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Review

## “Wages of Fear”: transient threefold decrease in intracellular ATP level imposes apoptosis

Denis S. Izyumov<sup>a</sup>, Armine V. Avetisyan<sup>a</sup>, Olga Yu. Pletjushkina<sup>a</sup>, Dmitrii V. Sakharov<sup>b</sup>,  
Karel W. Wirtz<sup>b</sup>, Boris V. Chernyak<sup>a,\*</sup>, Vladimir P. Skulachev<sup>a</sup>

<sup>a</sup>Department of Bioenergetics, Belozersky Institute of Physico-Chemical Biology, Moscow State University, Khokhlova Street 4, Moscow 119992, Russian Federation

<sup>b</sup>Department of Biochemistry of Lipids, Centre for Biomembranes and Lipid Enzymology, Institute of Biomembranes, Utrecht University, The Netherlands

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### Abstract

In HeLa cells, complete inhibition of oxidative phosphorylation by oligomycin, myxothiazol or FCCP combined with partial inhibition of glycolysis by DOG resulted in a steady threefold decrease in the intracellular ATP level. The ATP level recovers when the DOG-containing medium was replaced by that with high glucose. In 48 h after a transient (3 h) [ATP] lowering followed by recovery of the ATP level, the majority of the cells commits suicide by means of apoptosis. The cell death does not occur if DOG or an oxidative phosphorylation inhibitor was added separately, treatments resulting in 10–35% lowering of [ATP]. Apoptosis is accompanied by Bax translocation to mitochondria, cytochrome *c* release into cytosol, caspase activation, reactive oxygen species (ROS) generation, and reorganization and decomposition of chromatin. Apoptosis appears to be sensitive to oncoprotein Bcl-2 and a pancaspase inhibitor zVADfmk. In the latter case, necrosis is shown to develop instead of apoptosis. The cell suicide is resistant to cyclosporine A, a phospholipase inhibitor trifluoroperazine, the JNK and p38 kinase inhibitors, oligomycin, *N*-acetyl cysteine and mitoQ, differing in these respects from the tumor necrosis factor (TNF)- and H<sub>2</sub>O<sub>2</sub>-induced apoptoses. It is suggested that the ATP concentration in the cell is monitored by intracellular “ATP-meter(s)” generating a cell suicide signal when ATP decreases, even temporarily, below some critical level (around 1 mM).

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**Keywords:** ATP; Intracellular ATP-meter; Apoptosis; Necrosis; Reactive oxygen species

### 1. Introduction

The living cell must always guarantee proper functioning of its main metabolic systems and structures, among which intactness of its genome seems to be the most important. If it did not, the appearance of monsters becomes possible, which can ruin a tissue, an organ, an organism or even a population and a species. To avoid such a possibility, a cruel but radical

principle called the “Samurai law of biology” was postulated to operate in complex living systems: “It is better to die than to be wrong” [1,2].

The ATP level is a crucial parameter of cell homeostasis. It seems reasonable to assume that cells are equipped with ATP-monitoring devices (“ATP-meters”). Such mechanisms should (i) coordinate energy-supplying and energy-consuming processes, and (ii) initiate cell suicide if the cell fails to maintain [ATP] above a certain critical level during some period of time.

If the abovementioned relationships really exist, one may predict that it is possible to induce a programmed cell death by keeping ATP at a decreased level even if this level is later normalized. The postulated system is assumed to purify the population of cells from those that survive after an energetic disorder during which, however, they could not guarantee intracellular homeostasis and, hence, intactness of the genome and/or other essential cellular components.

**Abbreviations:** CM-DCF, 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate; DOG, 2-deoxyglucose; FCCP, trifluoromethoxycarbonyl cyanide phenylhydrazone; Hsp 72, 72 kDa heat shock protein; mitoQ, 10-(6'-ubiquinolyl)decyltriphenylphosphonium; mTOR, mammalian target of rapamycin; ROS, reactive oxygen species; sGC, soluble guanylate cyclase; TNF, tumor necrosis factor  $\alpha$ ; zVADfmk, carbobenzoxy-Val-Ala-Asp-fluoromethyl ketone

\* Corresponding author. Tel.: +7-95-9395530; fax: +7-95-9390338.

E-mail address: [skulach@belozersky.msu.ru](mailto:skulach@belozersky.msu.ru) (V.P. Skulachev).

Depletion of ATP is known to prevent execution of critical steps of the apoptotic program (primarily, formation of the apoptosome) [3]. This is why a decrease in [ATP] is usually attributed to necrotic stimuli and a switch from apoptotic to necrotic cell death [4,5]. Recently, Gabai et al. [6] and Yaglom et al. [7] have shown that more than 97% decrease in the ATP level in myogenic cells resulted in necrosis even if such a decrease was transient. The death was prevented by either switching-off a stress-activated protein kinase (JNK) with an inhibitor or overexpression of Hsp72, a fact pointing to the programmed character of this kind of necrosis. Transient but almost complete (99,8%) decrease in [ATP] probably was the major stimulus for apoptosis after hypoxia/reoxygenation of kidney cell cultures [8,9]. In both of the abovementioned models (necrotic and apoptotic), it was difficult to exclude that the death was a consequence of irreversible damage to some system(s) of vital importance during the period when [ATP] was far below  $K_m$  for the majority of ATP-consuming enzymes.

In this work, we used a milder procedure when ATP was decreased by no more than threefold. It is known [10] that normally various living cells maintain [ATP] around 3 mM. This means that a threefold lowering of [ATP] should result in the ATP level of about 1 mM, which is still higher than  $K_m$  for the great majority of ATP-dependent enzymes, including those involved in apoptosis [4]. As an experimental system, human cervical carcinoma cells of the HeLa line were employed. These cells can successfully maintain their ATP level and viability by either respiration or glycolysis [11,12]. We have found that transient threefold decrease in [ATP] followed by recovery of the ATP level induces apoptosis accompanied by Bax translocation to mitochondria, cytochrome *c* release to cytosol, caspase activation, reactive oxygen species (ROS) formation and other typical apoptotic events.

## 2. Materials and methods

### 2.1. Cell culture and Bcl-2 overexpression

Human carcinoma cells HeLa were grown in DMEM medium containing a high level of glucose (25 mM), gentamicin sulfate (0.08 mg/ml) and 10% FCS (Gibco) at 37 °C and 5% CO<sub>2</sub>. The human *bcl-2* gene was introduced into HeLa cells, using the pLPC-bcl-2 vector for transfection. A control clone was similarly prepared using an empty vector. These cell lines were provided by Dr. G.A. Belov (M.P. Chumakov Institute of Poliomyelitis and Viral Encephalitis, Moscow, Russia). Expression of Bcl-2 protein in transfected cells was confirmed by Western blot analysis, using anti-human Bcl-2 monoclonal antibody.

### 2.2. ATP depletion and recovery

For ATP depletion cells were incubated in DMEM with 5 mM glucose, 5 mM 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 inhibitor without DOG. Cell death was estimated after 18–24 and 48 h.

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

### 2.3. Cell viability measurement

Cell viability was determined by counting of apoptotic and necrotic cells. After incubation, the cells were stained with fluorescent dyes Hoechst 33342 (Sigma) (1 µg/ml, 25 min) and propidium iodide (Sigma, 2 µg/ml, 5 min). The percentage of apoptosis was calculated by counting of cells

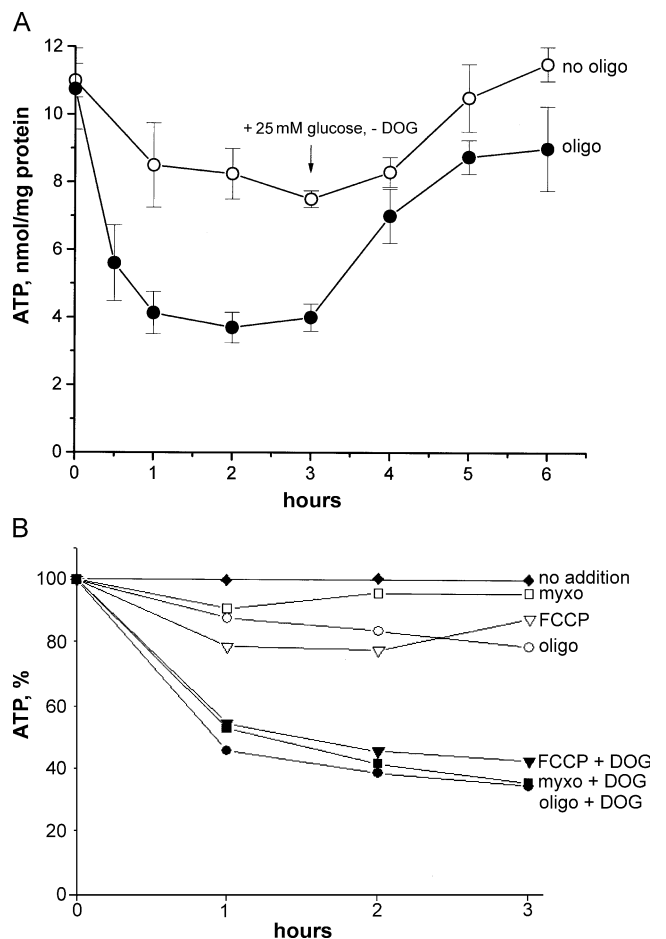


Fig. 1. Combined action of inhibitors of glycolysis (DOG) and oxidative phosphorylation (oligomycin, myxothiazol and FCCP) causes threefold decrease in the ATP level in HeLa cells. In A, the cells were incubated for 3 h in medium containing 5 mM glucose, 5 mM DOG with or without oligomycin (5 µg/ml, oligo). Then the medium was replaced with a fresh one, 25 mM glucose, no DOG, with or without oligomycin. Bars, S.D. In B, the cells were incubated with various oxidative phosphorylation inhibitors with or without DOG as in Fig. 1A. The concentrations of inhibitors were 5 mM DOG, oligomycin (5 µg/ml, oligo), 2 µM myxothiazol (myxo) and 10 µM FCCP.

with condensed and fragmented nuclei. Necrotic cells were detected by assessment of propidium iodide permeability. In some samples, a small (less than 20%) fraction of cells with the mixed staining (apoptotic nuclei and propidium iodide positive) was detected. These cases most probably represent switching over the cell suicide program from apoptosis to necrosis at a late stage of apoptosis. In the calculations, they were attributed to apoptosis.

#### 2.4. Fluorescent microscopy

The cells were fixed with formalin (4%) and permeabilized by 0.1% Triton X-100. The samples were incubated with monoclonal antibodies to cytochrome *c* and polyclonal rabbit antibodies to Bax (PharMingen) and stained with Oregon Green 488-conjugated anti-mouse and Texas Red-conjugated anti-rabbit secondary antibodies (Molecular Probes). The cells in Vectashield medium were analyzed under confocal or inverse fluorescent microscopes.

For detection of ROS production, cells were loaded with 5  $\mu$ M 5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate (CM-DCF) (Molecular Probes) (15 min) or with 1  $\mu$ M BODIPY-C11 (30 min) (for methods, see Refs. [12,14], respectively) and analyzed under the confocal microscope. BODIPY-C11 fluorescence was acquired using double excitation with Argon-ion laser (line 488 nm) and He–Ne laser (line 543 nm) and emission bandpass filters 515/30 and 585/30 (for oxidized and reduced form of BODIPY-11, respectively).

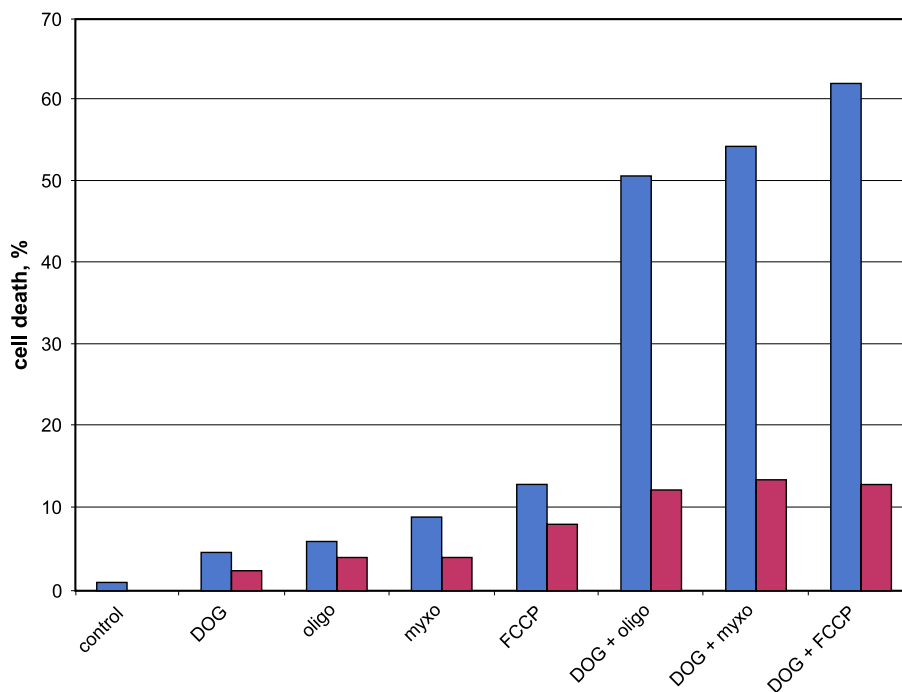


Fig. 2. Development of apoptosis (blue columns) and necrosis (red columns) during 48-h incubation of cells treated with various inhibitors. The same protocol as in Fig. 1 was used to induce an [ATP] decrease. Apoptosis and necrosis were estimated using the Hoechst 33342 and propidium iodide staining, respectively.

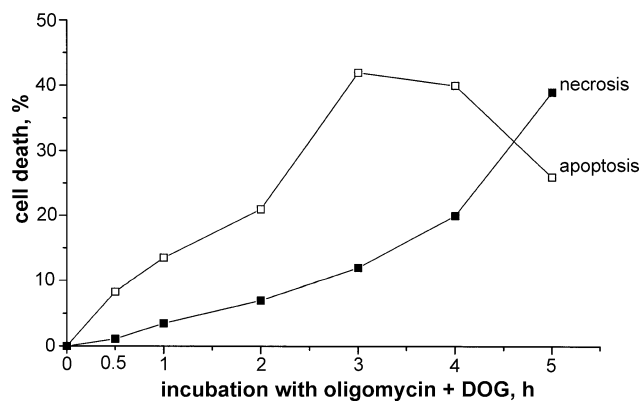


Fig. 3. Contribution of apoptosis and necrosis to the cell death as a function of duration of ATP depletion. After the ATP-depletion caused by treatment with DOG and oligomycin for hours indicated on abscissa (conditions as in Fig. 1), the cells were incubated with high glucose and oligomycin for 48 h.

In Fig. 1A, the S.D. values are given. In other figures, typical data of three to five experiments are shown.

### 3. Results

To decrease the ATP level, HeLa cells were incubated in low (5 mM) glucose medium supplemented with 5 mM 2-deoxyglucose (DOG) and an agent arresting oxidative phosphorylation, i.e. the  $H^+$ -ATP-synthase inhibitor oligomycin, respiratory chain inhibitor myxothiazol or uncoupler FCCP. In this (see below, Fig. 2) and preceding [11,12]

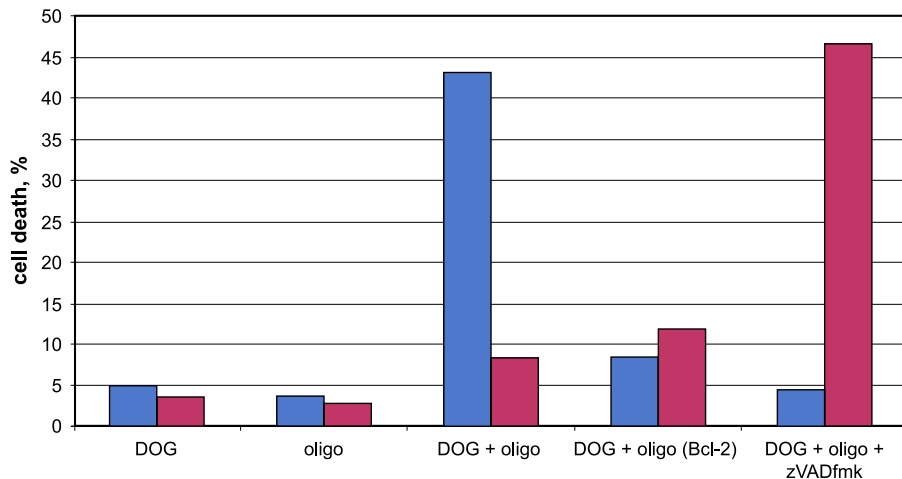


Fig. 4. Inhibition of the ATP decrease-induced apoptosis (blue columns) and necrosis (red columns) by Bcl-2 and zVADfmk. Bcl-2, the HeLa cell line overexpressing Bcl-2. The zVADfmk concentration, 50  $\mu$ M. Conditions as in Fig. 2.

studies, it was found that neither 5 mM DOG nor the abovementioned inhibitors, when added separately, were competent in initiating the HeLa cell death.

As is seen in Fig. 1A, a 1- to 3-h incubation of the cells in DOG-containing medium without or with oligomycin resulted in lowering of [ATP] to about 1/3 or 2/3 of the initial levels, respectively. After 3 h, the medium was replaced by fresh medium with high (25 mM) glucose, without DOG and with or without oligomycin. This gave rise to a recovery of the ATP level. Fig. 1B shows that

FCCP or myxothiazol can substitute for oligomycin if DOG is present. Without DOG, these inhibitors lowered the ATP level by less than 25%.

Effects of DOG and oxidative phosphorylation inhibitors on the cell death are shown in Figs. 2–4. As one can see in Fig. 2, any one of the tested inhibitors failed to kill the great majority of the cells during 48-h incubation. On the other hand, the treatment with combination of DOG and either oligomycin, myxothiazol or FCCP for 3 h resulted in the death of the majority of the cells during

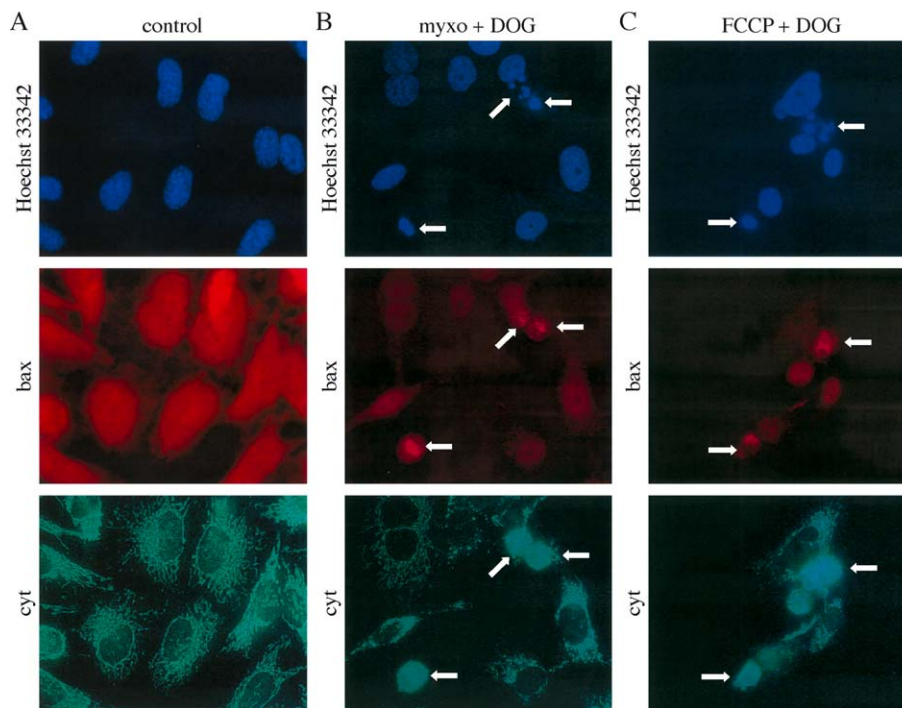


Fig. 5. Bax translocation to mitochondria and cytochrome *c* release from mitochondria induced by a transient decrease in the ATP level. For inhibitor concentrations, see Fig. 1. Duration of incubation with high glucose after the [ATP]-decreasing treatment, 24 h. (A) Control cells; (B) cells treated with myxothiazol and DOG; (C) cells treated with FCCP and DOG. Apoptotic cells are indicated by arrows.

48 h, apoptosis being strongly dominating over necrosis. Necrosis became dominating when the combined treatment with DOG and the inhibitors of oxidative phosphorylation lasted 5 h (Fig. 3).

Overexpression of the anti-apoptotic oncoprotein Bcl-2 or treatment with the pancaspase inhibitor zVADfmk completely abolished the effect of DOG + oligomycin on apoptosis. As to necrosis, it remained low in the Bcl-2-overproducing cells and was strongly increased by zVADfmk (Fig. 4). The latter observation indicates that, under conditions used, caspase(s) not only promotes apoptosis, but also inhibits necrosis.

In the next series of experiments, we characterized apoptosis induced by DOG and the inhibitors of oxidative phosphorylation. Apoptosis was found to result in translocation of pro-apoptotic protein Bax from cytosol to mitochondria and release of cytochrome *c* from mitochondria into cytosol (Fig. 5). Combined treatment with cyclosporine A and trifluoroperazine, as well as with oligomycin, prevented the tumor necrosis factor  $\alpha$  (TNF)-induced permeability transition pore formation and apoptosis [12] but failed to arrest cytochrome *c* release and apoptosis caused by decrease in ATP level. Apoptosis was accompanied by (i) appearance of a “DNA ladder”, (ii) marginal (see Ref. [13]) and total blebbing, (iii) a cell shrinkage, and (iv) activation of caspases (DEVDase activity) (not shown in figures). Such a typical feature of apoptosis as generation of ROS was proved to also be inherent in apoptosis described in this paper. This was shown when both water-soluble (CM-DCF) and membrane-linked (BODIPY-C11 [14]) ROS-sensitive fluorescent probes were employed (Figs. 6 and 7, respec-

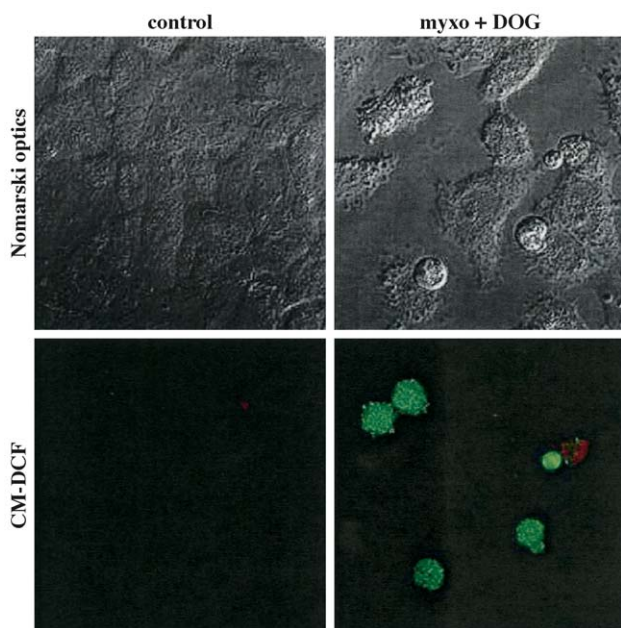


Fig. 6. ROS formation reported by the water-soluble probe CM-DCF (green staining). For inhibitor concentrations, see Fig. 1. Duration of incubation with high glucose after the [ATP]-decreasing treatment, 24 h. Propidium iodide was added to identify necrotic cells (red staining).

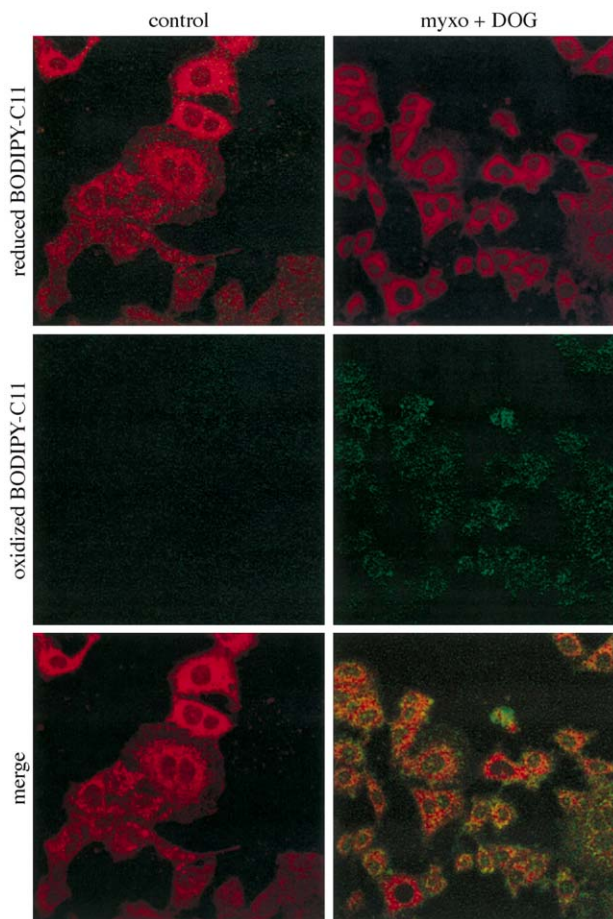


Fig. 7. ROS formation reported by oxidation of the lipid-soluble probe BODIPY-C11. For inhibitor concentrations, see Fig. 1. After the [ATP]-decreasing treatment, the cells were incubated with high glucose for 8 h.

tively). Oxidation of CM-DCF developed in 18–20 h after DOG + myxothiazol treatment and was detected only in the cells of the clearly apoptotic morphology. This effect was completely prevented by Bcl-2 and zVADfmk (not shown). On the other hand, oxidation of BODIPY-C11 was detected much earlier (after 8 h) in the majority of cells, which did not reveal any morphological signs of apoptosis. Neither general (*N*-acetyl cysteine) nor mitochondrion-targeted (mitoQ) antioxidants affected the apoptosis studied, so the role of ROS in this process remained unclear. Inhibition of JNK with SP600125 and p38 kinase with SB203580, separately or in combination, as well as a protein synthesis inhibitor emetine were also without effect (not shown in figures).

#### 4. Discussion

The data described above indicate that a threefold decrease in the intracellular [ATP] imposes apoptosis in HeLa cells. Such a decrease was induced by a cocktail of inhibitors (DOG and oligomycin, myxothiazol or FCCP).

Neither DOG nor the inhibitors listed were apoptogenic when tested separately, while [ATP] was decreased by less than 1/3.

The observed effect cannot be accounted for either by an increase [15] or by a decrease [16] in mitochondrial membrane potential since both oligomycin (induces hyperpolarization) and FCCP or myxothiazol (induce depolarization) proved to be apoptogenic when added with DOG, being without effect in the absence of DOG (Fig. 2). Similarly, the effect was not due to a change in the intracellular redox state, which should be shifted to a more reduced state by oligomycin and to a more oxidized state by FCCP. As to myxothiazol treatment, it is known to reduce the initial span of the respiratory chain and to oxidize its terminal span.

The apoptosis described in this paper is hardly a result of nonspecific damage to cellular enzyme systems or structures due to transient lowering in [ATP] from 3 to 1 mM since the great majority of ATP-dependent enzymes have  $K_m$  for ATP much lower than 1 mM. Rather, this is a result of the operation of an intracellular “ATP-meter” generating a suicide signal when the ATP level decreases by a factor  $\geq 3$ .

The apoptotic pathway actuated by the [ATP] decrease includes Bax translocation to mitochondria, which seems to initiate cytochrome *c* release from their intermembrane space (Fig. 5). This did not require permeability transition pore opening (for review see Ref. [17]) since cyclosporin A did not arrest apoptosis. In this respect, the ATP lowering resembles the apoptogenic effect of the nonspecific protein kinase inhibitor staurosporine, differing from the effect of TNF, which is cyclosporin A-sensitive. Also like staurosporine-induced apoptosis, our effect was not arrested by oligomycin, which inhibits TNF-induced apoptosis [12]. In contrast to the  $H_2O_2$ -induced apoptosis and like the staurosporine and TNF apoptosis, it was resistant to *N*-acetyl cysteine and the mitochondrially addressed antioxidant mitoQ [18,19].

An attractive possibility is that a putative ATP sensor is a protein kinase requiring, in contrast to other enzymes of this kind, millimolar ATP concentrations to operate. An example of such a kinase might be mTOR kinase [20,21]. Alternatively, this might be the AMP-activated kinase (AMPK) [22,23] if it is [AMP], rather than [ATP], which is monitored by the system in question. Both mTOR and AMPK were shown to be involved in gene regulation under starvation when some decrease in [ATP] and increase in

[AMP] are probable. However, effects described in our paper proved to be resistant to emetine, the protein synthesis inhibitor. This means that in this case, mTOR and/or AMPK, if involved, should operate in other, gene regulation independent fashion.

Recently, Ruiz-Stewart et al. [24] revealed that the NO-sensitive soluble guanylate cyclase (sGC) is strongly inhibited by ATP,  $K_i$  being about 2.2 mM. On the other hand, Takuma et al. [25] showed that cGMP has an anti-apoptotic effect. One may suggest that a small (from 3 to about 2 mM) decrease in [ATP], caused in our experiments by either DOG or some oxidative phosphorylation inhibitors, when they were added separately, did not induce apoptosis since the apoptogenic signal was arrested by the sGC–cGMP system. Simultaneously, a number of cGMP-controlled enzymes change their activity adjusting the energy-producing and energy-consuming processes to changed conditions [24]. If, nevertheless, this does not help and [ATP] fall continues down to 1 mM, the apoptogenic effect becomes stronger than the anti-apoptotic, so the cell is self-eliminated by apoptosis, a delicate suicide mechanism, affecting a given cell and perhaps its closest neighbors (the bystander effect). If the ATP fall becomes too deep (e.g., more than 30-fold [6]) or lasts too long (e.g., more than 3 h, this paper), necrosis is activated, which may be regarded as a visible (“public”) suicide. Now the cellular content is released to the intercellular space, serving as a long-distance attractant for macrophages and potent inducer of inflammation. It is remarkable that in both cases (apoptosis and necrosis), recovery of [ATP] after its fall fails to prevent the cell death (Fig. 8). For necrosis induced by a more than 20-fold [ATP] decrease [6], this might also be explained by irreversible damage to intracellular structure(s) during the period of ATP exhaustion. This is obviously not the case in our experiments when ATP was maintained at level not lower than 1 mM, which is high enough to saturate the great majority of the ATP consumers. Apparently, a threefold decrease in the ATP level is a signal for the cell that something is quite *wrong* with its homeostasis, and such a cell commits suicide, following the abovementioned principle “it is better to die than to be wrong”. Thus, in this case, suicide looks like the “Wages of Fear”.

Probably, similar situation takes place when cells commit suicide due to short-term anoxia, high temperature, ionized radiation, etc. However, in all the cases listed, it is impossible to discriminate between signal and direct damaging

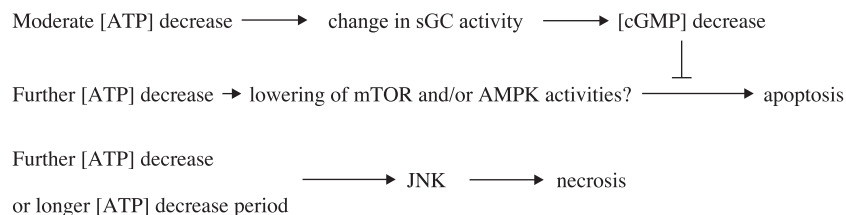


Fig. 8. A tentative scheme of regulation of programmed cell death mechanisms by the intracellular ATP level.

effect of the apoptogenic stimuli. On the other hand, it seems improbable that the moderate and transient decrease in the ATP level inducing apoptosis in our experiments caused any irreversible damage to the cell.

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