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Inhibition of mitochondrial bioenergetics: the effects on structure of mitochondria in the cell and on apoptosis★✪

Konstantin G. Lyamzaev[1], Denis S. Izyumov[1], Armine V. Avetisyan[1], Fuyu Yang[2], Olga Yu. Pletjushkina[1] and Boris V. Chernyak[1]✉

[1]*A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow, Russia,* [2]*Institute of Biphysics, Beijing, China*

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The effects of specific inhibitors of respiratory chain, F_oF_1ATP synthase and uncouplers of oxidative phosphorylation on survival of carcinoma HeLa cells and on the structure of mitochondria in the cells were studied. The inhibitors of respiration (piericidin, antimycin, myxothiazol), the F_1-component of ATP synthase (aurovertin) and uncouplers (DNP, FCCP) did not affect viability of HeLa cells, apoptosis induced by TNF or staurosporin and the anti-apoptotic action of Bcl-2. Apoptosis was induced by combined action of respiratory inhibitors and uncouplers indicating possible pro-apoptotic action of reactive oxygen species (ROS) generated by mitochondria. Short-term incubation of HeLa cells with the mitochondrial inhibitors and 2-deoxyglucose followed by 24–48 h recovery resulted in massive apoptosis. Apoptosis correlated to transient (3–4 h) and limited (60–70%) depletion of ATP. More prolonged or more complete transient ATP depletion induced pronounced ne-

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✉Correspondence to: B.V. Chernyak, A.N. Belozersky Institute, Moscow State University, 119899 Moscow, Russia; tel.: (70 95) 939 5549; fax: (70 95) 939 3181; e-mail: Bchernyak@yahoo.com

Abbreviations: AMPK, AMP activated kinase; DMEM, Dulbecco's modified Eagle's medium; DNP, 2,4-dinitrophenol; DOG, 2-deoxyglucose; Drp1, dynamin-related protein; FCCP, trifluoromethoxycarbonylcyanide phenylhydrazone; FCS, fetal calf serum; JNK, c-Jun N-terminal kinase; mTOR, mammalian target of rapamycin; PTP, permeability transition pore; ROS, reactive oxygen species; STS, staurosporine; TNF, tumor necrosis factor α; zVADfmk, carbobenzoxy-Val-Ala-Asp-fluoromethyl ketone; YFP, yellow fluorescent protein.

crosis. The inhibitors of respiration and uncouplers caused fragmentation of tubular mitochondria and formation of small round bodies followed by swelling. These transitions were not accompanied with release of cytochrome *c* into the cytosol and were fully reversible. The combined effect of respiratory inhibitors and uncouplers developed more rapidly indicating possible involvement of ROS generated by mitochondria. More prolonged (48–72 h) incubation with this combination of inhibitors caused clustering and degradation of mitochondria.

In the past decade the public view on mitochondria has changed dramatically. The clear description of "power plants" supplying energy (ATP) in the text-books was displaced with the mysterious image of "Pandora's box" determining the fate of a cell. In the previous paradigm involvement of mitochondria in pathology was limited to impairment of cellular energetics in genetic diseases, hypoxic and toxic insults. The recent explosion of experimental work demonstrated that various cases of apoptosis critically depend on the release of specific mitochondrial proteins into the cytosol (Zamzami & Kroemer, 2001). The most important usually is the release of cytochrome *c* (one of the components of the respiratory chain) catalyzing assembly of a large cytosolic complex "apoptosome" involved in activation of caspases, the major executioners of cell death (Adams & Cory, 2002). In contrast to the basic bioenergetic principles, the mechanisms of sensing of apoptotic signals by mitochondria are not well understood.

Almost simultaneously the traditional small round-shaped organelles appeared to be artifacts of preparations (thin slices) for electron microscopy originated from the variable and dynamic mitochondrial reticulum (Amchenkova *et al.*, 1988). These cable-like structures were suggested to be an energy (trans-membrane electric potential) transporting system of the cell (Skulachev, 2001). In agreement with this idea fragmentation of the mitochondrial reticulum (observed under various stressful conditions) was attributed to a safety fuse preventing possible short-circuit collapse of the whole network (Severina *et al.*, 1998). Recently (Frank *et al.*, 2002) it was shown that fragmentation of mitochondria not only accompanied apoptosis but was a necessary event preceding the release of cytochrome *c* into the cytosol. The molecular mechanisms of mitochondrial fragmentation were studied for a long time in yeast as a model of organelle division (Rube & van der Bliek, 2004) but application of these results to the dynamics of mitochondria related to apoptosis needs very careful studies.

The change in the paradigm of mitochondrial structure and function in the cell coincided with the change in the basic methods in this field. The bioenergetics of mitochondria was investigated using a wonderful arsenal of specific inhibitors. Starting with the pioneering work by Lardy and colleagues (Lardy *et al.*, 1964) the inhibitors (usually antibiotics) of almost every component of oxidative phosphorylation were found and the mechanisms of inhibition were deciphered. Later studies on chemiosmotic mechanisms became possible due to discoveries of different ionophores and membrane-permeable indicators (Skulachev, 1988). The new era in mitochondriology coincided with the great successes in genetic engineering and genomics. Hundreds of new proteins involved in mitochondria-related signaling were discovered in a very short time. The studies of their functions are based on powerful approaches using knock-out, siRNA or dominant-negative constructs. For a number of reasons these approaches are very difficult to apply to mitochondrial proteins involved in bioenergetic functioning. Even the nearly successful attempts, such as knock-out of cytochrome *c* (Li *et al.*, 2000), did not help to fill the gap between the two areas of research on mitochondria in energy transformation and in apoptosis.

An attempt for systematic application of the inhibitors of bioenergetic functions of mitochondria (referred below as "mitochondrial inhibitors") to studies on apoptosis is presented here.

MATERIALS AND METHODS

Cell culture. Human carcinoma cells HeLa and green monkey kidney epithelial cells CV-1 were grown in Dulbecco's modified Eagle's medium (DMEM) medium containing a high level of glucose (25 mM), gentamycin sulfate (0.08 mg/ml) and 10% fetal calf serum (FCS) (Gibco) at 37°C and 5% CO_2. To monitor the mitochondrial structure the cytochrome oxidase subunit VIII was expressed as a fusion with yellow fluorescent protein (Mito-YFP, Clontech, U.S.A.) in CV-1 cells. This cell line was a kind gift of Drs. F.K. Gioeva and A.A. Minin (Institute for Protein Research, Pushino, Moscow Region, Russia). HeLa cells were stained with mitochondrial specific dye Mitotracker Green (Molecular Probes). Cells were incubated with inhibitors at the following concentrations: 2 μM rotenone (Sigma), 2 μM myxothiazol (Sigma), 2 μM antimycin (Sigma), 0.4 mM 2,4-dinitrophenol (DNP) (Sigma), 10 μM trifluoromethoxycarbonylcyanide phenylhydrazone (FCCP) (Sigma).

ATP depletion and recovery. For ATP depletion cells were incubated in DMEM with 5 mM glucose, 5 mM 2-deoxyglucose (DOG), and alternatively oligomycin (5 μg/ml), 2 μM myxothiazol, or 10 μM FCCP for 3 h. Then the medium was changed to DMEM with 25 mM glucose and the same inhibitors without DOG. Cell death was estimated after 24 and 48 h.

The ATP level was measured by the luciferin–luciferase method with LKB reagent according to the manufacturer instructions. In these experiments bicinchoninic acid was used to measure the protein concentration.

Cell viability measurements. Cell viability was determined by counting apoptotic and necrotic cells. After incubation, cells were stained with fluorescent dyes Hoechst 33342 (1 μg/ml, 25 min) and propidium iodide (2 μg/ml, 5 min). The percentage of apoptosis was calculated by counting cells with condensed and fragmented nuclei. Necrotic cells were detected by assessment of propidium iodide penetrability. In some samples a small fraction of cells with mixed staining (apoptotic nuclei and propidium iodide positive) was detected. These cases were attributed to apoptosis.

RESULTS

Mitochondrial inhibitors do not induce or affect apoptosis: the rule and exceptions

Inhibitors of respiration (piericidin, antimycin, myxothiazol), ATP synthase (oligomycin, aurovertin) and uncouplers (DNP, FCCP) did not cause any loss in viability of HeLa cells during 48 h in the traditional cell culture medium DMEM supplemented with fetal serum (10%) and glucose (25 mM) (Fig. 1). An important exception was found in experiments with rotenone. This classic inhibitor of Complex I of the respiratory chain induced cell cycle arrest and following apoptosis in HeLa at 2 μM, a concentration which was necessary for complete inhibition of uncoupled respiration of these cells in the culture medium. The effect of rotenone was not related to inhibition of respiration or a specific effect on Complex I, since another inhibitor with identical specificity, piericidin, did not affect the cell cycle or kill the cells. The effect of rotenone was probably targeted on the cytoskeleton, as it was reported earlier (Brinkley *et al.,* 1974).

Tumor necrosis factor (TNF) and the general inhibitor of protein kinases staurosporine (STS) induced different apoptotic signaling pathways and both programs were

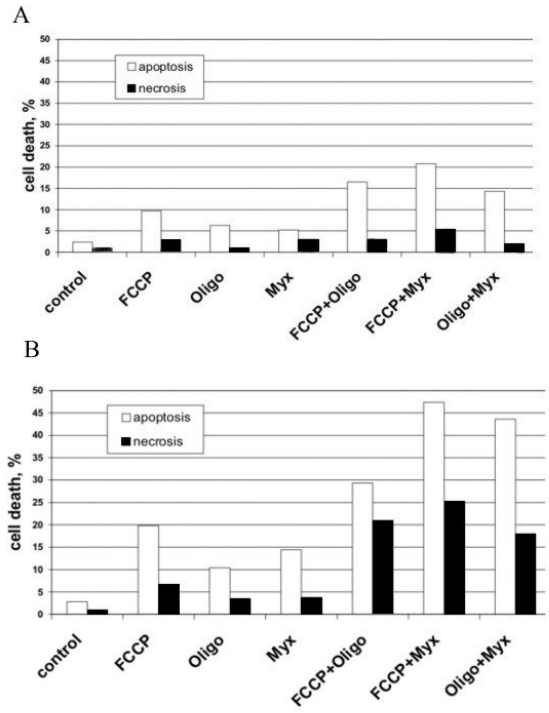

Figure 1. Cell death induced by mitochondrial inhibitors in HeLa cells.

Cells were incubated for 24 h in the presence (A) or absence (B) of 10% FCS with the following inhibitors: 10 μM FCCP, 5 μg/ml oligomycin (oligo), 2 μM myxothiazol (myx) or their combinations as indicated. Apoptosis and necrosis were determined as described in Materials and Methods.

completely executed and resulted in ordered DNA cleavage ("ladder" formation), fragmentation of nuclei and formation of apoptotic bodies in the presence of the mitochondrial inhibitors (Shchepina *et al.*, 2002a; 2002b). The release of cytochrome *c* into the cytosol, which is a central mitochondria-related event in both cases, also was not affected. The important anti-apoptotic mitochondria-located protein Bcl-2 inhibited the release of cytochrome *c* in the presence of the inhibitors as well as in control.

One interesting exception was represented by oligomycin. This inhibitor of the proton channel (F_o) in mitochondrial F_oF_1ATPase was found to inhibit TNF-induced release of cytochrome *c* and apoptosis (Shchepina *et al.*, 2002b). STS-induced apoptosis in HeLa was not affected by oligomycin (Shchepina *et al.*, 2002b), but in other models this pathway was also oligomycin-sensitive (Matsuyama *et al.*, 1998). The effect of oligomycin was not related to inhibition of oxidative phosphorylation (since inhibitors of respiration and uncouplers were ineffective) or to hyperpolarization of the membrane (since depolarization with uncouplers did not relieve the inhibition). Moreover, the effect was not directly linked to inhibition of ATPase since another specific inhibitor aurovertin B did not affect the release of cytochrome *c* and apoptosis. In contrast to oligomycin this inhibitor was targeted to the catalytic (F_1) component of the enzyme. The well-known non-mitochondrial target of oligomycin Na^+/K^+-ATPase of the plasma membrane was not responsible for the effects described. It was shown that a selective inhibitor of this enzyme, ouabain did not inhibit apoptosis. Interestingly, the selective effect of oligomycin on TNF-induced apoptosis correlated with the effect of cyclosporine A, an inhibitor of the permeability transition pore (PTP). This agent (in combination with trifluoperazine which enhance the effect) inhibited TNF-induced release of cytochrome *c* and apoptosis while it did not affect STS-induced apoptosis in HeLa. These data allow one to suggest that oligomycin inhibited the concerned action of F_o and PTP in release of cytochrome *c* from the intermembrane space of mitochondria during TNF-induced apoptosis.

The effective inhibition of oxidative phosphorylation with the inhibitors in use was confirmed with measurements of cellular respiration (Shchepina *et al.*, 2002a; 2002b), so these data indicated that glycolysis in HeLa cells can completely satisfy the demand in energy supply for survival of the cell or apoptotic cell death. It was suggested also that mitochondrial bioenergetic functioning (respiration, ATP synthesis, generation of high membrane potential) is not critical for apoptosis in general and for the mitochondria-related events of the program in particular.

To reveal possible specific induction of apoptosis with the mitochondrial inhibitors we have excluded serum from the culture medium suppressing growth factor dependent anti-apoptotic systems (Krasilnikov, 2000). Apoptosis was increased only slightly during 24 h and more prolonged incubation resulted in significant necrosis. Significant apoptosis was observed when a respiratory inhibitor (myxothiazol) was combined with uncoupler (FCCP) or with oligomycin in serum-free medium (Fig. 1B). This effect was probably caused by excessive production of reactive oxygen species (ROS) in the initial segments of the respiratory chain. ROS generation by Complex I was suggested earlier to be important for cell death in various pathologies including ischemia/reperfusion, Parkinson disease, etc. (Fleury *et al.*, 2002).

Programmed cell death induced by energy deprivation

The high concentration of glucose used in the cell culture medium was significantly higher than the physiological level. Presumably the limited supply of glycolytic substrates is especially important for rapidly growing solid tumors. To model these conditions we decreased the concentration of glucose to 5 mM and supplied the medium with 5 mM 2-deoxyglucose (DOG), a non-metabolized analog. These conditions did not cause any decrease in viability of HeLa cells indicating the high capacity of oxidative phosphorylation. As expected, mitochondrial inhibitors caused almost complete necrotic cell death during 24 h in this model. A general caspase inhibitor carbobenzoxy-Val-Ala-Asp-fluoromethyl ketone (zVADfmk) or overexpression of Bcl-2 did not inhibit the cell killing indicating that the necrotic morphology did not appear as a result of incomplete execution of apoptosis. However, if combined treatment with DOG and mitochondrial inhibitors for 3 h was followed by 24 h recovery in high-glucose medium (without DOG) significant (30–50%) apoptotic cell death was observed. Cultivation for 48 h resulted in almost complete cell death with strong prevalence of apoptosis over necrosis (Fig. 2A).

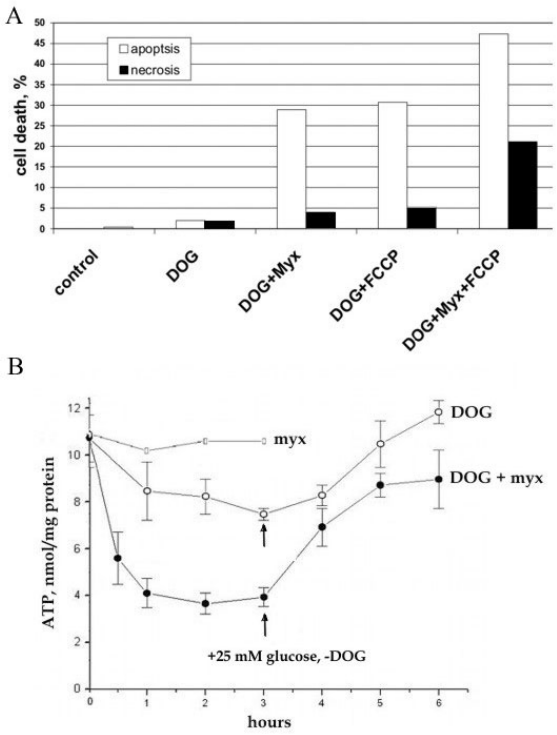

Figure 2. Apoptosis induced in HeLa cells by temporary and limited ATP depletion.

A. Cells were incubated for 3 h in low glucose (5 mM) DMEM with the following additions: no additions (contr), 5 mM 2-deoxyglucose (DOG), 10 μM FCCP, 2 μM myxothiazol (myx) or their combinations as indicated. Then the medium was changed to high-glucose (25 mM) DMEM supplemented with the same inhibitors without DOG. Apoptosis and necrosis were determined after 24 h. **B**. ATP content was determined in the same assays as in **A** using luciferase kit as described in Materials and Methods.

In the described model cell death did not significantly depend on the nature of the mitochondrial inhibitor indicating that apoptosis was induced by the temporary energy deprivation. The content of ATP dropped to 30–40% of initial level in 0.5 h and remained constant for the next 3–5 h. Removal of DOG caused a rapid restoration of ATP level even when the mitochondrial inhibitors were present (Fig. 2B). The temporary limited decrease

in ATP appears to be insufficient to cause significant damage to the cellular structures. Even the lowest concentration of ATP remained in the millimolar range, which is well above the saturation of the majority of ATP-consuming systems indicating the existence of specific ATP-meter(s). In this model combined action of myxothiazol and an uncoupler was significantly stronger (Fig. 2A) in agreement with the results obtained in serum-free glucose-rich medium (Fig. 1B). It could be suggested that generation of ROS by the respiratory chain improved apoptotic signaling triggered by temporary ATP depletion.

When the ATP depletion procedure was prolonged to 5 h the following recovery still restored the ATP level but resulted mostly in necrotic cell death after 24 or 48 h. A similar switch to necrosis was observed when ATP depletion was improved by more complete inhibition of glycolysis. The mitochondrial inhibitors in combination with DOG added to the medium depleted of glucose caused a rapid fall in cellular ATP to less then 10% of initial level. After 3 h the medium was changed to complete glucose-rich DMEM and cellular ATP was restored almost completely; the following 24 or 48 h cultivation resulted in massive necrotic death (not shown). These data indicate that temporary ATP depletion can be a trigger of programmed cell death with both necrotic and apoptotic features.

Apoptosis induced by ATP depletion was prevented by inhibition of caspases with zVADfmk or by overexpression of Bcl-2. Translocation of Bax from the cytosol to mitochondria and release of cytochrome *c* from mitochondria into the cytosol were observed in this model resembling the typical stress-induced apoptosis. Interestingly, zVADfmk stimulated necrosis under the same conditions suggesting that caspases not only catalyzed apoptosis but also inhibited necrosis. In contrast to apoptosis, the signaling resulting in necrosis remained poorly characterized. An example of necrosis induced by rotenone and glucose deprivation in myogenic cells was recently found to be dependent on activation of the stress-activated protein kinase (JNK) (Gabai *et al.*, 2000; Yaglom *et al.*, 2003). In HeLa cells neither apoptosis nor necrosis was sensitive to inhibitors of JNK or p38 (another stress-activated protein kinase). The nature of the putative ATP-meter(s) also remained mysterious. The candidate sensors includes mammalian target of rapamycin (mTOR) (Dennis *et al.*, 2001), a protein kinase with exceptionally high K_m (ATP) and AMPK (Rutter *et al.*, 2003), a kinase that is allosterically activated by AMP. The both kinases are involved in regulation of gene expression in response to nutrient starvation but their role in induction of apoptosis is purely speculative.

Inhibition of the bioenergetic functions causes morphological changes and degradation of mitochondria

Mitochondrial inhibitors in the presence of glucose induced dramatic changes in mitochondrial structure independently of ATP depletion or apoptotic events. The details of this process were visualized using CV-1 cells expressing yellow fluorescent protein (YFP) fused to subunit VIII of cytochrome oxidase (Fig. 3). It was shown that rotenone, myxothiazol or the uncouplers induced fission of the long mitochondrial filaments after a significant lag phase (2–3 h). At the next step the short fragments were transformed to small round bodies. This process included further fission combined with the change of the shape of the organelles. The following swelling of the mitochondria was clearly visible in the case of uncouplers. A similar sequence of events was observed in HeLa cells where mitochondria were stained with Mitotracker Green (not shown). The wash out of the uncouplers caused slow (24 h) complete restoration of the original mitochondrial network (Fig. 3A). Exceptionally rapid and intense changes were induced in mitochondria of CV-1 by oligomycin. This effect was mostly related to inhibition of Na^+/K^+-ATPase of the

plasma membrane (since ouabain caused similar changes) and was not observed in HeLa.

The most rapid fission and the following transitions of the mitochondrial network were induced by combined treatment with respiratory inhibitors and uncouplers indicating a possible role of ROS produced by the respiratory chain (Fig. 3B). This suggestion is in good agreement with the observations of mitochondrial transitions induced by hydrogen peroxide (0.1–0.4 mM) in the same cell lines. Similar steps of mitochondrial fragmentation were described (Skulachev *et al.*, 2004) but the initial lag-phase was significantly

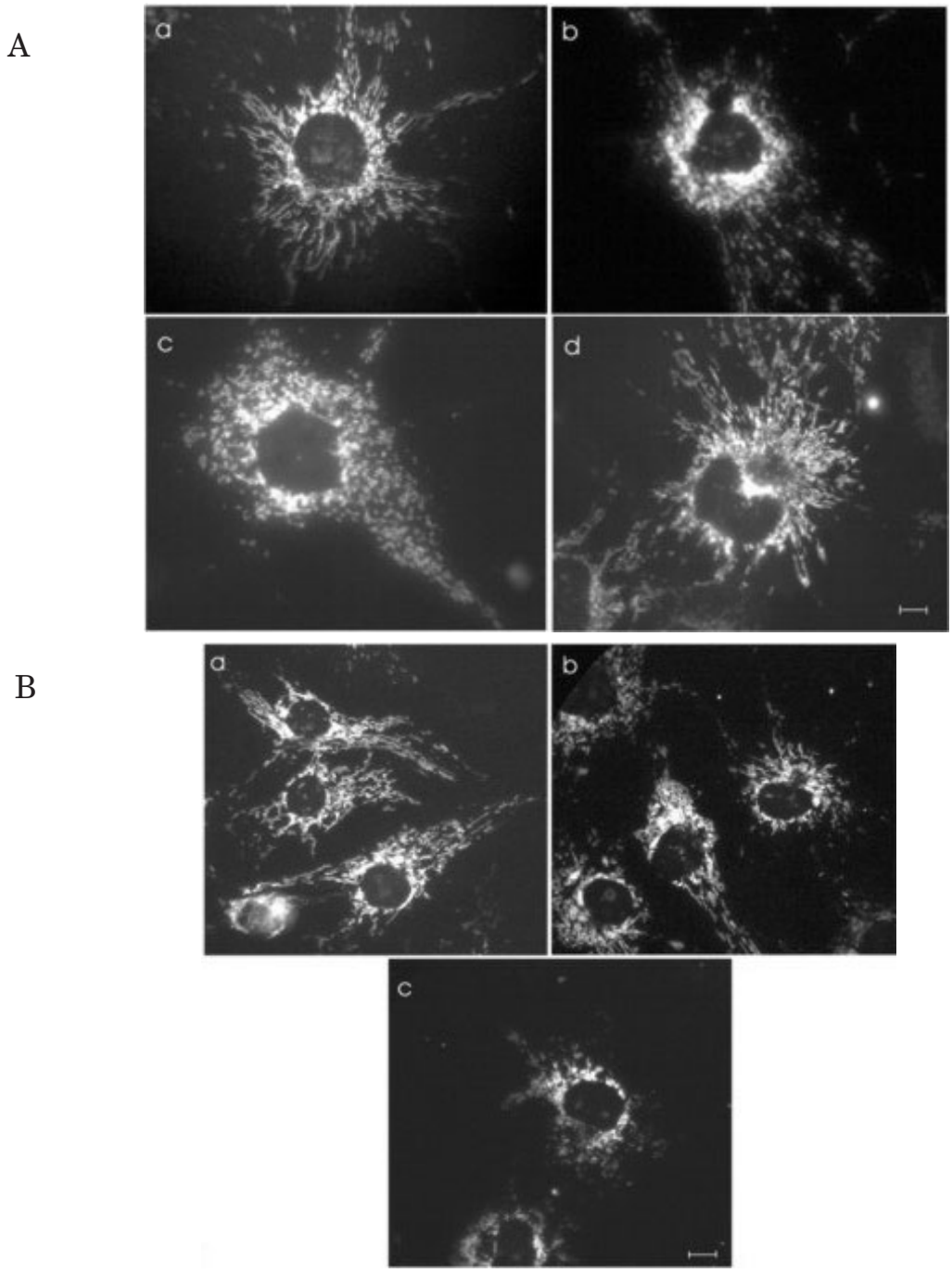

Figure 3. Fragmentation of mitochondria in CV-1 cells incubated with mitochondrial inhibitors.

A. Cells were incubated with 0.4 mM DNP for 0 h (**a**), 2 h (**b**) and 6 h (**c**). After incubation with DNP (6 h) cells were washed and cultivated for 24 h (**d**). Bar 10 μm. **B**. Cells were incubated with 2 μM myxothiazol (myx) for 6 h (**a**), or with 0.4 mM DNP and 2 μM myxothiazol (DNP + myx) for 45 min (**b**) or 6 h (**c**). Bar 10 μm.

shorter. In both models no visible signs of apoptosis were observed until the final steps of mitochondrial transitions. The release of cytochrome *c* from mitochondria was not observed even when mitochondria were swollen.

Similar changes in morphology of mitochondria were described in various models of apoptosis (Desagher & Martinou, 2000). A detailed study of staurosporine-induced apoptosis in CV-1 revealed a clear time gap between fission of mitochondria and release of cytochrome *c* (Skulachev *et al.*, 2004). Interestingly both events were well synchronized in mitochondrial population of a single cell and proceeded very rapidly in comparison with the lag phase. Recently, it was found that fission of mitochondria during apoptosis depended on translocation of dynamin-related protein (Drp1) from the cytosol to some local sites at the surface of mitochondria (Frank *et al.*, 2002). Bax targeted the same sites and Drp1 dominant-negative mutant protein not only prevented fission but also inhibited apoptosis (Karbowski *et al.*, 2002). There is no evidence that mitochondrial transitions induced by the inhibitors or by hydrogen peroxide included the same molecular mechanisms; however, it appears that fission of mitochondria can be necessary but is not sufficient for apoptosis.

At the final steps of the treatment with uncouplers alone or in combination with respiratory inhibitors fragmented mitochondria gathered near the nucleus and formed several clusters (Fig. 4). A similar picture was observed in cells treated with hydrogen peroxide and in apoptotic cells. In fibroblasts treated with TNF this effect was attributed to specific modification and inhibition of kinesin, the molecular motor responsible for anterograde movement of mitochondria along microtubules (De Vos *et al.*, 2000). Electron microscopy revealed swollen and partially degraded mitochondria in these clusters (L. Bakeeva, unpublished). This observation was in agreement with the significant decrease of the total mass of mitochondrial ma-

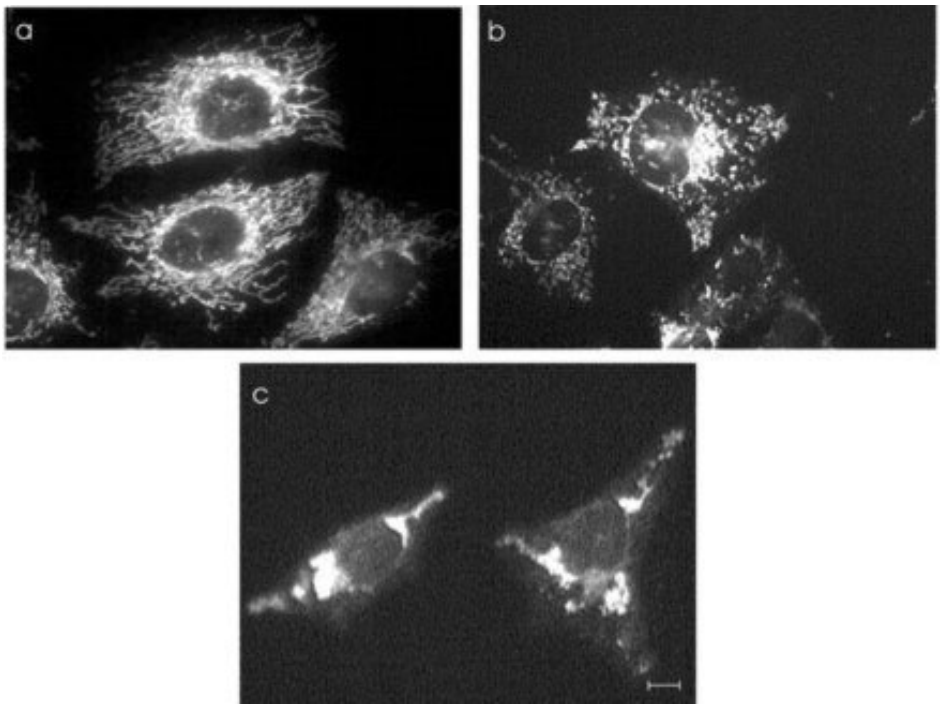

Figure 4. Clusterization of mitochondria in HeLa cells after prolonged treatment with mitochondrial inhibitors.

Cells were incubated with 0.4 mM DNP and 2 μM antimycin for 0 h (**a**), 24 h (**b**) and 72 h (**c**). Mitochondria were stained with Mitotracker Green. Bar 10 μm.

terial and content of specific mitochondrial proteins (not shown). Selective elimination of mitochondria from apoptotic cells was recently described (Xue *et al.*, 2001) and the major role of autophagy in this process was suggested. We did not observe accumulation of autophagosomes in our model, so probably the mechanism of depletion of the cells of mitochondria was different. It should be stressed that in apoptotic models inhibition of caspases was necessary to prevent the cellular collapse and to observe elimination of mitochondria.

When HeLa cells were treated for 48–72 h with uncouplers in combination with antimycin or myxothiazol a significant (50–70%) fraction of the cells died but the rest of the population was viable, without any signs of apoptosis and with very low mitochondrial content (Fig. 4). The cell death was not related to energy deprivation, since the same respiratory inhibitors caused complete cessation of oxidative phosphorylation but did not cause cell killing. Complete depolarization of the mitochondrial membrane and (or) hyperproduction of ROS were the most probable reasons for cell death. The viable cells depleted of mitochondria probably had a selective advantage due to low content of pro-apoptotic mitochondrial proteins and elimination of the major source of ROS production. Thus both the induction of an unknown mechanism(s) of mitochondrial elimination and the selective pressure could be responsible for the observed phenomena. It could be suggested that similar processes are responsible for depletion of mitochondria in some rapidly growing tumors (Cuezva *et al.*, 2002).

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