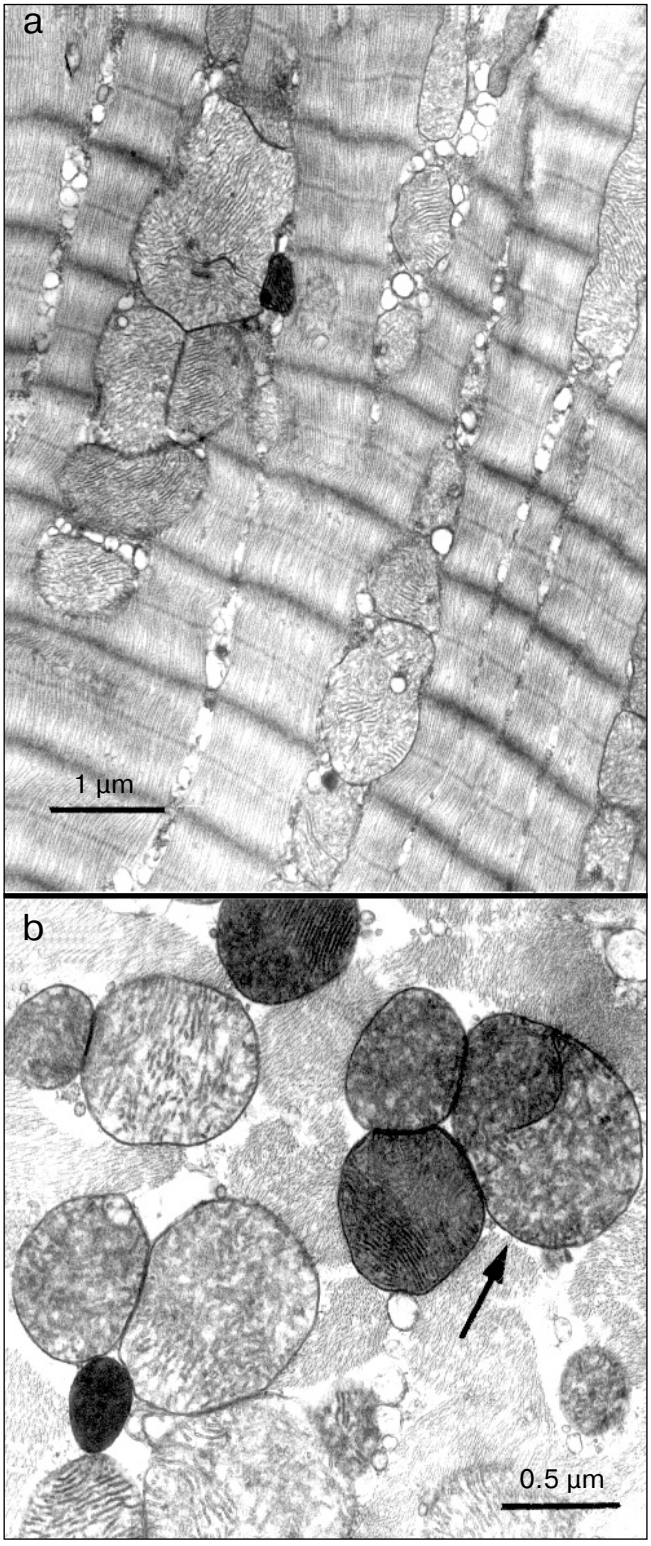

Fig. 1. Electron microphotograph of a fragment of isolated myocardial slice incubated under anoxic conditions at 20°C for 72 h: a) general view. Myofibrils are well preserved and mitochondria are in the right order along myofibrils; b) ultrastructure of separated mitochondria: electron-dense, irrigated with light matrix. Arrow shows a septate mitochondrion.

RESULTS

Figure 1a shows an electron microphotograph of isolated myocardial tissue incubated under anoxic conditions at 20°C for 72 h. Myofibrils are well preserved, Z-disks and H-zones are distinguished; mitochondria are located along myofibrils. Figure 1b shows heterogeneity of mitochondrial ultrastructure. The main fraction of the mitochondrial population is mitochondria with irrigated cleared matrix; they are contrasted, with well distinguished membranes and numerous native intermitochondrial junctions. Fewer mitochondria are characterized by condensed electron-dense matrix. At the same time, an unusual (for cardiomyocytes) population of small electron-dense mitochondria appears; these mitochondria are localized within 3-4 ultrathin serial sections. On Fig. 1b these mitochondria (as well as others) are localized among myofibrils; however, sometimes these mitochondria were distinguished inside mitochondria with irrigated matrix (Fig. 2b). Mitochondria with irrigated matrix are often characterized by unusual types of rearrangements of the inner mitochondrial membrane. Under lower magnification they look like zones with increased electron density mimicking electron-dense mitochondria; however, at higher magnification (Fig. 2a) it is clearly distinguished that in these electron-dense zones the inner mitochondrial membrane forms unusual structure. Manifestation of its morphology depends on the direction of the slice plane. This is either spongy structure with cells of equal size or stacks of membrane layers. Analysis of serial sections revealed that such zones are located within 13-15 sections. Besides these previously unknown ultrastructural changes, other changes in the ultrastructure of anoxic mitochondria were also noted. For example, there were septate mitochondria and electron-dense inclusions in the intracristate space of mitochondria (Fig. 1b).

Figure 3a shows an electron microphotograph of myocardial slice incubated aerobically with TNF-α for 6 h. Cell ultrastructure is preserved, myofibrils are well defined, and there is no signs of lysis and mitochondria are located in correct rows along myofibrils. Mitochondrial ultrastructure of myocardium aerobically incubated with TNF-α for 6 h insignificantly differed from mitochondria of myocardium incubated under anoxic conditions for 72 h (Fig. 3b, high magnification). Mitochondria of round shape with irrigated matrix and well-defined membranes are characteristic of the major proportion of these organelles. Large and small mitochondria with electron dense matrix were also distinguished within cells. As in the case of the anoxic condition, cardiomyocytes of myocardium aerobically incubated with TNF-α were characterized by the presence of separated small electron-dense mitochondria located inside larger irrigated mitochondria (Fig. 2d). In mitochondria with light matrix, local rearrangements of the inner mitochondrial membrane similar to that described above (Fig. 2c) were also observed.

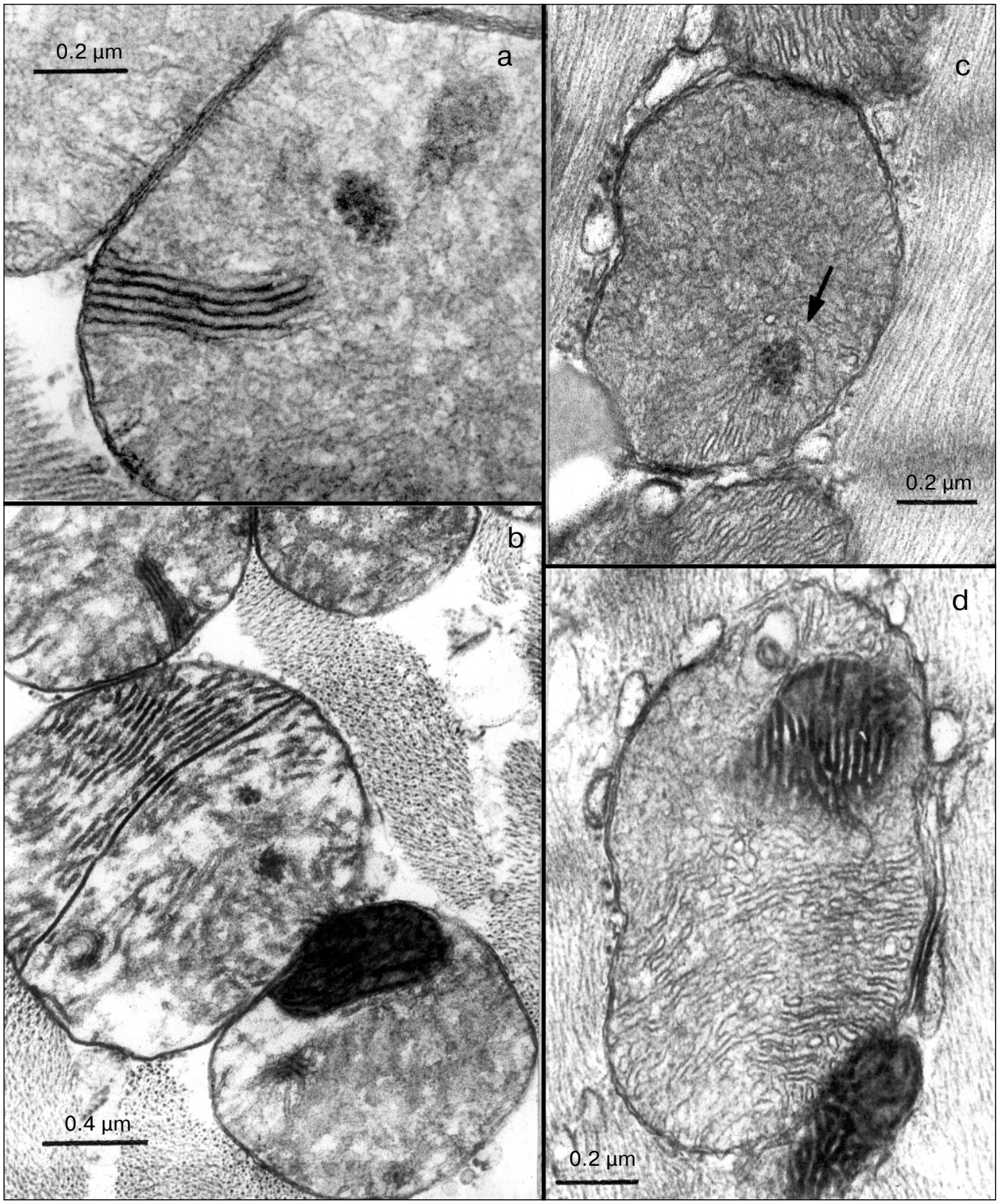

Fig. 2. Electron microphotographs of mitochondria of myocardial slice incubated under anoxic conditions at 20°C for 72 h and aerobically in the presence of TNF-α for 6 h. Incubation under anoxic conditions at 20°C for 72 h: a) local rearrangement of the inner mitochondrial membrane on longitudinal and cross sections; b) various ultrastructural changes of mitochondria: location of a small electron-dense mitochondrion inside larger mitochondrion with irrigated matrix; septate mitochondria; local rearrangement of the inner mitochondrial membrane at longitudinal section. Aerobic incubation for 6 h in the presence of TNF-α; c) arrow shows cross section with visible rearrangement of the inner mitochondrial membrane; d) location of a small electron-dense mitochondrion inside larger mitochondrion with irrigated matrix.

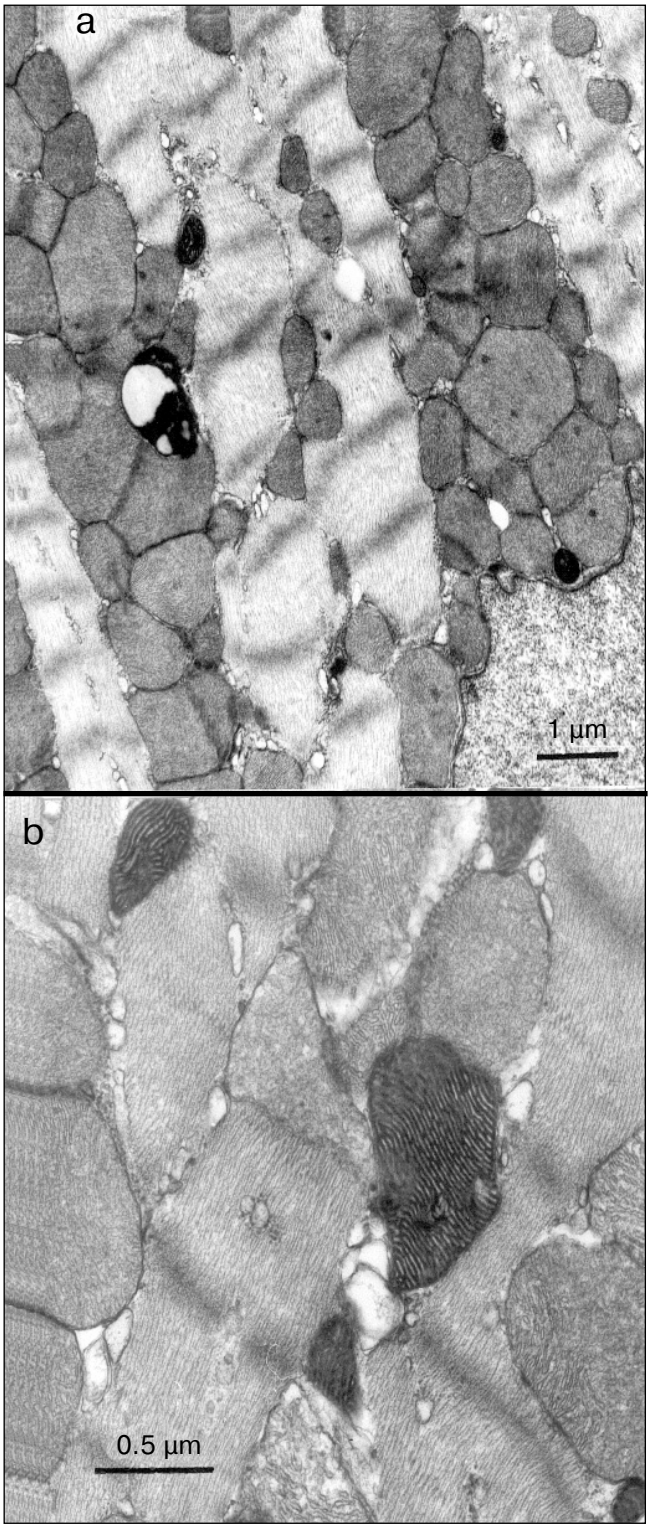

Fig. 3. Electron microphotograph of a myocardial slice incubated aerobically with TNF-α for 6 h: a) general electron microphotograph. Myofibrils are well preserved and mitochondria are in proper order along myofibrils; b) ultrastructure of various mitochondria: electron-dense, irrigated with light matrix, and swollen organelles.

Figure 4 shows an electron microphotograph of a myocardial slice incubated with TNF-α under anoxic conditions for 6 h. The morphology sharply differs from samples incubated aerobically. Cell ultrastructure remains intact, myofibrils are well defined, and there are no signs of lysis. However, in contrast to cardiomyocytes incubated aerobically for 6 h with TNF-α, electron-dense mitochondria of angular shape predominate. In many of them there are myelin-like structures formed by the inner mitochondrial membrane. Similar ultrastructural changes were also observed in cardiomyocytes of control samples incubated aerobically for 6 h without TNF-α.

Figure 5 shows an electron microphotograph of isolated myocardial slice incubated under anoxic conditions for 72 h at 20°C and pH 5.9. All mitochondria were collapsed and myofibrils were lysed. Such morphology is typical for cardiomyocyte necrosis.

Figure 6 shows electrophoregrams of DNA. Electrophoresis of DNA from myocardial slices incubated under anoxic conditions for 72 h yielded a typical apoptotic ladder with a step of 200 base pairs (bp) (Fig. 6, lane *1*). Lack of high polymer DNA suggests apoptotic DNA fragmentation. Electrophoresis of DNA from myocardial slices incubated under anoxic conditions at pH 5.9 revealed total DNA fragmentation to stretches of ≤50 bp (Fig. 6, lane *2*).

The table shows the effect of anoxia on respiration of myocardial slices. After the first day of incubation the respiration rate with succinate as substrate is increased. This suggests uncoupling of oxidative phosphorylation. However, after two and three days of incubation under anoxic condition this respiration parameter decreased, possibly due to impairments of the respiratory enzymes. Endogenous respiration was limited by substrate availability. Stimulation of endogenous respiration observed after two- and three-day incubation can be attributed to accumulation of oxidizing substrates. The initial respiration rate of the isolated ventricular pieces with endoge-

Effect of anoxic incubation on respiration of myocardial slices

Time of anaerobic incubation, h	Rate of endogenous respiration, μmol O_2/min per mg tissue	Rate of succinate oxidation, μmol O_2/min per mg tissue
1	0.065	0.233
29	0.067	0.544
52	0.247	0.357
72	0.177	0.256

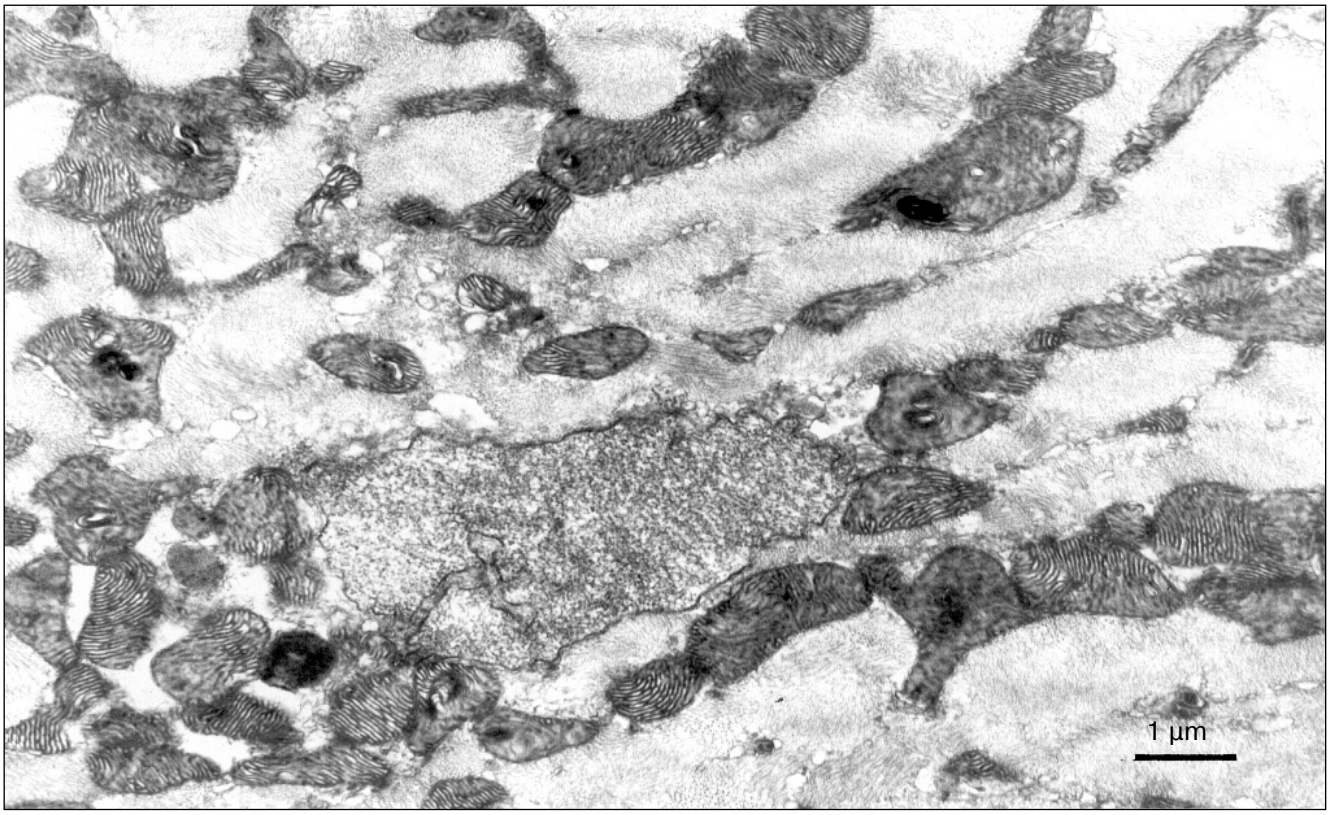

Fig. 4. Electron microphotograph of a myocardial slice incubated with TNF-α under anoxic conditions for 6 h.

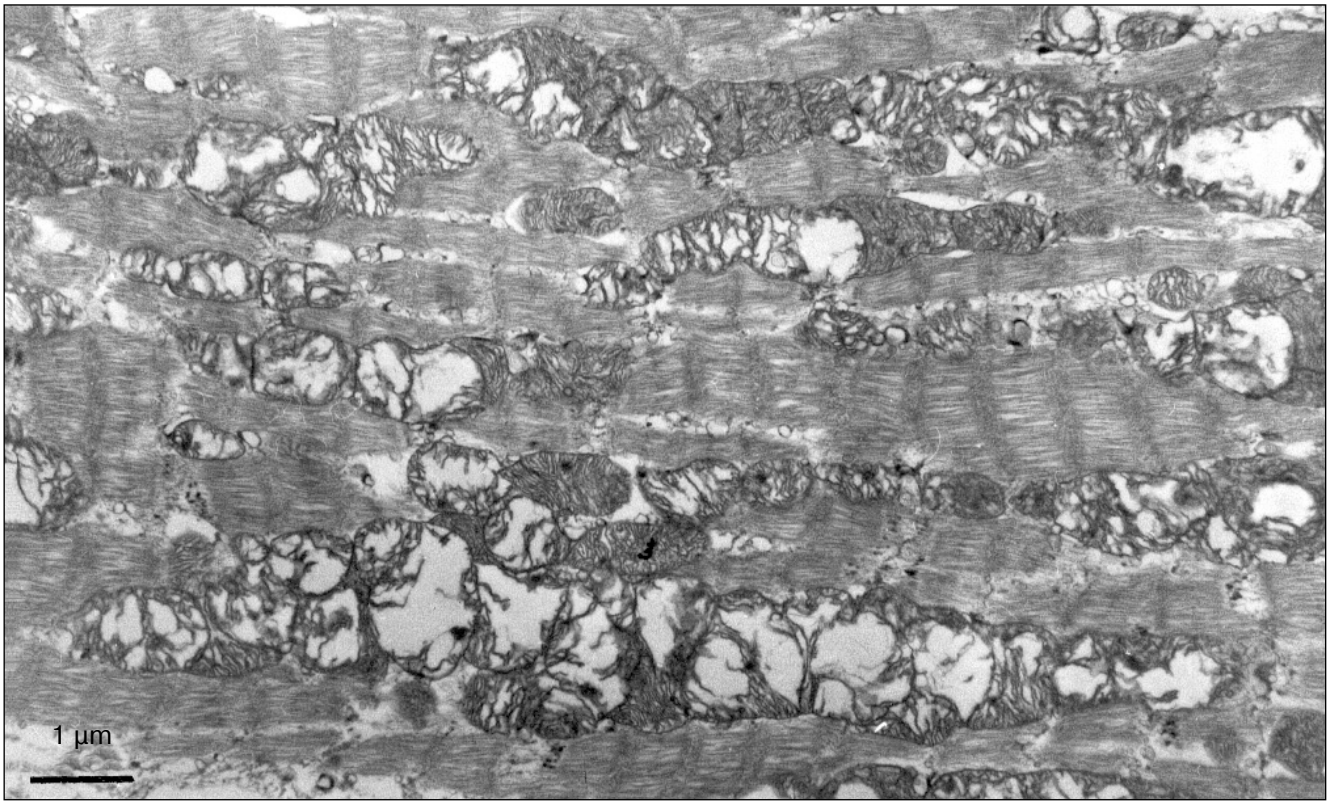

Fig. 5. Electron microphotograph of a myocardial slice incubated with TNF-α under anoxic conditions at pH 5.9 for 72 h.

Fig. 6. Electrophoresis of DNA isolated from myocardial slices. Lanes: *1*) incubation under anoxic conditions at pH 7.4 for 72 h. This resulted in a typical apoptotic ladder with two well-defined low molecular weight fractions; high molecular weight fraction is absent; *2*) incubation under anoxic conditions at pH 5.9 for 72 h. A picture shows total DNA fragmentation; M) molecular weight markers.

nous substrates was 0.065 μmol O_2/min per mg wet tissue. Calculations revealed that oxygen concentration in the system utilizing oxygen at such rate should be reduced to 1 μM within 5-15 min.

DISCUSSION

The development of apoptosis under anoxic conditions has not yet been described. We found only one report where the authors modeled anoxia at the final stages of their experiment and at the initial stages (during the first 2 h) the system contained at least 2% oxygen [17]. In the present study hypoxia was modeled by passing nitrogen through the incubation system. Special experiments on the measurement of respiration rate of myocardial slices and calculations revealed that remaining oxygen should be consumed within 5-15 min. Anoxic incubation for 72 h resulted in internucleosomal DNA fragmentation (Fig. 6, lane *1*). This suggests that anoxia is accompanied by induction of apoptosis. Lack of apoptotic bodies suggests that the process did not reach the terminal stage. We did not find any ultrastructural signs of necrosis in myocardium, such as damage of myofibril Z-line acquiring "spotty"-like structure [18]. Consequently, anoxic incubation of myocardial slices can be employed as a model of apoptosis.

Anoxic incubation of myocardial preparations caused heterogeneity of the mitochondrial population, which was characterized by a wide spectrum of changes in the ultrastructure of cardiomyocyte mitochondria. The effect of prolonged anoxia caused local rearrangements of the inner mitochondrial membrane with formation of ordered cellular-like structures (Fig. 2a) and unusual location of electron-dense small mitochondria inside larger mitochondria with light matrix (Fig. 2b). Many well-defined intermitochondrial junctions and formation of less electron-dense mitochondria were observed (Fig. 1b). For elucidation of possible relationship between these unusual changes in mitochondrial ultrastructure and apoptosis, we incubated myocardial pieces with TNF-α, a known inducer of apoptosis [3]. Similar morphology of cardiomyocyte mitochondria observed after aerobic incubation for 6 h with TNF-α and anoxic incubation for 72 h (Fig. 2) suggests that changes in mitochondria under anoxia resemble changes observed during development of apoptosis. Under aerobic incubation for 6 h, TNF-α did not cause changes in cardiomyocyte morphology typical for apoptosis. The latter suggests oxygen requirement for induction of TNF-α-dependent apoptosis.

Similarity in morphology of cardiomyocytes incubated under anoxic conditions for 72 h and under aerobic conditions for 6 h in the presence of TNF-α suggests the development of a common scenario of apoptosis irrespective of the mechanisms of its induction. Under our experimental conditions apoptosis was induced via oxygen-dependent and oxygen-independent mechanisms. It is generally accepted that apoptosis induced by anoxia requires subsequent re-oxygenation [15]. The results of the present study suggest that the development of anoxia-induced programmed cell death may occur without the involvement of oxygen.

We believe that the heterogeneity of ultrastructure of mitochondria subjected to the effect of anoxia may reflect involvement of three types of cell signaling: anoxia-induced destruction, specific apoptosis-initiating signal, and signal for compensatory reactions mobilizing rescue mechanisms under these conditions. Similarity of cell

morphology after aerobic incubation with TNF-α (for 6 h) and prolonged anoxic incubation (for 72 h) suggests that the observed changes cannot be attributed to the destructive effect of anoxia. It is possible that changes in morphology of mitochondria are related to the development of apoptosis. However, we cannot exclude the possibility that some changes in mitochondria ultrastructure represent consequences of mitochondrial destruction during apoptosis and some changes reflect triggering of defense reactions of the cell during apoptosis.

Apoptosis is a pH-dependent process [19]. Acidification of the incubation medium caused the development of apoptosis. Ischemia (anoxia) results in cytosol acidification due to activation of glycolysis [19]. In our experimental model pH buffer prevented strong pH decrease, but local pH changes in some intracellular compartments cannot be ruled out. Perhaps, such local changes can act as the apoptotic signal. In our experiments clear signs of necrosis were found after anoxic incubation and incubation at pH 5.9 (Fig. 5). Electrophoresis revealed DNA fragmentation to oligonucleotides of ≤50 bp and lack of an apoptotic ladder (Fig. 6, lane *2*). This suggests that appearance of necrosis during ischemia of heart [6] perfused with saline was due to pH decrease in cells and intercellular space. During perfusion of the whole heart, the diffusion rate of pH buffer is rather too low to compensate pH decrease during glycolysis. According to our observations apoptosis did not reach the terminal stage during 72 h of anoxic incubation. Interestingly, apoptosis induced by shorter aerobic incubation with TNF-α (for 6 h) reached the same stage as after prolonged aerobic incubation. In this connection we should stress that low rate Fas-dependent apoptosis occurs when mitochondria are not involved in the process.

Thus, in the present report we have described a new model of apoptosis induced by total anoxia. We have also shown that the induction of apoptosis via two different mechanisms (aerobic TNF-α-dependent and anoxic) is accompanied by essentially identical changes in the ultrastructure of cardiomyocyte mitochondria. Decrease of pH during anoxia under standard conditions resulted in necrosis rather than apoptosis.

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